Effects of Testosterone on Cytokines and Left Ventricular Remodeling Following Heart Failure

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Key Words

Testosterone • Heart failure • Myocardial infarction • Remodeling • Rats

TNF- α , suppresses ventricular remodeling and improves cardiac function.

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Abstract

Aims: To investigate the effects of testosterone treatment on cytokines, ventricular remodeling in rats with heart failure. **Materials and methods:** Sprague-Dawley male rats with heart failure were divided into testosterone (n = 22) and placebo (n = 22) group. Pseudo surgery was performed on a third group of 15 male rats as control. **Results:** Compared with the placebo group, the testosterone group had a greater LVEF ($P < 0.05$), a higher serum IL-10 level ($P < 0.05$) and a lower serum TNF- α level ($P < 0.05$). The expression of TNF- α mRNA, MMP-9 mRNA and the myocardial hydroxyproline contents in the testosterone group were also lower than in the placebo group ($P < 0.05$). The mortality rate in the testosterone and placebo group was 31.8% and 68.2%, respectively ($P < 0.05$). **Conclusion:** Serum testosterone levels were decreased significantly in male rats with heart failure. Testosterone treatment diminishes the imbalance between interleukin-10 and

Introduction

Chronic heart failure (CHF) is a leading cause of morbidity and mortality. [1] The mainstay of therapy for CHF includes diuretics, vasodilators and inotropic agents. In addition, neuro-hormonal manipulations such as angiotensin converting enzyme inhibitors, beta-blockers and aldosterone receptor antagonists have also been used. These drugs improve the clinical symptoms, elevate the left ventricular ejection fraction (LVEF) and reduce mortality and hospitalization to some degree. However, even with the optimal pharmacological therapy the annual mortality of heart failure remains high. [1].

The role of cytokine network in the pathogenesis of CHF has been recently investigated. Serum levels of pro-inflammatory cytokines such as serum tumor necrosis factor (TNF- α) are increased in CHF patients, whereas anti-inflammatory cytokines such as interleukin-10 (IL-10) are decreased. This imbalance in the

inflammation and anti-inflammation pathways may result in decreased myocardial contractility, ventricular dilation, myocardial remodeling, increased cardiac myocyte apoptosis, and cardiac cachexia. [2] TNF- α levels are independent predictors of mortality in patients with advanced heart failure. [2] Moreover, TNF- α is one of the most important stimulus of matrix metalloproteinases (MMP), which are the most important proteolysis system in the process of extra cellular matrix degradation. [3] Activation of MMP that induces extra cellular matrix fibrosis is an important character of ventricular remodeling. [3].

Recently, immunomodulatory therapy has been applied to restore the inflammatory imbalance in patients with CHF. The immunomodulatory regimens include some broad-spectrum anti-inflammatory agents such as testosterone, immunoglobulin, growth hormone and statins, which have been shown to improve the symptoms and prognosis of CHF by modulating inflammatory cytokine network. [4, 5].

Men with CHF appear to have relatively low testosterone level. [6] Several animal studies and small clinical trials have suggested that testosterone treatment may increase cardiac output and reduce peripheral vascular resistance. [7-10] Our study aimed to investigate the effects of low dose testosterone on the imbalance of pro- and anti-inflammatory pathways and cardiac function in rats with post-infarct heart failure.

Materials and Methods

Surgical procedures

This study was approved by the institutional review board of Zhengzhou University. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). One hundred Sprague-Dawley male rats (8 week old, average weight 270 ± 40 g) were obtained from Experimental Animal Center of Henan Province. They were housed in groups in an air-conditioned room with a 12:12 h dark-light cycle and given standard feed and free access to tap water.

Induction of myocardial infarction (MI) was performed in 85 rats whereas pseudo surgery was performed in 15. Under general anesthesia (pentobarbital 40 mg/kg, ip), rats were anesthetized, intubated and ventilated with oxygen using a positive-pressure respirator. A left thoracotomy was performed via the fourth intercostal space, the lungs were retracted to expose the heart and the pericardium was opened. The left anterior descending coronary artery was ligated near its origin, between the pulmonary outflow tract and the edge of the left

atrium. Acute myocardial ischemia was considered successful when the anterior wall of the left ventricle turned pale, and ST segment elevation was observed on the electrocardiogram.

After the establishment of myocardial infarction, the lungs were inflated by increasing positive end-expiratory pressure and the thoracotomy site was closed. In another 15 rats, pseudo surgery was performed by detaching a silk suture under the coronary artery but not ligating it.

Six weeks after the surgery, 44 of the 85 rats underwent coronary ligation survived. They were randomly divided into testosterone group ($n = 22$), which was treated with testosterone (Xianju Pharmaceutical Inc. Zhejiang, China) 5mg/ kg (im) every two weeks for 12 weeks; and placebo group ($n = 22$). At the 18th week following the surgery, the chest was opened and the heart was stopped at diastole by intraventricular injection of 15% KCl. After the heart was weighed, the left ventricle was sectioned transversely into three slices, rapidly frozen in isopentane and stored at -70°C for tissue analysis.

Echocardiography

Transthoracic echocardiography was performed at the 6th week and the 18th week following the surgery, using a Vivid-7 ultrasound machine (GE Corp) with an 11.4 MHz phased array transducer under anesthesia and mechanical ventilation as previously mentioned. The transducer was placed in gentle contact with the mid-precordial area through a transmission medium. M-mode echocardiograms, guided by 2-dimensional long-axis images, were obtained through the anterior and posterior left ventricular walls at the level of the papillary muscles. The left ventricular end-systolic diameter (LVEDs), the left ventricular end-diastolic diameter, (LVEDd) was measured from the M-mode tracing by the leading-edge method of the American Society for Echocardiology. Left ventricular ejection fraction (LVEF) was determined by formula $\text{LVEF} = (\text{LVEDd} - \text{LVEDs}) / \text{LVEDd} \times 100\%$. LVEF = 45% was defined as heart failure.

The echocardiographer who performed the assessment of the left ventricular function was blinded to the animal grouping. The intra-observer variations of LVEDs, LVEDd and LVEF were less than 4%.

Radioimmunoassay of serum testosterone level

Serum testosterone levels were measured preoperatively and at 6 weeks and 18 weeks following the surgery, using an antitestosterone antibody-coated tube (RIA kit, Kemeidongya Research Center of Biotechnology, Beijing, China) according to the manufacturer's instructions. The intra-assay and the inter-assay coefficient of variation was 7.4% and 9.5%, respectively.

Serum TNF- α and IL-10 levels

Serum TNF- α and IL-10 levels were determined at the 18th week after the surgery by ELASE (enzyme-linked immunosorbent assay), using an enzyme-linked immunosorbent assay kit (Senxiong technology Inc. Shanghai, China). Rats who died during the observation period were excluded from the blood testing.

Items	Pseudo surgery (n=15)	Placebo (n=22)	Testosterone (n=22)
Body weight (g)			
Before	266.1±19.1	262.4±16.1	264.6±15.5
6 th week	365.8±33.9	311.7±13.9*	308.9±13.9**
18 th week	517.6±19.2 [§]	300.6±10.6*	438.9±32.7** [§]
Serum T levels (ng/dl)			
Before	466.6±237.1	512.6±211.5	510.0±201.0
6 th week	495.6±183.4	331.20±82.3*	317.2±92.5**
18 th week	475.3±161.4	172.1±59.8 [§]	415.5±59.2**
LVEF (%)			
6 th week	71.9±7.2	36.1±9.3*	35.8±9.0
18 th week	69.1±6.7	33.2±7.5 [§]	41.9±10.5**
Mortality	0	15 (68.2%)*	7 (31.8%)**

Table 1. Difference in each group: T: testosterone, HYP: hydroxyproline. *: Placebo group compared with pseudo-surgery group, $P < 0.05$. **: Testosterone group compared with placebo group, $P < 0.05$. [§]: Comparison of 6th and 18th week within the same group, $P < 0.05$.

RT-PCR for TNF- α mRNA and MMP-9 mRNA

The RT-PCR analyses for mRNA and MMP-9 mRNA in myocardial tissues were performed as follows: total TNF- α RNA was extracted using RNeasy Micro Kit or RNeasy Mini Kit (Qiagen, Australia) and cDNA synthesized with AMV reverse transcriptase according to the manufacturer's instructions (Promega, USA), using oligo dT (Invitrogen, USA). Primer sequences for detecting TNF- α were 5'-GGC CTC CAG AAC TCC AGG CG-3' for forward and 5'-GTC CCT TGA AGA GAA CCT GG-3' for reverse primers respectively. The size of the amplicon was 470 bp. Relative expression of TNF- α mRNA was analyzed by expression of smooth muscle β -actin using primer sequences as 5'-CGG CTC CGG CAT GTG CAA GG-3' for forward and 5'-GCT CCT CAG GGG CCA CAC GC-3' for reverse and the size of the amplicon was 256bp.

RT-PCR for MMP-9 mRNA was performed as the same as that for TNF- α mRNA. Forward primer was 5'-CGA AGG CGA CCT CAA GTG GC-3' and reverse primer was 5'-TGG CCC TCG AAG ATG AAT GG-3'. The size of the amplicon was 550bp. Relative expression of MMP-9 mRNA was analyzed by expression of GAPDH using primer sequences as 5'-CGG CTC CGG CAT GTG CAA GG-3' for forward and 5'-ATC CCC CAT TTG ATG TTA G-3' for reverse and the size of the amplicon was 259bp.

Hydroxyproline contents

Hydroxyproline contents in myocardial tissues were detected by staining method, using detection agents manufactured by Jiancheng Research Center of Biological Engineering (Nanjing, China).

Statistical methods

All experimental data are expressed as Means $\pm$ SD and processed by SPSS12.0. The comparison of serum testosterone and cytokine levels, the expression of TNF- α mRNA and MMP-9 mRNA, hydroxyproline contents and LVEF between groups was performed by ANOVA. Chi-square test was used to compare the mortality rate between the testosterone and placebo group. Pearson correlation analysis was used to assess the association between LVEF and serum testosterone levels. $P < 0.05$ was considered statistically significant.

Results

Changes in body weight

In all groups, there was an increase in body weight during the study. However, the mean body weight of the placebo group was lower than the pseudo surgery group 6 and 18 weeks after the surgery. The mean body weight in the testosterone group was greater than the placebo group 18 weeks after the surgery (Table 1, $P < 0.05$).

Left ventricular function and mortality

There was a significant reduction in the LVEF in the placebo and testosterone group 6 weeks following the surgery (Table 1, $p < 0.05$). At the end of the study, the LVEF in the placebo group remained low but there it was improved in the testosterone group (Table 1, $P < 0.05$). At the end of the study, the mortality rate in the testosterone group was lower than the placebo group (Table 1, $P < 0.05$).

Serum testosterone levels

The mean testosterone level in the placebo group 6th and 18th weeks after the surgery was significantly lower than the pseudo surgery group (Table 1, $P < 0.05$). Within the placebo group, the mean serum testosterone level at 18th week of the surgery was lower than the 6th week (Table 1, $P < 0.05$). The serum testosterone levels in this group were positively correlative with LVEF (Fig 1, $r = 0.781$, $P = 0.004$).

In the testosterone group, the mean testosterone level at 18th week was higher than at 6th week, and was higher than the mean testosterone level of the placebo group (Table 1, $p < 0.05$).

Serum cytokines and myocardial expression of cytokine mRNA

At the end of the study, the mean serum TNF- α level in the placebo group was significantly higher than the pseudo surgery group (Table 2, $P < 0.05$). The mean

Fig. 1. Relationship of testosterone and left ventricular ejection fraction (LVEF) at the 18th week following the surgery (placebo group, n=7).

Table 2. Comparison of serum cytokines and myocardial expression of cytokine mRNA: T: testosterone, HYP: hydroxyproline. *: Placebo group compared with pseudo-surgery group, *P*<0. 05. **: Testosterone group compared with placebo group, *P*<0.05.

Items	Pseudo -surgery (n=15)	Placebo (n=22)	Testosterone (n=22)
Serum IL-10 levels (pg/ml)	7.30±2.14	4.90±1.32*	6.55±2.11**
Serum TNF-α levels(pg/ml)	5.29±2.30	8.59±3.22*	5.97±1.81**
Expression of TNF-α mRNA	0.26±0.09	0.82±0.19*	0.50±0.12**
Expression of MMP-9 mRNA	0.27±0.09	0.75±0.11*	0.60±0.11**
HYP (ig/g of myocardium)	396.46±54.52	538.42±82.32*	469.00±55.54**

serum TNF-α level in the testosterone group was markedly lower than that in placebo group (Table 2, *P*<0.05).

The mean serum IL-10 level in the testosterone group was similar to the pseudo surgery group (Table 2, *P*>0.05) but higher than the placebo group (Table 2, *P*<0.05).

Compared with the placebo group, the testosterone group had a lower myocardial expression of TNF-α mRNA and MMP-9 mRNA, Fig 2 and 3, Table 2, *P*<0.05).

The mean myocardial hydroxyproline contents in the testosterone group was significantly lower than the placebo group (Table 2, *P*<0.05).

Discussion

Catabolic/anabolic disturbance and hormonal activation are common in patients with chronic heart failure and is responsible for the development of cardiac cachexia. In male patients with chronic heart failure, anabolic hormone depletion is common, and a deficiency of total blood testosterone or free testosterone is an independent marker of poor prognosis. [11] Significant reduction in blood testosterone identifies groups with a higher mortality. [11] Our study has clearly demonstrated

that in male rats, the serum testosterone levels are decreased following heart failure. The reduction in serum testosterone correlates well with the severity of left ventricular dysfunction. Treatment with synthesized testosterone for up to 12 weeks reduced the weight loss following heart failure. There was a reduced TNF-α synthesis and diminished ventricular remodeling following testosterone treatment. More importantly, testosterone therapy diminished the reduction in testosterone and improves the left ventricular function. The treatment group also had a lower mortality rate than the placebo group. Because left ventricular ejection fraction is directly related to the mortality rates,[1] the mortality benefits seen in the treatment group are likely due to improved left ventricular function.

Influence of testosterone therapy on cytokine levels

We have detected that in rats with heart failure, the levels of TNF-α are elevated in serum and myocardial expression of TNF-α mRNA is enhanced. On the other hand, the serum level of IL-10, a prominent anti-inflammatory factor, is decreased. The changes in TNF-α and IL-10 are likely due to the presence of left ventricular dysfunction because when left ventricular

Fig. 2. Difference in expressions of TNF- α mRNA in each group. Column M: DNA marker; Column 1, 2: expressions in the pseudo surgery group; Column 3, 4: expressions in the testosterone group; Column 5, 6: expressions in the placebo group.

Fig. 3. Difference in expressions of MMP-9 mRNA in each group. Column M: DNA marker; Column 1, 2: expressions in the pseudo surgery group; Column 3, 4: expressions in the testosterone group; Column 5, 6: expressions in the placebo group.

function is partially preserved by testosterone treatment, the alterations in serum levels of TNF- α and IL-10 are also largely prevented. Our findings are supported by a recent study which shows that decrease in IL-10 or IL-10-to-TNF- α ratio correlates well with the reduction in cardiac function. [11].

In the present study we did not attempt to clarify the signaling pathways between the left ventricular dysfunction and the changes in cytokines. However, the increase in TNF- α following heart failure may have been facilitated by the reduction in IL-10, because IL-10 down-regulates the production of TNF- α . [12] IL-10 also induces the production of specific cytokine inhibitors, such as IL-1 receptor antagonist (IL-1RA) and TNFRs. [12].

TNF- α is one of the key cytokines in the development of catabolism, causing skeletal muscle wasting. [13]. It also produces left ventricular dysfunction, cardiomyopathy and pulmonary edema when overexpressed in human subjects. [14]. In recent years, smaller animal or human studies to antagonize the effects of endogenous TNF- α in heart failure have achieved some favorable results. [15, 16]. However, the results from large-scale clinical trials on anti-TNF- α therapy with etanercept (a fusion protein of TNFR-2 which can combine with TNF- α) and infliximab (a monoclonal antibodies directed against TNF- α) have been disappointing. [17, 18]. Whether broad-spectrum immunomodulators, such as immunoglobulin or testosterone may offer a positive outcomes remains to

been seen. [19].

The present study has clearly shown that treatment with testosterone is associated with an increase in interleukin-10 and decrease in TNF- α , restoring the imbalance between the two factors in the failing heart. The favorable effects of testosterone on these cytokines are likely through suppression of macrophage production of TNF- α , while increasing CD⁴⁺T lymphocytes production of IL-10 production. [20, 21, 22]. In men, testosterone levels correlate negatively with serum cytokine levels. [20]. Testosterone replacement therapy induces a reduction in TNF- α and an increase in IL-10 in hypogonadal men. [6].

Effect of testosterone therapy on ventricular remodeling

An important character of ventricular remodeling during heart failure is extra cellular matrix fibrosis or over degradations. MMP is the most important proteolysis system in the process of extra cellular matrix degradation and play a vital role in ventricular remodeling in chronic heart failure. [23] TNF- α is one of the most important stimulus of MMP. [23].

The present study found that the expression of MMP-9 mRNA in myocardial tissue of the placebo group is increased significantly. Testosterone therapy diminishes the myocardial expression of MMP-9.

Hydroxyproline mainly resides in collagen protein. The hydroxyproline contents in the myocardium reflect

the information of deposited collagen protein. Our study shows that the myocardial hydroxyproline contents are increased following heart failure. Testosterone therapy reduces the myocardial hydroxyproline contents, diminishing excessive proliferation of collagen protein, thus preventing ventricular remodeling. These beneficial effects of testosterone are likely to be mediated by suppressing TNF- α or by down-regulating MMP-9 directly. [23].

Conclusions

There was a reduced level of serum testosterone level in male rats with heart failure. The reduction of testosterone is accompanied by increase in TNF- α and MMP-9, but a decrease in serum interleukin-10. Low dose testosterone therapy improves the interleukin-10 and TNF- α ratio, and suppresses ventricular remodeling. These changes are accompanied by significant improvement in left ventricular function and a reduction in mortality. Further studies are warranted to assess if the anatomical, functional and mortality benefits following testosterone therapy can be maintained in long-term.

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