

The expression of transforming growth factor- β 1 in myocardial tissue and concentration of serum B-type natriuretic peptide in myocardial remodeling of Sprague-Dawley rats treated with carvedilol

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Abstract. – OBJECTIVE: The objective of this study was to observe the expression of transforming growth factor- β 1 (TGF- β 1) in myocardial tissue and the concentration of serum B-type natriuretic peptide (BNP) in myocardial remodeling of Sprague-Dawley rats induced by isoproterenol (ISO) and the effects of carvedilol intervention.

MATERIALS AND METHODS: Thirty rats were divided randomly into three groups: (1) Control group: rats were injected with 5 mL/(kg·d) of saline for 10 days, followed by 10 mL/(kg·d) of saline by gavage for 4 weeks. (2) Model group: rats were injected with 5 mg/(kg·d) ISO for 10 days, followed by 10 mL/(kg·d) of saline by gavage for 4 weeks. (3) Treatment group: rats were injected with 5 mg/(kg·d) ISO for 10 days, followed by 10 mg/(kg·d) carvedilol by gavage for 4 weeks. Following treatments, the Cardiac Weight Index (CWI) was measured. The pathological changes to myocardial tissue were observed by HE staining and Masson's trichrome staining. The mRNA expression of TGF- β 1 was determined by RT-PCR. The protein expression of TGF- β 1 was detected by immunohistochemistry and Western blot. The concentration of serum BNP was measured by ELISA.

RESULTS: According to our results, no significant pathological changes were observed in myocardial tissue of the control group. The denaturation, hypertrophy, edema and necrosis of myocardial cells as well as increased collagen fibers in myocardial tissue of the model group, were more pronounced compared to the treatment group. The CWI, level of TGF- β 1 in myocardial tissue, and the concentration of serum BNP of the model group, were significantly higher than that of the treatment group, and those of the treatment group were significantly higher than in the control group. There were significant differences among the three groups. There were also significant differences between any two groups.

CONCLUSIONS: The expression of TGF- β 1 in myocardial tissue was upregulated and the concentration of serum BNP was increased in myocardial remodeling of SD rats induced by ISO. Carvedilol intervention can downregulate the expression of TGF- β 1 and decrease the concentration of BNP, inhibiting myocardial remodeling, and improve cardiac function.

Key Words:

Transforming growth factor- β 1, B-type natriuretic peptide, Myocardial remodeling, Carvedilol.

Introduction

Dilated cardiomyopathy (DCM), evolved from myocarditis, is a chronic myocardial ischemic disease, which is a serious health concern among children, as well as one of the most common forms of pediatric primary cardiomyopathy. Based on studies in recent years, myocardial remodeling with major changes, such as increases in heart weight, is the final pathologic change and the pathological basis of complications such as cardiac dilatation, heart failure, and even sudden cardiac death. Transforming growth factor- β 1 (TGF- β 1) is one of the most significant initial factors in the process of myocardial remodeling and myocardial fibrosis, and has the ability to stimulate the synthesis of myocardial collagen fibers from cardiomyocytes and cardiac fibroblasts¹. B-type natriuretic peptide (BNP) is a polypeptide, which is of great significance in maintaining the body's salt and water balance, blood pressure, and cardiovascular system. Clinically, BNP can serve as the diagnostic indicator of heart failure evolved from myocardial remodeling, and can determine prognosis². The objective of this study was to obser-

ve the expression of TGF- β 1 and the concentration of serum BNP in myocardial remodeling of Sprague-Dawley rats induced by isoproterenol (ISO) and the effects of carvedilol intervention on TGF- β 1 and the concentration of BNP. The effects of carvedilol ability to inhibit myocardial remodeling and improve heart failure are explored.

Materials and Methods

Materials

30 healthy male Sprague-Dawley rats, body mass of 180.00 ± 28.36 g, were provided by the Laboratory Animal Centre of Xuzhou Medical University, Xuzhou, China (clean grade, certification number: SCXK(Su)2003-0003). Animal feeding and sampling for all experiments were complied with the relevant provisions on experimental animal management and protection.

Reagents and Equipment

ISO hydrochloride injection (1 mg/2 mL, produced by Shanghai Harvest Pharmaceutical Co, batch No. H31021344); Carvedilol (Jinluo, batch No. H20000100, Qilu Pharmaceutical Co., Ltd); agarose, TRizol, reverse transcription kit, reverse transcription-polymerase chain reaction (RT-PCR) kit, BNP kits, PCR reagents, DL 600 Marker and $5 \times$ TAE were from Tiangen Biotechnology Co., Ltd; rabbit high sensitivity two-step immunohistochemical detection reagent, TGF- β 1 polyclonal antibodies, and DAB color reagent were from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd; primers were from Sangon Biotech (Shanghai, China) Co., Ltd.; primer sequences: TGF- β 1 forward: GAACCAAGGAGACGGAATACAG, reverse: AACCCAGGTCCTTCCTAAAGTC, size of amplified product is 299 bp; β -actin forward: CCAA-AAGGGTCATCATCTCC, reverse: CAACCTG-GTCCTCAGTGTAGC, size of amplified product is 498 bp. Bicinchoninic acid (BCA) protein assay kit and goat anti-mouse antibody were from Beijing Zhongshan Golden Bridge Biotechnology Co., Ltd, (Beijing, China); TGF- β 1 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, USA); actin primary antibody was from Wuhan Boster Biological Engineering Co., Ltd; nitrocellulose membranes (NC film) were from Sigma-Aldrich (St. Louis, MO, USA).

Animal Groupings and Treatments

The myocardial remodeling model of SD rats was achieved through continuous hypodermic

injection of ISO in low doses. Thirty healthy male Sprague-Dawley rats were divided randomly into three groups: 1- Control group: rats were injected with 5 mL/(kg·d) of saline for 10 days, followed by 10 mL/(kg·d) of saline by gavage for 4 weeks (n=10). 2- Model group: rats were injected with 5 mg/(kg·d) ISO for 10 days, followed by 10 mL/(kg·d) of saline by gavage for 4 weeks (n=10). 3. Treatment group: rats were injected with 5 mg/(kg·d) ISO for 10 days, followed by 10 mg/(kg·d) carvedilol by gavage for 4 weeks (n=10).

Specimen Collection and Testing Methods

Rats were anesthetized with chloral hydrate, and immobilized on the operating table after measuring their body weight. Blood from the heart cavity was collected and centrifuged at 8000 rpm for 5 min with a centrifugal radius of 3 cm. Serum was frozen in a -80°C refrigerator, which was spared to detect BNP concentrations. Hearts were removed immediately and surrounding vessels were cut. Hearts were washed in pre-cooled saline, dried on sterile filter paper, and weighed with an electronic balance to calculate cardiac weight index ($\text{CWI} = \text{heart weight} / \text{body weight}$). As for the subsequent RT-PCR and Western blot analyses, the apex, right ventricle and septum were taken, placed in pre-sterilized and diethyl pyrocarbonate (DEPC)-processed cryogenic vials and then in liquid nitrogen. The remaining part of the myocardium (left and right ventricles and interventricular septum) were fixed immediately in 40 g/L paraformaldehyde for conventional sampling, dehydration, paraffin embedding, sectioning, HE staining and Masson's trichrome staining.

Detection of TGF- β 1 Protein Expression in Myocardial Tissue by Immunohistochemistry

After tissue underwent paraffin embedding, sectioning and conventional processing, they were placed and incubated in 3% hydrogen peroxide for 15 min, and blocked with normal goat serum at room temperature for 15 min. An appropriate amount of diluted primary antibody: TGF- β 1 (1:200) was placed on sections to incubate at 4°C overnight; sections were then incubated with biotin labeled IgG at 37°C for 30 min followed by horse radish peroxidase (HRP) labeled streptavidin working solution (S-A/HRP) at 37°C for 30 min. Diaminobenzidine (DAB) counterstain was applied mildly with hematoxylin after the appearance of color development. Sections were dehy-

drated and clear graded with ethanol and xylene, and mounted with neutral balsam. Yellow granules appearing in myocardial tissue were considered a positive performance of the protocol. Image pro plus analysis system software was applied in immunohistochemical imaging for detection of optical density.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) Method to Detect TGF- β 1 mRNA of Myocardial Tissue

After total RNA was extracted and identified, RNA purity and concentration were measured and reverse transcription was performed. The PCR reaction was carried out according to PCR kit instructions. The reaction conditions were as follows: denaturation at 94°C for 3 min, denaturation at 94°C for 30 s, annealing at 54°C (TGF- β 1) or 57°C (β -actin) for 30 s, extension at 72°C for 1 min, re-extension at 72°C for 5 min. The product after 30 cycles underwent 10 g/L agarose gel electrophoresis. Image pro plus analysis system software was applied to calculate the gray values and record relative values normalized to β -actin.

Western Blot to Detect the Expression of Myocardial TGF- β 1-Protein

Ventricular tissue preserved in liquid nitrogen was cut into pieces to extract cardiac tissue protein. Protein was quantified with bicinchoninic acid (BCA) protein assay kit, then 50 μ g of total protein were separated by 7% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gel at room temperature. Electrophoresis was ended when bromophenol blue electrophoresis was at the bottom of the resolving gel. Wet transfer method was applied for membrane transfer. A nitrocellulose membrane was immersed in 20 ml blocking buffer solution containing 1 g of skim milk powder; next, it was confined in a greenhouse for 2 h on a horizontal shaker. After that, it was washed three times for 5 min. Membranes were incubated in primary antibody TGF- β 1 (1:200) in blocking buffer, or actin (1:400) at 4°C overnight. The next day, membranes were allowed to warm again for 30 min, washed three times for 5 min. Next, we added alkaline phosphatase (AP) conjugated secondary antibody IgG (goat anti-mouse antibody, 1:500) at room temperature for 2 h, washed three times with buffer, and one time in double distilled water. After luminochemical detection by ECL detection reagents (Millipore, Billerica, MA, USA), membranes were exposed with a Bio-Rad 2000 gel imaging system (Hercules,

CA, USA). The gray scale of Western blot bands was quantified by ImageJ analysis software. We calculated the ratio between TGF- β 1 band intensity and corresponding β -actin bands.

BNP Detection by ELISA

To determine BNP level, we used the ELISA method. In detail, the purified antigens were diluted to 5 μ g/ml in phosphate buffered saline (PBS) and ELISA plates (Nunc, Immuno Medisorp FH) were coated with 100 μ l/well of the diluted antigen for 1 h at 37°C. Plates were washed three times with phosphate buffered saline (PBS) containing 0.05% Tween 20 (PBST) and blocked by 200 μ l of 1% bovine serum albumin (BSA) and 0.05% Tween 20 diluted in PBS (BSAT) for 2 h at 37°C. After washing three times, sera (1 μ l diluted in 100 μ l BSAT) were added and incubated for 1 h at room temperature (RT). After subsequent wash with PBST, the plates were incubated with peroxidase-conjugated anti-swine IgG (whole molecule, Sigma-Aldrich, St. Louis, MO, USA, diluted 1:30,000 in BSAT) for 1 h at RT. Following the three washing steps, chromogenic substrate (3,3',5,5'-Tetramethylbenzidine) was added and incubated in the dark for 20 min. The enzymatic reaction was stopped by 50 μ l of 2N H₂SO₄ and the color reaction was detected in an ELISA plate reader at 450 nm. The cut-off for the assay was established by Receiver Operating Characteristic (ROC) analysis using a confidence level of 95% and the SPSS 10.0 software package (SPSS Inc., Chicago, IL, USA) for calculations.

Statistical Analysis

SPSS 16.0 software (SPSS Inc., Chicago, IL, USA) was applied for statistical data analysis. All data are represented as $\bar{X} \pm s$. Single-factor analysis of variance was applied in the comparison of the 3 groups. LSD method was used in pairwise comparison of the homogeneity of variance between groups and Dunnett's T3 method in heteroscedasticity comparison. If $p < 0.05$, the differences were considered statistically significant.

Results

Comparison on the Results of the Rats in Each Group

CWI, myocardial tissue TGF- β 1 and serum BNP of the model group were significantly higher than that of the treatment group. Furthermore, the above parameters in the treatment group were si-

Table I. The comparisons of CWI, TGF- β 1 and BNP among the control group, the model group and the treatment group.

		TGF- β 1				
	No of rats	CWI (mg/g)	Protein (IHC)	mRNA (RT-PCR)	Protein (Western blot)	BNP (ng/L)
Control group	10	2.98 $\pm$ 0.10	0.12 $\pm$ 0.01	0.65 $\pm$ 0.04	0.76 $\pm$ 0.21	51.56 $\pm$ 1.80
Model group	10	3.31 $\pm$ 0.07 ^a	0.15 $\pm$ 0.02 ^a	1.19 $\pm$ 0.04 ^a	1.22 $\pm$ 0.16 ^a	61.13 $\pm$ 2.00 ^a
Treatment group	10	3.03 $\pm$ 0.04 ^b	0.13 $\pm$ 0.01 ^b	0.86 $\pm$ 0.01 ^b	0.95 $\pm$ 0.15 ^b	57.08 $\pm$ 1.52 ^b
<i>F</i> value		54.383	13.62	115.14	18.07	72.81
<i>p</i> -value		0.000	0.000	0.000	0.000	0.000

CWI: Cardiac Weight Index; TGF- β 1: transforming growth factor- β 1; BNP: B-type natriuretic peptide; RT-PCR: reverse transcription-polymerase chain reaction. ^aAll indices of the model group were significantly higher than that of the treatment group and the control group (all $p < 0.01$); ^bAll indices of the treatment group were significantly higher than that of the control group (all $p < 0.01$).

significantly higher than in the control group. There were significant differences among the three groups. There also were significant differences between any two groups (Table I).

Result of HE Staining

Cardiac structure was clear and neatly arranged in the control group. Myocardial cells were round or oval in the central part of the cells. Cytoplasm was abundant and with normal intercellular space. No myocardial cell degeneration, necrosis or fibrosis was observed (Figure 1A1). In the model group, myocardial cell degeneration and hypertrophy was observed. Areas of myocardial ischemia and necrosis were observed mainly under the endocardium and some that extended through the full thickness of myocardium. Meanwhile, we observed multifocal sarcoplasm of cardiac muscle fiber that was fuzzy and some of which was dissolved. Some cardiomyocytes were hypertrophic, had edema and varying degrees of granular degeneration and vacuolar degeneration. Cardiomyocyte gaps widened significantly. Interstitial edema and large amounts of proliferated collagen fibers were observed (Figure 1B1). Myocardial structure in the treatment group was significantly improved over the model group, but there were still some areas of myocardium that were spotty and with focal myocardial necrosis, myocardial hypertrophy, degeneration, and a small amount of collagen fiber proliferation (Figure 1C1).

Masson's Trichrome Staining of Cardiac Myocytes

Myocardial cells were red and collagen fibers were green in the control group. The content of collagen fibers in the control group were mainly distributed in the perivascular compartment, and there

was relatively less than in the model group (Figure 1A2); collagen fiber proliferation was significant in the model group with part of the myocardium replaced by collagen fibers. Myocardial interstitial fiber collagen in the left ventricle was increased significantly with myocardial intercellular collagen proliferation the most significant. Myocardial cells were not arranged neatly and there were large confluent focal necrotic collagen fibers (Figure 1B2). Compared to the model group, the degree of cardiomyocyte fibrosis in the treatment group was lower. The arrangement of cardiomyocytes was relatively neat, and spotted and focal collagen fibers were still visible (Figure 1C2).

Expression of Myocardial Tissue TGF- β 1 Protein by Immunohistochemistry

The expression of myocardial TGF- β 1 protein by immunohistochemistry is shown in brown. The expression of TGF- β 1 in the model group increased significantly compared to the treatment group (Figure 1C3) and the expression in the treatment group increased significantly over the normal group (Figure 1A3).

Myocardial Tissue TGF- β 1 mRNA Detection by RT-PCR

The expression of myocardial TGF- β 1 mRNA produced an intense band. The expression in the model group greatly increased over the treatment group and the expression in the treatment group was increased over the normal group (Table I and Figure 2).

Myocardial tissue TGF- β 1 Protein Expression by Western Blot

The expression of TGF- β 1 protein in the model group increased compared to the treatment group

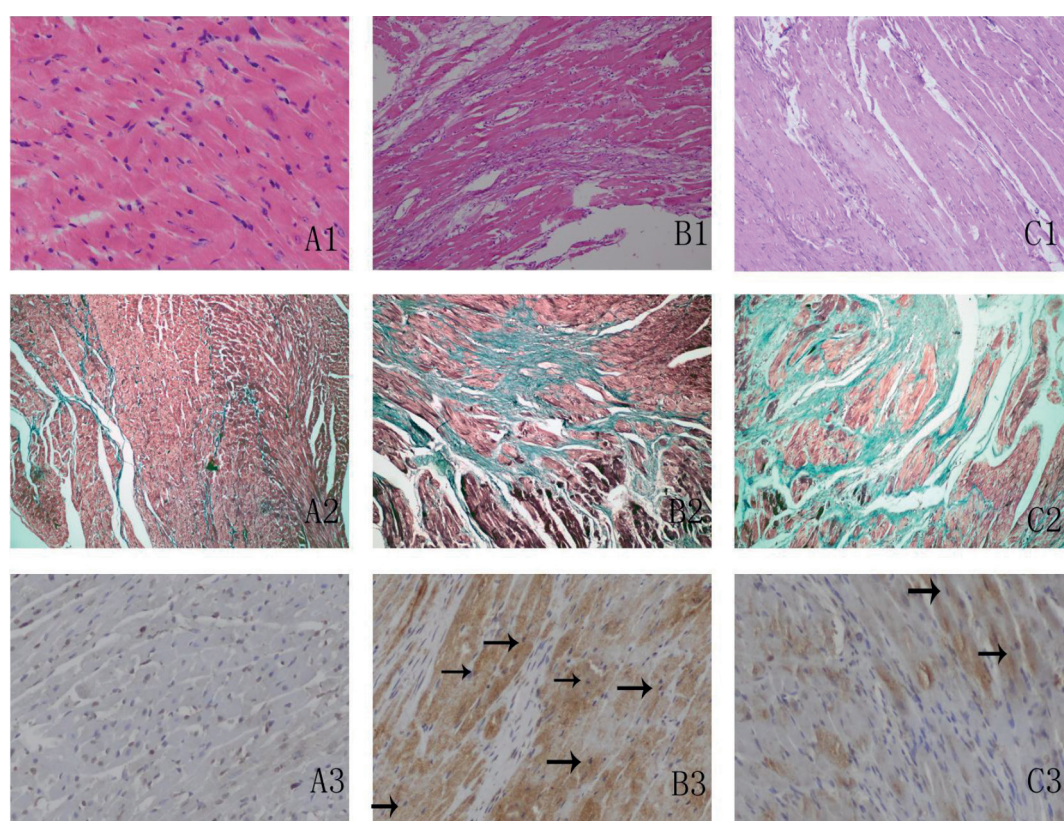


Figure 1. Immunohistochemical analysis of TGF- β 1 expression in myocardial tissue. TGF- β 1: transforming growth factor- β 1. A1: the control group (HE $\times$ 200); B1: the model group (HE $\times$ 400); C1: the treatment group (HE $\times$ 200); A2: the control group (Masson $\times$ 200); B2: the model group (Masson $\times$ 400); C2: the treatment group (Masson $\times$ 400); A3: TGF- β 1 of the control group (immunohistochemistry $\times$ 200); B3: TGF- β 1 of the model group (immunohistochemistry $\times$ 200); C3: TGF- β 1 of the treatment group (immunohistochemistry $\times$ 200). Arrow: immunohistochemically positive cells.

and the expression in the treatment group was increased compared to the normal group (Table I and Figure 3).

Discussion

ISO is a β -receptor agonist, which may induce myocardial remodeling such as left ventricular hypertrophy and myocardial fibrosis under long-term stimulation. Its working mechanism is mainly related to its effect on blood vessel contraction and enhancement of myocardial contractility. Subcutaneous injection of ISO will stimulate the cardiac β 1 receptor, resulting in heart rate acceleration, myocardial contractility strengthening, increased cardiac output and increased myocardial oxygen consumption. It will further lead to platelet aggregation and accelerate thrombosis, leading to myocardial damage, an imbalance in myocardial oxygen supply and demand.

This will result to cardiac ischemia and hypoxia, and the cardiac ischemia will be worsened by oxygen free radicals produced in self oxidation and stress reactions. Long-term ISO injection in rats can cause a weight increase of the left ventricle, an increase of left ventricular protein content and the level of β -myosin heavy chain, resulting in myocardial fibrosis^{3,4}. In view of the stability, ease of imitation and high maneuverability of the animal model, myocardial remodeling with ISO is a simple method of inducing this condition. This study applied continuous subcutaneous ISO injections in small doses to establish myocardial remodeling. CWI in the model group was significantly higher than the control group, indicating a significant increase in the degree of myocardial remodeling; myocardial HE staining and Masson's trichrome staining in the model group also demonstrates myocardial cell degeneration, hypertrophy, and fibrosis. Therefore, the latter also supports the above-mentioned view.

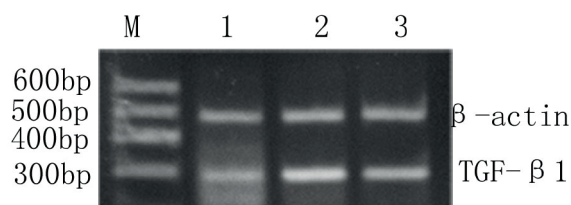


Figure 2. The expression of TGF- β 1 by RT-PCR. RT-PCR: reverse transcription-polymerase chain reaction; TGF- β 1: transforming growth factor- β 1; 1: the control group; 2: the model group; 3: the treatment group

TGF- β 1 is a multifunctional cytokine which can regulate the proliferation and differentiation of cells, stimulate the synthesis and secretion of various cytokines, inflammatory mediators and other active substances, and engage in the composition and degradation of extracellular matrix. It was found in animal and human studies that the expression of TGF- β 1 is significantly higher in the progression of myocardial remodeling⁵. Meanwhile, TGF- β 1 expression can increase in cardiac cells and fibroblasts, stimulate fibroblast proliferation and provide positive feedback on the process of myocardial remodeling through production of profibrotic markers produced by myocardial stimulation and extracellular matrix produced by fibroblast stimulation⁶. Direct stimulation of cardiac fibroblasts cultured *in vitro* by TGF- β 1 can induce cell proliferation and collagen synthesis while gene transfer of TGF- β 1 *in vivo* can induce myocardial fibrosis. Inhibiting the expression of TGF- β 1 can reduce myocardial fibrosis⁷. Previous studies have shown that TGF- β 1 plays a key role in the development process of myocardial remodeling in diseases such as acute myocardial infarction, diabetic cardiomyopathy⁸, coronary heart disease and viral myocarditis. In this study, three methodologies were used to evaluate TGF- β 1 expression in myocardial tissue during remodeling. Our results indicate that TGF- β 1 expression in myocardial tissue in the model group is higher than in the control group. Significant degeneration of myocardial cells, hypertrophy and fibrosis is shown in the model group by 2 staining methods, which further demonstrated the vital positive feedback effect of TGF- β 1 on the myocardial remodeling process. These observations are consistent with other previous studies⁵⁻⁸, which has provided a theoretical basis focusing on TGF- β 1 as a marker in clinical diagnosis, treatment and tests of myocardial remodeling-related diseases.

BNP is a cardiac hormone mainly synthesized and secreted by ventricular cells. It can antagonize the renin-angiotensin-aldosterone system, effect diuresis, reduce ventricle volume, cause vasodilation, increase ventricular volume and/or increase tension of the ventricular wall. Therefore, sensitive and specific evaluations of changes in cardiac function (left ventricular function in particular), by measuring BNP levels, can be applied in the diagnosis of heart failure and in making a prognosis^{9,10}. Heart failure guidelines, outlined by the European Society of Cardiology, have considered BNP level as one of the objective indicators of heart failure. Recent studies have shown that BNP value was increased in the early diagnosis of left ventricular dysfunction, which has no symptoms but can also be used to determine infarct size. Thus, it can work as an independent risk factor index in cardiovascular diseases^{11,12}. The results of this study have shown that the concentration of serum BNP in the model group was significantly higher than that of the control group, indicating that myocardial damage such as degeneration, hypertrophy and fibrosis of cardiomyocytes, as well as deterioration of heart function caused by cardiac remodeling or the myocardium in the model group. Tamura et al¹³ showed that, adding BNP to cultured cardiac fibroblasts, inhibited their proliferation. In rat models with BNP gene deletion, myocardial fibrosis could be found when pressure load was increased. Therefore, the indication of elevated serum BNP could reflect more than increased ventricular load and deterioration of heart function, which is consistent with the present meaning of the widely applied clinical BNP detection.

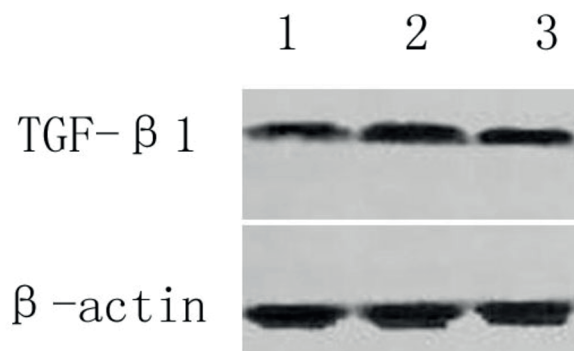


Figure 3. The expression of TGF- β 1 by Western blot. TGF- β 1: transforming growth factor- β 1; 1: the control group; 2: the model group; 3: the treatment group.

As a third generation β -blocker, carvedilol has several features of blockers including, anti-oxidation, anti-apoptotic, anti-arrhythmic and can inhibit neutrophil infiltration, which has provided the theoretical foundation for the prevention of cardiovascular disease and has become one of the pivotal myocardial protective agents^{14,15}. In a prospective randomized controlled trial, carvedilol significantly improved the heart function of pediatric patients and delay myocardial remodeling in the treatment of children with heart failure caused by dilated cardiomyopathy¹⁶. According to Zhang et al¹⁷, carvedilol can inhibit collagen synthesis of cultured cardiac fibroblasts in a concentration-dependent manner and its mechanism is related to β -blockers. More studies have shown that carvedilol can correct the imbalance of matrix metalloproteinases/tissue inhibitor of metalloproteinases¹⁸, inhibit the activation of the p38 MAPK signal transduction pathway, TGF- β 1/Smads pathway, ERK signaling pathway, and connective tissue growth factor and endothelin-1. Carvedilol can affect TGF- β 1 directly or indirectly through the above mechanisms, thus playing its role in inhibiting myocardial hypertrophy and remodeling, improving myocardial fibrosis as well as other functions. The results of this study have shown that CWI in the treatment group decreased significantly compared to the model group after carvedilol intervention, indicating that the degree of myocardial remodeling was significantly decreased compared to the model group while cardiac hypertrophy was suppressed and reduced. Three methods were applied to determine TGF- β 1 expression in myocardial tissue.

Conclusions

Our results indicate that myocardial TGF- β 1 in the control group decreased significantly compared to the model group. By 2 staining methods, we show that degeneration of myocardial cells, hypertrophy and fibrosis in the treatment group were all significantly improved and reduced compared to the model group. Serum BNP in the treatment group also decreased significantly compared with the model group, suggesting improved heart function. Therefore, these results confirm that carvedilol can inhibit cardiac hypertrophy and remodeling, improve myocardial fibrosis and cardiac function.

The cardiac weight index (CWI) was increased; the expression of TGF- β 1 in myocardial tissue

was upregulated and the concentration of serum BNP was increased in myocardial remodeling of SD rats induced by ISO. Carvedilol intervention can decrease CWI, downregulate the expression of TGF- β 1, decrease the concentration of BNP, and inhibit myocardial remodeling, thereby improving cardiac function. We provide a theoretical basis for the clinical application of carvedilol for the improvement of cardiac function.

Conflict of interest

The authors declare no conflicts of interest.

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