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Original Article

Synergistic effect of estradiol and testosterone protects against IL-6-inducedcardiomyocyte apoptosismediated by TGF-β1

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Abstract: Objective: Basic studies have verified that estradiol or androgen is influential on cardioprotection, howeversynergistic effects of estradiol and testosterone on heart failure (HF) are still unknown. This study aimed to evaluate the association among sex hormones and heart failure risk factors. Methods: 142 controls and 196 patients with HF were selected for this study. Serum levels of estradiol, testosterone, Brain natriuretic peptide precursor, transforming growth factor-β1, β2 and β3, lipid-lipoprotein profile, glucose, high-sensitivity C-reactive protein, serum creatinine, microglobulin, uric acid, alanine aminotransferase, aspartate aminotransferase were determined. H9c2 cardiac myocytes were used to investigate the effect of estradiol and testosterone on cardiomyocytes apoptotic involved in TGF-β1. Signaling pathway of caspase 3, Bax, Bcl-2, caspase 8 and TGF-β was determined during the IL-6 induced apoptotic. Results: First, our results showed that compared with the control, the E2/T ratio decreased from 6.32±9.89 to 3.43±3.16 (P<0.001) in female, from 4.00±8.14 to 7.80±11.35 (P<0.001) in male with heart failure, and the level of TGF-β1 increased. What's more, these changes were favorably associated with the cardiac function classification. Univariate and multivariate logistic regression analysis showed that serum E2/T ratio, TGF-β1 and NT-proBNP were independent risk factor in heart failure patients. Second, we found that TGF-β1 was upregulated in rat H9c2 cardiomyocyte induced by IL-6, and TGF-β1 regulates the apoptosis of rat H9c2 cardiomyocyte. Furthermore, we verified the beneficial effects of the defined appropriate E2/T ratio on cardiomyocyte apoptotic mediated by TGF-β1. Conclusion: The balance of the serum E2/T ratio was broken in patients with heart failure, and an imbalanced E2/T ratio showed a strong association with heart failure risk factors, and E2 combined with T play a synergistic effect on anti-apoptosis involved in TGF-β1.

Keywords: Estrogen, testosterone, heart failure, transforming growth factor-β1, H9c2 cells

Introduction

Heart failure (HF) results from the inability of heart to function properly, which is seen in a number of cardiovascular disorders. It is estimated that more than 23 million people will die due to cardiovascular disorders annually by the year 2030 [1]. HF is a growing health problem in the world [2]. Cardiomyocyte apoptosis is an important contributor to myocardial dysfunction and heart failure due to a systematic reduction in the number of cardiomyocytes [3, 4]. The effective restoration of cardiomyocyte apoptosis is an effective way to protect myocardial function. Typically, the stimulation of ischemia, hypoxia and inflammation factor may result in cardiomyocyte apoptosis.

Basic studies have verified that estradiolor androgen is influential on cardioprotection, including regulating inflammation, energy metabolism, homeostasis balance and cardiomyocyte aging. Although increasing evidence supports an association between single sex hormones and heart failure, the results still remain controversial [5-8]. Studies have also shown that, androgen involved in estrogen synthesis, estrogen involved in androgen removal [9], and estrogen plays a physiological role depended on androgen [10]. These findings prompted us to postulate that it may not be enough to investigate the association between a single hormone and heart failure risk factors. Therefore, we should combine estrogens and androgens

together, i.e. E2/T ratio. To further study the association between sex hormones and heart failure risk factors, this study was designed to investigate the role of synergistic effect of estradiol and testosterone on cardiomyocyte apoptosis.

Estradiol and androgen can interact with transforming growth factor-β1 (TGF-β1). Studies have also demonstrated that estrogenregulates the secretion of TGF-β1 in cultured human dermal fibroblasts [11] and Inhibits the activation of TGF-β1 in Mast Cell [12]. In addition, androgen broadly influences TGF-β1 responses by intercepting an additional step in the TGF-β1 signaling pathway, i.e., by suppressing TβRII expression in prostate epithelial cells [13]. Therefore, we speculated that TGF-β1 may be a common downstream target of estrogen and testosterone that is involved in cardioprotection. Of the three different TGF-β isoforms (1, 2, and 3) that have been identified, TGF-β1 is the most prominent and most often analyzed form. In the heart several cell types are identified as source of TGF-β release, as cardiomyocytes, endothelial cells, fibroblasts and macrophages can release TGF-β1. Interestingly, the cytokine TGF-β1 has been described to influence each of the single components of the remodeling process, i.e., TGF-β1 promotes myocardial fibrosis [14, 15], cardiomyocyte apoptosis [16], or cardiac hypertrophy [17].

Here we hypothesized that estradiol and testosterone (E2/T) combination confers cardioprotection against cardiomyocyte apoptosis-mediated by TGF-β1. In this study, we evaluated serum estradiol, testosterone and TGF-β1 levels in heart failure patients. To further elucidate whether estradiol and testosterone (E/T) combination suppress cardiomyocyte apoptosis, this study examined the effects of either E2 or T alone or E2-T combination on isolated cardiomyocyte subjected to inflammation cytokine IL-6. To clarify whether the cardioprotective effect mediated by TGF-β1, we inhibited TGF-β1 using TGF-β1 receptor inhibitor SB431542, and changed the expression of TGF-β1 by si-r-TGF-β1 and p-EGFP-TGF-β1. We assessed the above effects and ascertained the level of expression of TGF-β1 and apoptosis related protein using western blotting analysis.

Material and methods

Patients and healthy subjects

We consecutively enrolled 196 patients with HF (patient group) who were hospitalized at the Department of Cardiology of Renmin Hospital of Wuhan University and 142 apparently healthy volunteers. A total of 196 subjects were recruited from March 2014 to August 2015. All of the subjects were diagnosed as heart failure. Subjects with other serious illnesses were excluded, such as cancer, acute coronary syndrome and pulmonary embolism.

Ethics statement

This study was approved by the Medical Ethics Review Committee of Renmin Hospital, Wuhan University. All of the participants in this study were required to provide written informed consent in accordance with Renmin Hospital of Wuhan University Ethics Committee; patients were placed under the supervision of a lawful caregiver if necessary.

Samples preparation and analytical methods

All samples were obtained by venipuncture in the morning after subjects had fasted for at least 8 h. A minimally traumatic venipuncture was performed, using a 21-gauge butterfly needle, with the subject seated. After standardized processing at the clinical site, the samples were aliquoted into 2 ml tubes. Within 30 min all blood samples were centrifuged at 3,000 g for 10 min and the serum was stored at -80°C until assayed.

To measure the soluble form of human TGF-β1, 2 and 3 levels in serum, we used a commercial enzyme-linked immunosorbent assay (ELISA) kit purchased from CUSABIO. (Wuhan, China). All serum samples were obtained to determine Serum lipid profiles, glucose, high-sensitivity C-reactive protein (hs-CRP), serum creatinine (CR), Microglobulin (β2-MG), Uric acid (UA), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) by an SIEMENS ADVIA2400 automatic biochemistry analyzer; Testosterone (T) and Estradiol (E2) were determined by SIEMENS Advia Centaur CP, E2/T was used to make the resulting ratio unit free [18]. Brain natriuretic peptide precursor (NT-proBNP) measurement was performed with

Table 1. Baseline clinical characteristics of 196 cases

NYHA class	I, n (%)	17 (8.8%)
	II, n (%)	48 (24.8%)
	III, n (%)	61 (31.6%)
	IV, n (%)	66 (34.2%)
Treatment, n (%)		
ACE inhibitors		133 (69%)
Diuretics		125 (65%)
Bate-blocks		83 (43%)
Digoxin		42 (22%)
Ang II blocks		33 (17%)
Spironolactone		31 (16%)
Previous MI, n (%)		44 (23%)
Previous CABG, n (%)		35 (18%)
Diabetic, n (%)		14 (7%)
Hypertension, n (%)		44 (23%)
Atrial fibrillation, n (%)		11 (6%)
Echocardiogram data		
LVEF, %		46.77±12.01
LAD, mm		39.02±8.78
LVDD, mm		53.54±10.58
RAD, mm		38.63±7.49
RVD, mm		20.62±4.36
LVSD, mm		9.27±1.40
LVPWD, mm		9.18±1.33

Note: NYHA, New York Heart Association; MI, myocardial infarction; CABG, coronary artery bypass Grafting; DM, diabetes mellitus; ACE, angiotensin converting enzyme; LVEF, left ventricular ejection fraction; LAD, left atrial diameter; LVDD, left ventricular diastolic dimension; RAD, right atrial diameter; RVD, right ventricle diameter; LVSD, left ventricular systolic diameter; LVPWD, left ventricular posterior wall diameter.

a commercially available immunoassay on the Dimension® EXL™ with LM automatic biochemistry analyzer. All samples were determined blind to patient data and assayed in duplicate.

Cell culture and plasmid transfection

H9c2 cardiac myocytes purchased from the American Type Culture Collection (ATCC) were used in this study. Cells were cultured in Dulbecco's modified essential medium (DMEM) (Gibco, catalog number: 11995-065) with 10% fetal bovine serum (FBS), 100 units/ml penicillin, 100 μg/ml streptomycin. Cells were maintained at 37°C with 5% CO_2.

When the cells were full, washed with PBS buffer, digested by trypsin (without EDTA) and seeded into either 6-well plates (Corning, USA) with 20 thousand cells per well. When cells covered 80-90% proportion of plates, si-r-TGF-β1, si-RNA, p-EGFP-TGF-β1 plasmid and p-EGFP plasmids were transfected into H9c2 cardiac myocytes using Lipofectamine® 2000 transfection reagent according to the manufacturer's instructions. To screen out the transfected p-EGFP-TGF-β1 and p-EGFP plasmid cells, 1 mg/ml G418 was added into the medium after transfection.

RNA extraction and reverse transcription PCR

Total RNA was extracted using the Total RNA Isolation System from Promega according to the manufacturer's instructions. Reverse transcription and PCR amplification were done according to Revert Aid first strand cDNA synthesis kit manufacturer's instructions supplied by Thermofisher. Based on the information of TGF-β1, GAPDH supplied on PubMed, two pairs of gene-specific primers were designed: TGF-β1 sense 5'-GGACTACTACGCCAAAGAAG-3' and anti-sense 5'-TCAAAAGACAGCCACTCAGG-3'; GAPDH sense 5'-CAAGGTCATCCATGACAACTTTG-3' and anti-sense 5'-GTCCACCACCCTGTTGCTGTAG-3'. Reverse transcription was carried out in a reaction mixture containing 10 μl RNA, 1 μl oligo dT primer, 1 μl ddH_2O, 4 μl reaction buffer (5×), 1 μl RiboLock™ RNA reverse transcriptase inhibitor, 2 μl dNTP Mix, 1 μl RevertAid™-MuLV reverse transcriptase inhibitor. Quantitative PCR was carried out in a reaction mixture containing 2 μl cDNA, 0.5 μl reverse primer (6.25 pmol/ml), 10 μl SYBR Green PCR mix and 7.5 μl ddH_2O. The cycling conditions were as follows: denaturation step at 95°C for 3 min, followed by 35 cycles at 94°C for 30 s, 56°C for 30 s, 72°C for 30 s, and a final extension step at 72°C for 7 min. Results are normalized relative to the amount of GADPH mRNA and are plotted by the amount relative to the reference sample.

Annexin-V FITC assay

According to the manufacturer's instructions, the Annexin-V FITC apoptosis detection kit (Beyotime, Shanghai, China) was used to measure apoptosis in H9C2 cell. After various incubation periods, cells were trypsinized, and the cells and culture medium were collected. Following centrifugation at 1,000×g for 10 min, the supernatant was discarded. The cells were resuspended in 1 ml PBS, and transferred to an Eppendorf tube. Annexin-V FITC was added and

Table 2. Characteristics of controls and cases

Characteristics	Cases (n = 193)	Controls (n = 50)	*p* value
Age, years	67.54±12.84	65.17±13.26	0.249
Man, n (%)	106 (54.9%)	28 (56%)	0.833
Woman, n (%)	87 (45.1%)	22 (44%)	
BMI	22.45±2.43	22.13±1.67	0.453
Smokers, n (%)	67 (0.347)	15 (0.3)	0.434
Alcohol drinkers, n (%)	82 (0.425)	21 (0.21)	0.541
TGF-β1, ng/ml	22.31, 16.99-29.45	15.49, 12.01-19.32	<0.001
TGF-β2, pg/ml	32.95, 25.63-47.31	36.92, 31.38-42.90	0.264
TGF-β3, pg/ml	32.63, 22.61-46.55	28.83, 22.70-35.56	0.04
NT-proBNP, pg/ml	2316.00, 447.00-5930	76, 36.25-114.75	<0.001
Estradiol, pg/ml	27.11, 14.11-40.98	34.19, 26.01-64.04	0.001
Testosterone, ng/dl	169.36, 40.65-371.24	195.55, 51.37-486.92	0.21
E2/T (men)	7.80±11.35	4.00±8.14	0.058
E2/T (women)	3.43±3.16	6.32±9.89	0.037
Glucose, mM	5.68±1.07	5.85±0.88	0.09
hs-CRP, mg/l	3.60, 0.68-11.55	1.45, 0.84-2.12	0.001
CR, μmol/l	74.25, 59.00-101.67	78.50, 60.60-100.50	0.741
β2-MG, mg/l	2.67, 2.19-3.90	2.09, 1.89-2.34	0.038
UA, mM	390.00, 303.20-530.00	218.05, 215.27-227.84	<0.001
Total cholesterol, mM	4.53±0.61	4.54±0.87	0.65
Triglycerides, mM	1.12, 0.82-1.50	1.55, 1.08-2.00	0.002
HDL-c, mM	0.99±0.39	1.28±0.32	<0.001
LDL-c, mM	2.33±0.91	2.60±0.65	0.052
ALT, U/L	22.00, 13.10-35.00	19.00, 11.00-24.25	0.12
AST, U/L	25.00, 20.00-35.00	23.50, 18.00-29.05	0.15

Note: BMI, body mass index; TGF-β, transforming growth factor β; NT-proBNP, plasma N-terminal pro-B-type natriuretic peptide; E2, Estradiol; T, Testosterone; Glu, glucose; hs-CRP, high-sensitivity C-reactive protein; CR, creatinine; β2-MG, β2-microglobulin; UA, uric acid; TC, total cholesterol; TG, triglycerides; HDL-c, high density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol; ALT, alanine aminotransferase; AST, aspartate transaminase.

mixed on ice and left in the dark for 15 min. Cell apoptosis was detected by flow cytometry. Experiments were performed in triplicate.

Western blot

H9c2 were lysed in RIPA buffer (Thermo Scientific) with 1 mM PMSF. Protein samples were separated in SDS-PAGE and transferred to PVDF membranes. Nonspecific protein binding was prohibited by a blocking buffer (5% milk, 20 mM pH 7.6 Tris-HCl, 150 mM NaCl, and 0.1% Tween-20) then probed overnight at 4°C with the following primary antibodies: Cleaved caspase 3 (1:1000), Bax (1:1000), Bcl-2 (1:500), caspase 8 (1:1000), TGF-β (1:1000) (Cell Signaling Technology, Danvers, MA). After incubations with a secondary antibody for 2 h, the densitometry of immunoblots was analyzed by Odyssey imaging system (Gene). β-actin (Cell signaling technology, 4970) was used as a loading control.

Statistical analysis

SPSS 19.0 was used to analyze experimental data. Continuous data are expressed as mean ± standard deviation (S.D.). Skewed data are expressed as median and interquartile range (IQR). Statistical analysis was based on the Student's *t*-test, the Mann-Whitney U test, the chi-square test for comparison of two groups or one-way analysis of variance for multiple comparisons. The logistic regression analysis and Pearson correlation analysis were used. $P<0.05$ was considered to indicate statistical significance.

Results

Serum marker of heart failure patients

A total of 196 cases of heart failure patients and 162 healthy person were collected from Renmin Hospital of Wuhan University between 2013 Jan to 2015 Feb. And detail information of all included heart failure patients were showed in **Table 1**. Serum specimens of all included patients were collected. The comparison of outcomes between heart failure patients and healthy person including age, sex, BMI, smoking, drinking, concentration of serum TGF-β, serum NT-proBNP, serum estradiol, serum testosterone, serum E2/T ratio, glucose, hs-CRP, CR, β2-MG, UA, TC, TG, HDL-c, LDL-c, ALT and AST were showed in **Table 2**. All data were showed as Means ± SD. We found significant difference between heart failure group and control group in TGF-β1, TGF-β3, NT-pro-

Table 3. The levels of E2, T, E2/T ratio, TGF-β1 and NT-proBNP expression between different NYHA classes of heart failure

Markers		I	II	III	IV	F value	*p* value
E2 (pg/ml)	m	32.08±13.71	28.27±10.84	23.32±11.26	14.82±5.21	3.777	<0.001
	f	35.05±8.38	27.52±6.82	20.86±5.92	14.82±3.16	4.691	<0.001
T (pg/ml)	m	35.16±10.82	26.37±8.51	9.76±3.21	2.32±1.15	7.823	<0.001
	f	6.84±3.25	6.35±2.51	6.17±1.71	5.76±1.24	1.345	0.352
E2/T	m	0.93±0.35	1.16±0.49	2.41±1.18	6.13±1.31	8.642	<0.001
	f	5.62±2.05	4.19±1.93	3.25±1.42	2.56±1.11	2.746	0.032
TGF-β1 (ng/ml)		14.52±2.61	17.37±5.52	23.93±6.99	28.18±7.99	6.793	<0.001
NT-proBNP (pg/ml)		612.2±196.6	2267.4±236.1	4793.3±1171.3	7213.5±1763.4	10.071	<0.001

Note: E2, Estradiol; T, Testosterone; E2/T ratio, estradiol/testosterone ratio; TGF-β1, transforming growth factor β1; NT-proBNP, plasma N-terminal pro-B-type natriuretic peptide.

Table 4. Logistic Regression Analysis for HF

Factor	Univariate			Multivariate		
	OR	95% CI	*p* value	OR	95% CI	*p* value
Age	1.278	0.989-1.040	0.258			
Sex	0.041	0.501-1.753	0.84			
TGF-β1	24.117	0.823-0.920	<0.001	5.97	0.981-0.998	0.011
TGF-β2	0.007	0.979-1.024	0.932			
TGF-β3	5.286	0.957-0.997	0.021	1.281	0.151-1.659	0.501
NT-proBNP	16.543	0.985-0.995	<0.001	8.707	1.016-1.135	0.003
Estradiol	8.721	1.003-1.016	0.003	3.569	0.992-1.562	0.059
Testosterone	1.569	1.000-1.002	0.21			
E2/T ratio	6.807	1.130-2.361	0.009	2.829	0.16-0.161	0.093
Glucose	1.112	0.918-1.329	0.292			
hs-CRP	10.459	0.576-0.873	0.001	4.048	0.001-0.796	0.044
CR	0.579	0.989-1.005	0.447			
β2-MG	14.591	0.139-0.530	<0.001	1.548	0.924-1.018	0.213
UA	29.515	0.979-0.990	<0.001	1.343	0.609-1.136	0.239
Total cholesterol	14.829	1.339-2.454	<0.001	1.514	0.053-4.383	0.218
Triglycerides	1.525	0.899-1.599	0.217			
HDL-c	48.106	27.138-365.530	<0.001	1.006	0.427-1.316	0.316
LDL-c	35.193	0.027-0.162	<0.001	1.654	0.849-2.204	0.198
Lp (a)	21.549	0.979-0.991	<0.001	1.387	0.917-1.413	0.247
ALT	2.532	0.967-1.003	0.112			
AST	0.171	0.994-1.004	0.679			

Note: TGF-β, transforming growth factor β; NT-proBNP, plasma N-terminal pro-B-type natriuretic peptide; E2, Estradiol; T, Testosterone; E2/T ratio, estradiol/testosterone ratio; Glu, glucose; hs-CRP, high-sensitivity C-reactive protein; CR, creatinine; β2-MG, β2-microglobulin; UA, uric acid; TC,total cholesterol; TG, triglycerides; HDL-c, high density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol; ALT, alanine aminotransferase; AST, aspartate transaminase.

BNP, female estradiol, male testosterone, E2/T ratio, hs-CRP, β2-MG, UA, TG and HDL-c. Among these data, we found serum E2/T ratio, TGF-β1 and NT-proBNP expression level were positive correlated with the NYHA class of heart failure, which suggested that serum E2/T ratio, TGF-β1 and NT-proBNP served as parameters in evaluating heart failure risk showed in **Table 3**. In order to accurately prove these parameters in evaluating heart failure risk, univariate and multivariate logistic regression analysis was done and the analysis result also showed that serum E2/T ratio, TGF-β1 and NT-proBNP were independent risk factor in heart failure patients

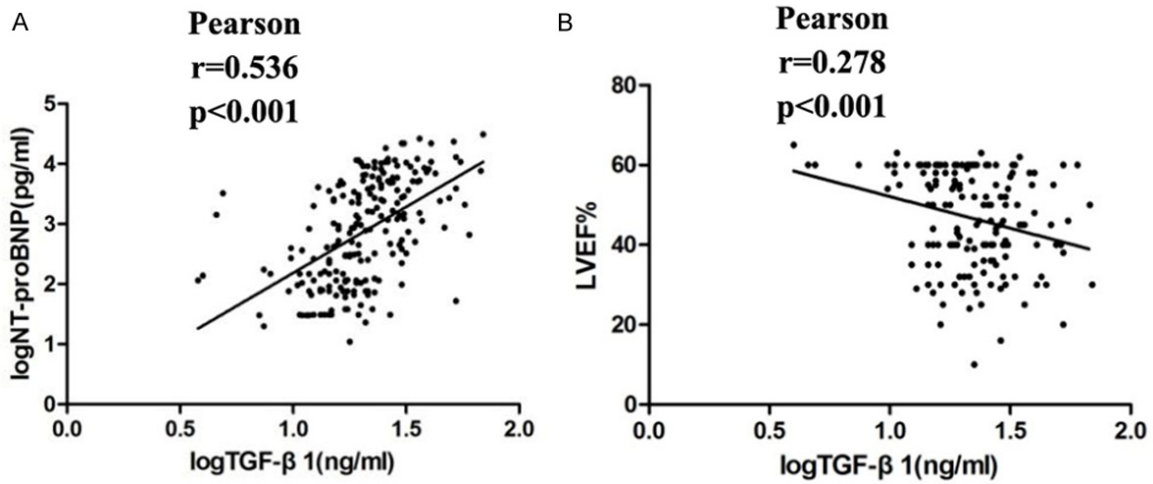

Figure 1. Correlation between serum TGF-β1 and serum NT-proBNP or LVEF% in 196 cases of heart failure patients. A. Correlation between serum TGF-β1 and serum NT-proBNP expression level; B. Correlation between serum TGF-β1 expression level and LVEF%. Statistical analysis was performed with Pearson's test.

showed in **Table 4**. As we known, NT-proBNP is golden standard which is used to assess heart function. Thus, we carried out a Pearson correlation test to analysis the correlation between serum TGF-β1 and NT-proBNP expression level in heart failure patients. And data showed a significant positive correlation between serum TGF-β1 and NT-proBNP expression level ($r = 0.536$, $P<0.001$, **Figure 1**). According to these serum level data, we speculated that E2/T ratio imbalance and high expression level of TGF-β1 may causeheart failure.

Cardiomyocyte apoptosis induced by IL-6

Pathological feature of heart failure include inflammation and myocardial fibrosis which lead cardiomyocyte apoptosis eventually. In order to further explore the mechanism of heart failure induced by serum markers, we constructed cardiomyocyte apoptosis model induced by exogenous IL-6. Using different concentration (0, 0.5, 1.0, 5.0, 10, 50 ng/ml) and treatment duration (0, 2, 4, 6, 8, 12 h) of IL-6 to treat rat H9c2 cardiomyocyte, and then tested apoptosis rate by Annexin-V-FITC flow cytometry and tested apoptosis associated protein by western blot. Annexin-V-FITC flow cytometry data showed that apoptosis rate significantly increased in IL-6 treatment groups compared with control group. And with IL-6 treatment concentration increased, apoptosis rate significantly increased (**Figure 2**). Western blot showed a significantly increased expression level of cleaved caspase 3 and caspase 8 proteins and decreased expression level of Bcl 2 with IL-6 treatment concentration increased in rat H9c2 cardiomyocyte (**Figure 2**). Treatment time gradient experiment showed that apoptosis rate significantly increased with the IL-6 (10 ng/ml) treatment time increased. Cleaved caspase 3, bax and caspase 8 expression levels increased when IL-6 treatment time increased (**Figure 3**).

TGF-β1 was upregulated in rat H9c2 cardiomyocyte induced by IL-6

To research the role of TGF-β1 played in cardiomyocyte apoptosis, we tested TGF-β1 expression level in rat H9c2 cardiomyocyte after a treatment of IL-6. The concentration gradient experiment showed that with the treatment concentration of IL-6 increased, TGF-β1 mRNA and protein expression level significantly increased (**Figure 4A**). And the time gradient experiment showed that with the treatment time of IL-6 increased, TGF-β1 mRNA and protein expression level significantly increased (**Figure 4B**). The result of this part demonstrated that TGF-β1 was unregulated in rat H9c2 cardiomyocyte induced by IL-6.

TGF-β1 can regulate the apoptosis of rat H9c2 cardiomyocyte

Since the serum data showed a significant higher expression level of TGF-β1 in heart failure patients, we suspected that TGF-β1 played an important role in heart failure. We construct-

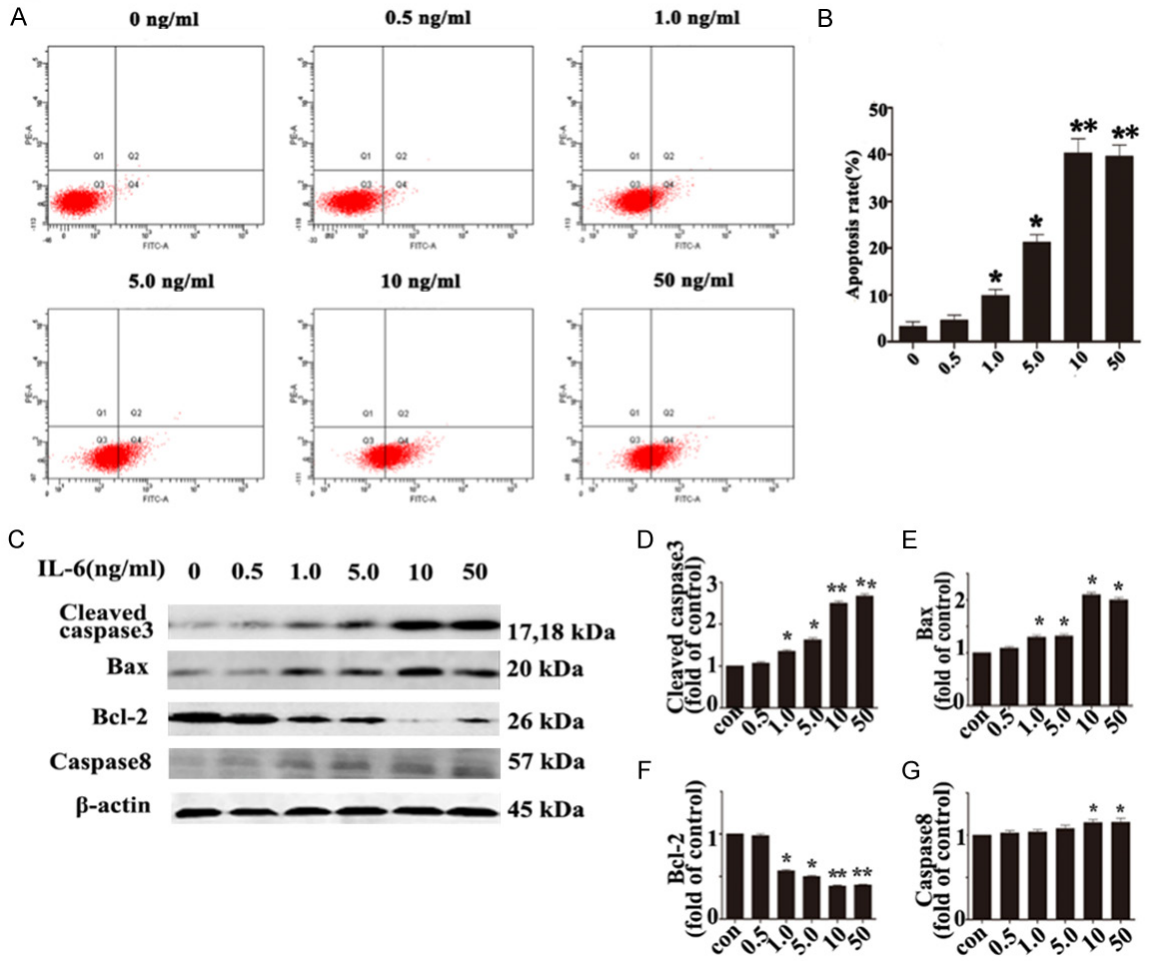

Figure 2. Apoptosis of cardiomyocyte induced by IL-6. Treatment concentrations of IL-6 were set to 0, 0.5, 1.0, 5.0, 10, 50 ng/ml. A. Flow cytometry of rat H9c2 cardiomyocyte with treatment of different concentration of IL-6. B. Percentage of apoptosis rate of cardiomyocyte with treatment of IL-6. C. Apoptosis associated proteins expression level was tested by western blot in rat H9c2 cardiomyocyte with treatment of IL-6. D. Fold changes of cleaved caspase 3 protein expression level in rat H9c2. E. Fold changes of Bax protein expression level in rat H9c2. F. Fold changes of Bcl 2 protein expression level in rat H9c2. G. Fold changes of caspase 8 protein expression level in rat H9c2. *stands for $P<0.05$, **stands for $P<0.01$.

ed rat H9c2 cardiomyocyte with upregulated and downregulated expression of TGF-β1 via TGF-β1 plasmid and siRNA transfection, and tested the expression of apoptosis associated proteins. Both of pEGFP-TGF-β1 plasmid and its control plasmid pEGFP were transfected into rat H9c2 cardiomyocyte, and then observed the expression of green fluorescent protein expression under inverted fluorescence microscope (**Figure 5A**). Compared with pEGFP plasmid transfection group, cleaved caspase 3, caspase 8 and Bax proteins and mRNA expression level significantly increased and antiapoptosis protein Bcl 2 and its mRNA expression level decreased in pEGFP-TGF-β1 plasmid transfection group in rat H9c2 cardiomyocyte (**Figure 5B**). We downregulated the expression of TGF-β1 in H9C2 cardiomyocyte by transfection of TGF-β1 siRNA and TGF-β1 inhibitor (SB431542) and tested apoptosis associated proteins expression level. The result showed that cleaved caspase 3, caspase 8 and Bax proteins and mRNA expression level significantly decreased and antiapoptosis protein Bcl 2 and its mRNA expression level increased after transfection of TGF-β1 siRNA or treatment with SB431542. This part of research results demonstrated that upregulation of TGFβ1 promoted apoptosis and downregulation of TGFβ1 inhibited apoptosis of cardiomyocyte.

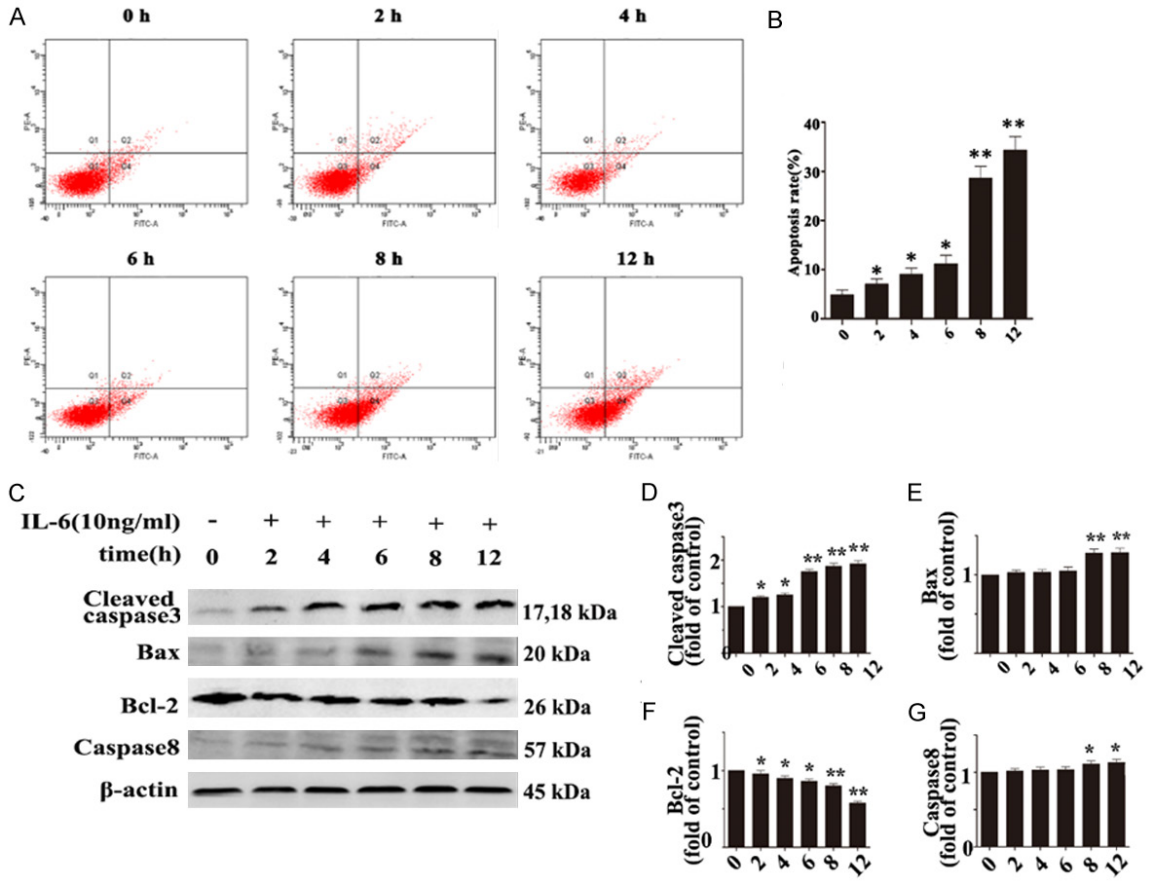

Figure 3. Apoptosis of cardiomyocyte induced by IL-6. Treatment duration of IL-6 was set to 0, 2, 4, 6, 8, 12 h. A. Flow cytometry of rat H9c2 cardiomyocyte with treatment of different duration of IL-6. B. Percentage of apoptosis rate of cardiomyocyte with treatment of IL-6. C. Apoptosis associated proteins expression level was tested by western blot in rat H9c2 cardiomyocyte with treatment of IL-6. D. Fold changes of cleaved caspase 3 protein expression level in rat H9c2. E. Fold changes of Bax protein expression level in rat H9c2. F. Fold changes of Bcl 2 protein expression level in rat H9c2. G. Fold changes of caspase 8 protein expression level in rat H9c2. *stands for $P<0.05$, **stands for $P<0.01$.

IL-6 promote the apoptosis of rat H9c2 cardiomyocyte via upregulation of TGF-β1

Our data showed that both of IL-6 and upregulation of TGF-β1 induced apoptosis of rat H9c2 cardiomyocyte and TGF-β1 can be upregulated by IL-6. Thus, we suspected that IL-6 promoted the apoptosis of rat H9c2 cardiomyocyte via upregulation of TGF-β1. To prove this suspection, reversal experimental was carried out by regulating the expression level of TGF-β1 in rat H9c2 cardiomyocyte. When rat H9c2 cardiomyocyte was treated with IL-6, TGF-β1 and apoptosis associated proteins were upregulated. And on the basis of IL-6 treatment, a following downregulation of TGF-β1 induced by TGF-β1-siRNA resulted in relative downregulation of apoptosis associated proteins (**Figure 6**).

Estradiol and testosterone can regulate the apoptosis of inflammatory cardiomyocyte induced by IL 6 and downregulation of TGF-β1 inhibited apoptosis of inflammatory cardiomyocyte

To research whether estradiol and testosterone can regulate the apoptosis of normal cardiomyocyte, we treated rat H9c2 cardiomyocyte with estradiol (50 nM) and testosterone (10 nM) and tested the apoptosis proteins. The result showed that cleaved caspase 3, caspase 8, Bax and Bcl 2 proteins expression level were not changed in rat H9c2 cardiomyocyte with treatment of estradiol, testosterone or combination of estradiol and testosterone, which reveals that estradiol and testosterone cannot regulate the apoptosis of normal cardiomyo-

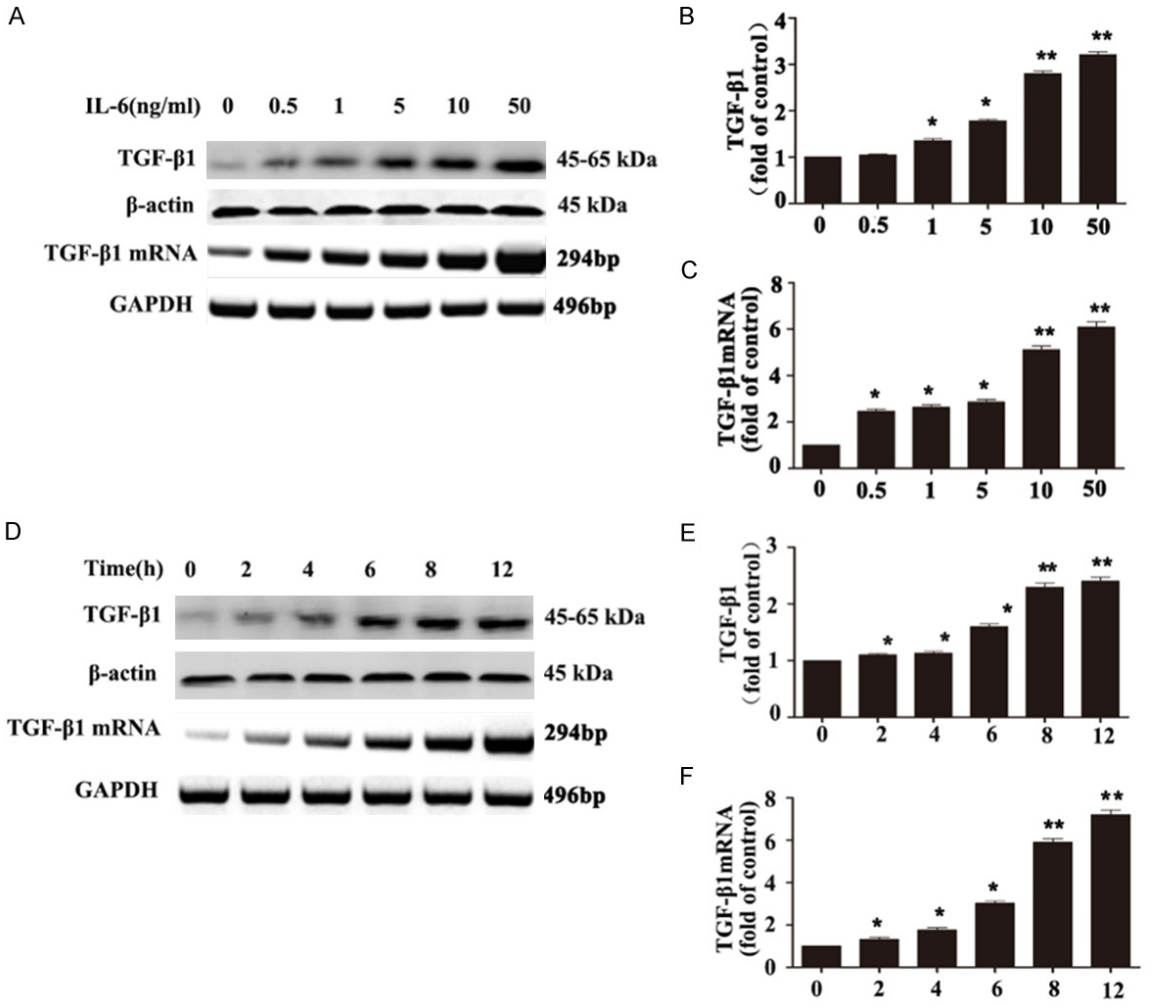

Figure 4. TGF-β1 protein and mRNA expression level in rat H9c2 cardiomyocyte with a treatment of IL-6. A. TGF-β1 protein and mRNA expression level were tested by Western blot and RT-PCR in rat H9c2 cardiomyocyte with different treatment concentration of IL-6. B. Fold changes of TGF-β1 protein expression level in rat H9c2 cardiomyocyte with different treatment concentration of IL-6. C. Fold changes of TGF-β1 mRNA expression level in rat H9c2 cardiomyocyte with different treatment concentration of IL-6. D. TGF-β1 protein and mRNA expression level were tested by western blot and RT-PCR in rat H9c2 cardiomyocyte with different treatment duration of IL-6. E. Fold changes of TGF-β1 protein expression level in rat H9C2 cardiomyocyte with different treatment duration of IL-6. F. Fold changes of TGF-β1 mRNA expression level in rat H9c2 cardiomyocyte with different treatment duration of IL-6. *stands for $P<0.05$, **stands for $P<0.01$.

cyte. However, when cardiomyocyte was treated by IL 6 and leaded to inflammatory, estradiol and testosterone can inhibit the apoptosis of inflammatory cardiomyocyte. And downregulation of TGF-β1 inhibited apoptosis of inflammatory cardiomyocyte (**Figure 7**).

Estradiol and testosterone partially reversed upregulation of TGF-β1 induced by IL-6

Compared to healthy person, we found that serum estradiol in female and serum testosterone in male were significantly lower in heart failure patients. We suspected that estradiol and testosterone played an important protective effect in cardiomyocyte and it may inhibit the apoptosis of cardiomyocyte with inflammation. To prove our suspicion, we constructed inflammatory cardiomyocyte induced by IL 6 (10 ng/ml) and then treated with estradiol (50 nM) and testosterone (10 nM). We found that on the base of upregulation of TGF-β1 induced by IL-6, treatment of estradiol alone or combination of estradiol and testosterone lead to partially decreased TGF-β1 expression level in rat H9c2 cardiomyocyte. However, treatment of testosterone alone has no effect on the expression of TGF-β1 (**Figure 8A**). It is worthwhi-

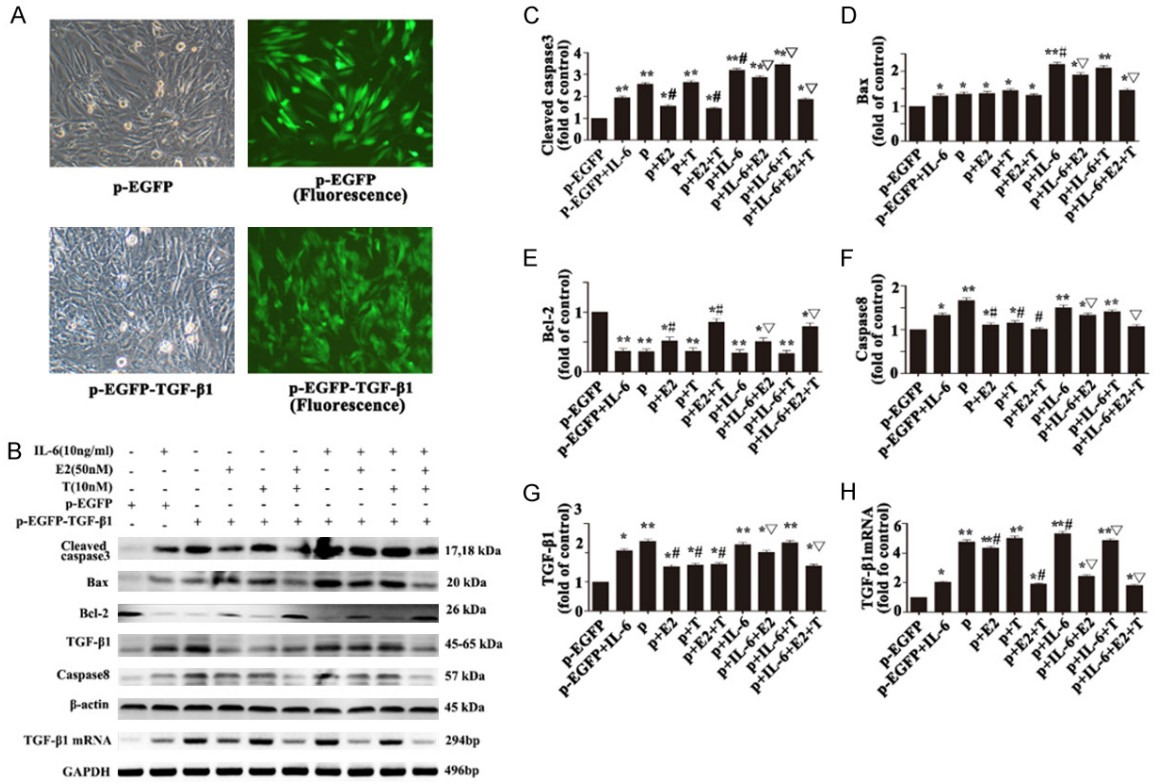

Figure 5. Upregulation of TGF-β1 promotes apoptosis of H9c2 cardiomyocyte induced by IL-6. A. Bright field and fluorescence images of H9c2 cardiomyocyte transfected by p-EGFP and p-EGFP-TGF-β1 plasmids. B. Apoptosis proteins expression level of H9c2 cardiomyocyte treated with IL-6, E2/T and transfection of p-EGFP and p-EGFP-TGF-β1 plasmids. C. Fold change of expression level of cleaved Caspase 3. D. Fold change of expression level of Bax. E. Fold change of expression level of Bcl 2. F. Fold change of expression level of Caspase 8. G. Fold change of expression level of TGF-β1 protein. H. Fold change of expression level of TGF-β1 mRNA. *stands for $P<0.05$, **stands for $P<0.01$.

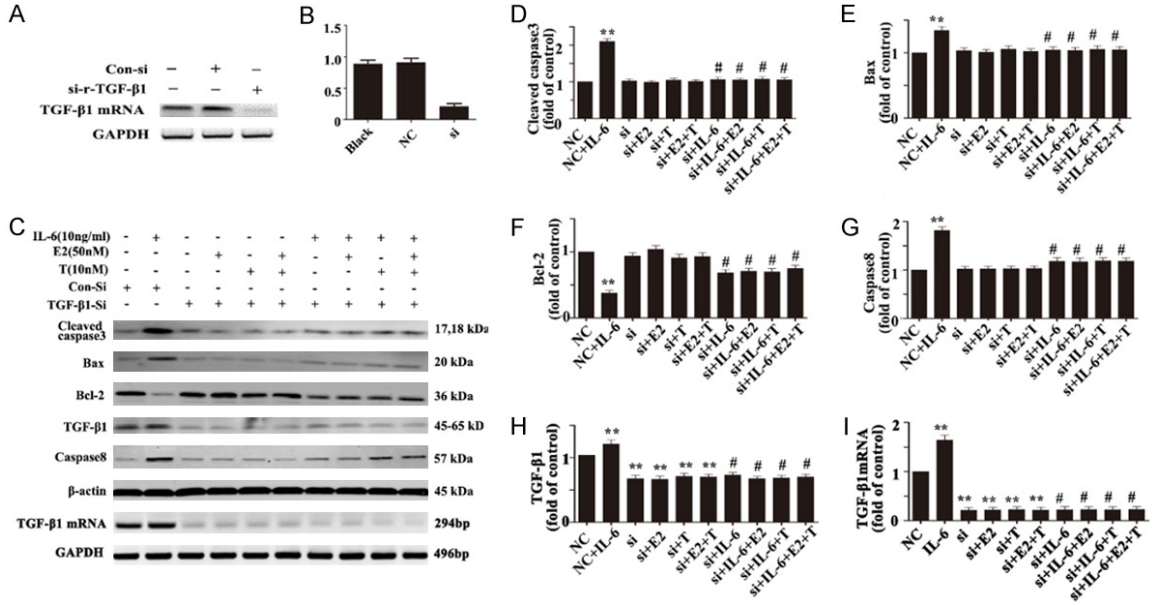

Figure 6. Downregulation of TGF-β1 promotes apoptosis of H9c2 cardiomyocyte induced by IL-6. A. TGF-β1 mRNA expression level of H9c2 cardiomyocyte with transfection of TGF-β1-siRNA and negative control siRNA. B. Fold change of expression level of TGF-β1 mRNA. C. Apoptosis proteins expression level of H9c2 cardiomyocyte treated with

IL-6, E2/T and transfection of p-EGFP and p-EGFP-TGF-β1 plasmids. D. Fold change of expression level of cleaved Caspase 3. E. Fold change of expression level of Bax. F. Fold change of expression level of Bcl 2. G. Fold change of expression level of Caspase 8. H. Fold change of expression level of TGF-β1 protein. I. Fold change of expression level of TGF-β1 mRNA. *stands for $P<0.05$, **stands for $P<0.01$.

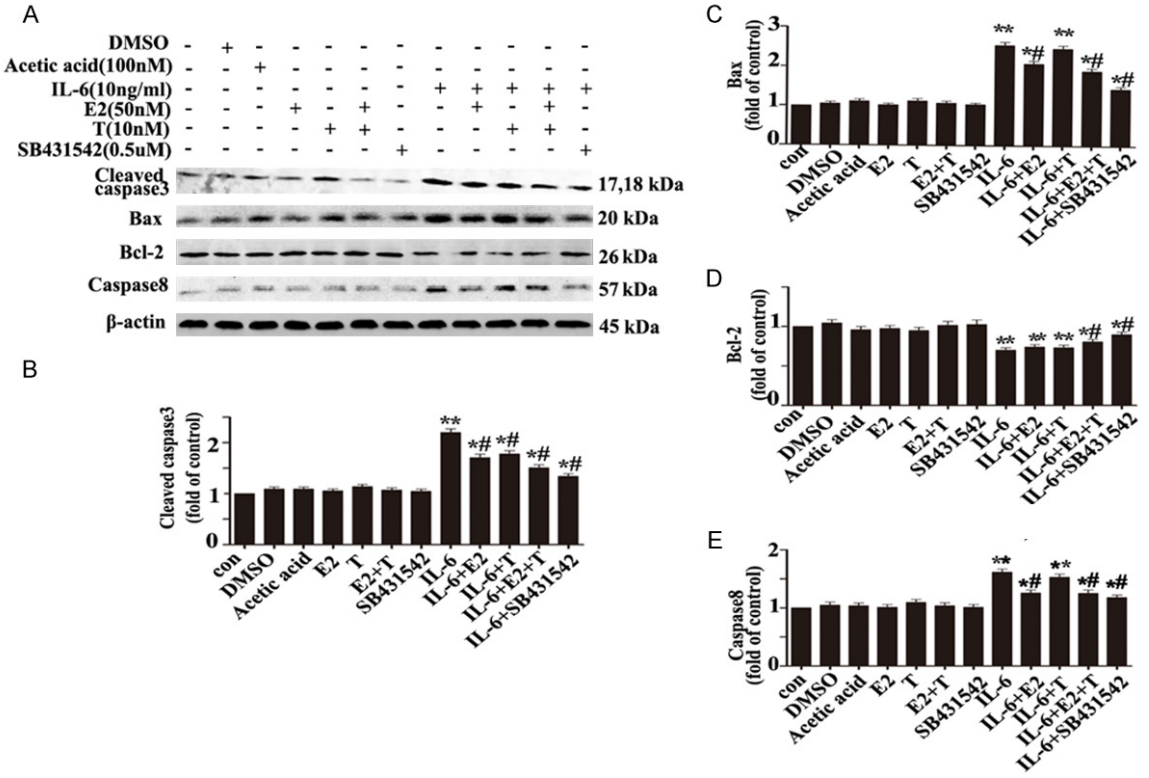

Figure 7. Estradiol and testosterone can regulate the apoptosis of inflammatory cardiomyocyte induced by IL 6 and downregulation of TGF-β1 inhibited apoptosis of inflammatory cardiomyocyte. A. Western blot result of apoptosis proteins expression of inflammatory cardiomyocyte induced by IL 6 after a treatment of estradiol and testosterone. B. Fold change of expression level of cleaved Caspase 3. C. Fold change of expression level of Bax. D. Fold change of expression level of Bcl 2. E. Fold change of expression level of Caspase 8. SB431542 is TGF-β1 inhibitor. *stands for $P<0.05$, **stands for $P<0.01$.

le pointing out that TGF-β1 expression level in the combination of estradiol and testosterone group was significantly lower compared to estradiol alone group ($P<0.05$). Since the combination of estradiol and testosterone reduced TGF-β1 expression level, we wonder the optimum E/T ratio and concentration of estradiol and testosterone. To reveal the optimum E/T ratio, we set four E/T ratios (1:1, 5:1, 10:1 and 15:1) and the corresponding estradiol concentration were 1×10^{-8} M, 5×10^{-8} M, 1×10^{-7} M and 1.5×10^{-7} M. Result showed that TGF-β1 expression level was lowest in the E/T ratio (5:1) group (**Figure 8D**). And then we fixed the E/T ratio in 5:1 and changed estradiol and testosterone treatment concentration (E: 5×10^{-10} M, 5×10^{-9} M, 5×10^{-8} M and 5×10^{-7} M; T: 1×10^{-10} M, 1×10^{-9} M, 1×10^{-8} M and 1×10^{-7} M), and tested TGF-β1 protein and mRNA expression level. Result showed that TGF-β1 expression level was lowest when estradiol and testosterone were respectively set to 5×10^{-8} M and 1×10^{-8} M (**Figure 8G**).

Estradiol and testosterone partially reversed upregulation of apoptosis protein induced by TGF-β1

We have proved that estradiol and testosterone partially reversed upregulation of TGF-β1 induced by IL-6, and estradiol and testosterone have no effect on normal cardiomyocyte. Thus we need to further our research to explore whether estradiol and testosterone have a syn-

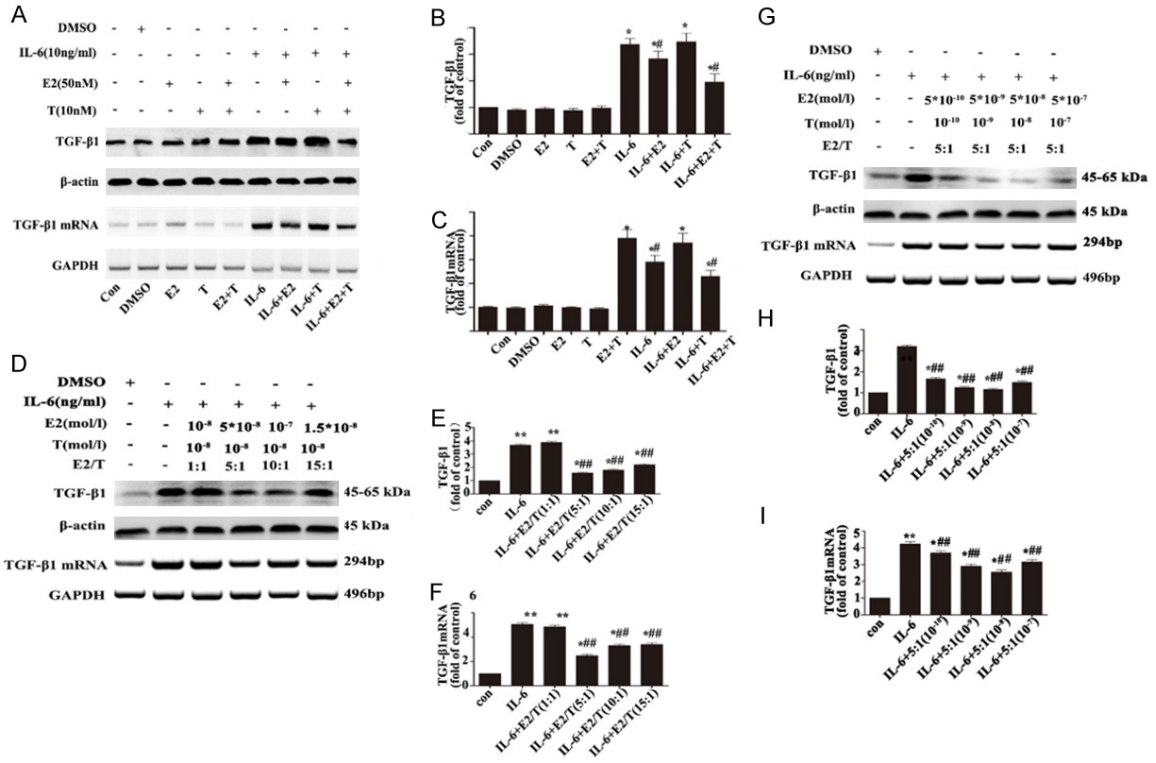

Figure 8. Estradiol and testosterone partially reversed upregulation of TGF-β1 protein and mRNA induced by IL-6. A. Upregulation of TGF-β1 protein and mRNA induced by IL-6 was downregulated by estradiol and testosterone. B. Fold change of TGF-β1 protein with the treatment of IL-6 and estradiol and testosterone. C. Fold change of TGF-β1 mRNA with the treatment of IL-6 and estradiol and testosterone. D. The influence of different concentration ratio of estradiol and testosterone on TGF-β1 protein and mRNA expression level. E. Fold change of TGF-β1 protein with the treatment of IL-6 and different concentration ratio of estradiol and testosterone. F. Fold change of TGF-β1 mRNA with the treatment of IL-6 and different concentration ratio of estradiol and testosterone. G. The influence of different concentration of estradiol and testosterone on TGF-β1 protein and mRNA expression level. H. Fold change of TGF-β1 protein with the treatment of IL-6 and different concentration of estradiol and testosterone. I. Fold change of TGF-β1 mRNA with the treatment of IL-6 and different concentration of estradiol and testosterone. *stands for $P<0.05$, **stands for $P<0.01$.

ergistic effect on TGF-β1 mediated rat cardiomyocyte apoptosis. To verify the effect of estradiol and testosterone is regulation of TGF-β1, we downregulated TGF-β1 by TGF-β1 siRNA transfection in inflammatory rat H9C2 cardiomyocyte induced by IL-6 and then treated with estradiol and testosterone, the apoptosis proteins expression level of H9C2 cardiomyocyte did not change. However, treatment with estradiol and testosterone in inflammatory cardiomyocyte partially reversed upregulation of cleaved caspase 3, caspase 8, Bax and Bcl 2 (**Figure 9**). Based on these findings, we draw a conclusion that estradiol and testosterone partially reversed upregulation of apoptosis proteins induced by TGF-β1.

Discussion

Heart failure (HF) is a growing health problem in developed nations. The cardiomyocyte plays a pivotal role in maintaining cardiac function, pathologically, cardiomyocyte apoptosis and fibrosis are early features of heart failure, replacement of lost cardiomyocytes by fibrotic material, generating a vicious cycle of further decline of cardiac function, so cardiomyocyte apoptosis is an important contributor to myocardial dysfunction and heart failure [3]. Apoptosis has been long established to be a regulated process, with limited inflammatory burden, activated by both extrinsic and intrinsic cell pathways [19]. IL-6, a well-known inflammatory cytokine, is an important factor in the pathogenesis of cardiovascular injury and cell death [20, 21].

In the present study, we first found that among a series of serum markers we detected in individuals with HF, TGF-β1 exhibited higher levels and TGF-β1 levels were positive correlated with

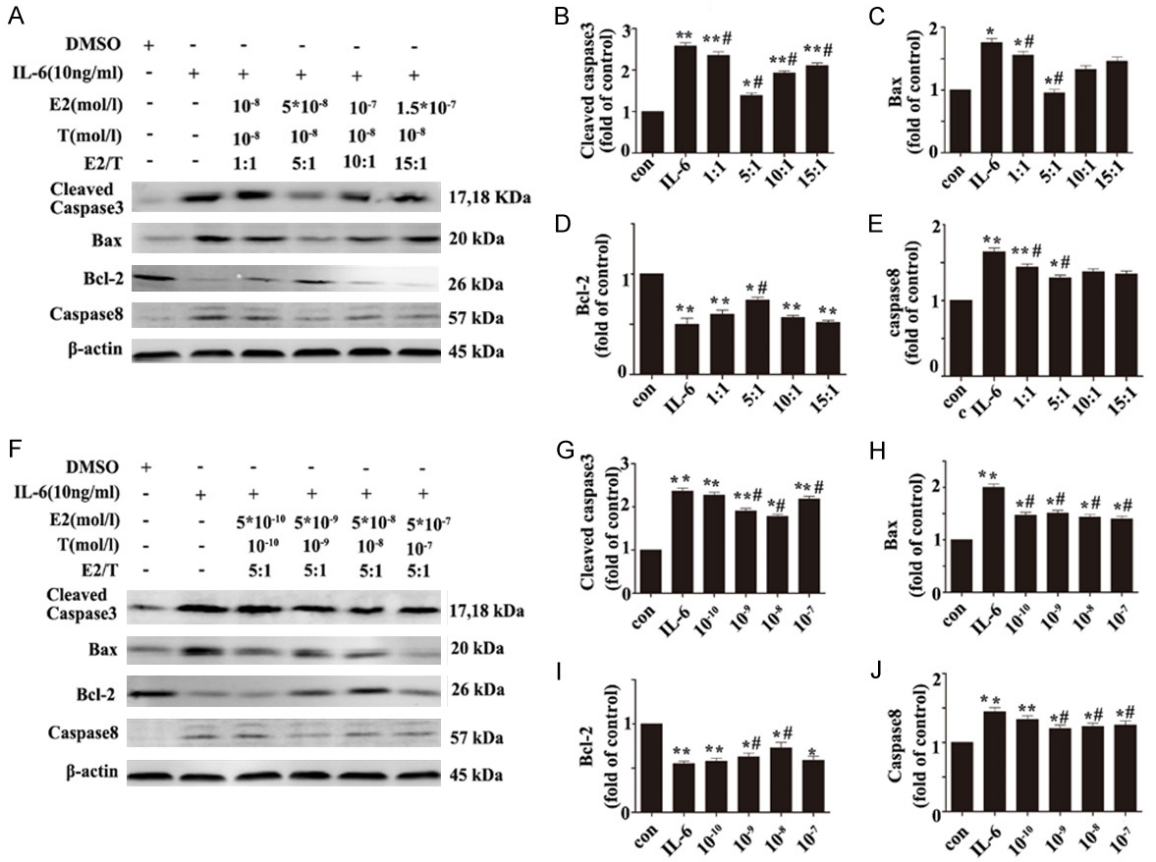

Figure 9. Estradiol and testosterone partially reversed upregulation of apoptosis protein induced by TGF-β1. A. Apoptosis associated proteins expression level with the treatment of different concentration ratio of estradiol and testosterone. B. Fold change of cleaved caspase 3 protein expression level with the treatment of different concentration ratio of estradiol and testosterone. C. Fold change of Bax protein expression level with the treatment of different concentration ratio of estradiol and testosterone. D. Fold change of Bcl 2 protein expression level with the treatment of different concentration ratio of estradiol and testosterone. E. Fold change of caspase 8 protein expression level with the treatment of different concentration ratio of estradiol and testosterone. F. Apoptosis associated proteins expression level with the treatment of different concentration of estradiol and testosterone. G. Fold change of cleaved caspase 3 protein expression level with the treatment of different concentration of estradiol and testosterone. H. Fold change of Bax protein expression level with the treatment of different concentration of estradiol and testosterone. I. Fold change of Bcl-2 protein expression level with the treatment of different concentration of estradiol and testosterone. J. Fold change of Caspase 8 protein expression level with the treatment of different concentration of estradiol and testosterone. *stands for $P<0.05$, **stands for $P<0.01$.

clinical variables reflecting heart failure severity, such as LVEF%, NT-proBNP, the NYHA class, suggesting that TGF-β1 reflect the decline of cardiac function, in addition, we found that E2 in female and T in male at the same age with heart failure were all markedly decreased. We also evaluated the E2/T ratio; our results indicate that the E2/T ratio decreased from 6.32/1 to 3.43/1 in female, while increased from 4.00/1 to 7.80/1 in male with HF. A striking observation from this study is these changes were favorably associated with the cardiac function classification, we supposed that an imbalanced E2/T ratio may have a strong association with heart failure risk participating in pathological processes. To confirm our hypothesis, we first investigated the appropriate E2/T ratio in resisting cardiomyocyte apoptosis induced by inflammatory cytokine IL-6.

TGF-β1, a polypeptide member of the transforming growth factor β superfamily of cytokines, is recognized as the most pivotal TGF-*β* isoform for the cardiovascular system, which are implicated in a wide variety of cell functions, such as cardiomyocytes, mesenchymal

and immune cells [22], critically regulating inflammation, extracellular matrix deposition, cell proliferation, differentiation and apoptosis [23]. TGF-β1 upregulation is closely associated with the development of cardiovascular diseases, including hypertension, cardiac hypertrophy [24], and cardiac fibrosis [25] leading to heart failure. Several studies have demonstrated that myocardial TGF-β1 synthesis is markedly and consistently upregulated in animal models of heart failure. Heterozygous TGF-β1 ± mice was protected from the development of aging-associated cardiac fibrosis and diastolic dysfunction [26]. In isolated cardiomyocytes of adult rat, TGF-β1 has been shown to induce apoptotic cell death [16]. This apoptosis induction could be blocked by prior transformation of cardiomyocytes with SMAD-decoy-oligonucleotides. In another study it has been shown that miR-24 prevents processing of latent TGF-β1 to its active form, indicating that reduction of TGF-β-levels may have contributed to reduced apoptosis [27]. However the relationship between TGF-β1 and heart failure remains controversial, reduced circulating levels of TGF-β1 have been reported in heart failure and correlate with increased markers of inflammation [28].

The relationship between sex hormones and HF has been widely described. Previous studies have demonstrated that associations between hormone parameters and autonomic status in men with mild systolic HF [29], in addition, not only androgens but also estrogens are important for the survival of men with HF [6, 30]. Cardiomyocytes apoptosis is an important contributor to myocardial dysfunction and heart failure, so preventing cardiomyocytes apoptosis is an effective way to protect myocardial function. Recently some published in vivo studies demonstrated that estrogens are able to prevent cardiomyocyte apoptosis [31] and Testosterone enhances estradiol's cardioprotection [32], while another research show that testosterone and 17b-estradiol have opposite effects on podocyte apoptosis that precedes glomerulosclerosis in female estrogen receptor knockout mice [33]. We previously reported that the serum E2 or T concentrations and E2/T ratio are adversely altered in postmenopausal women with CHD [18]. In addition, the combination of estradiol and testosterone at the appropriate ratio and concentration have a synergistic effect on early stage atherosclerosis [34].

Our study shows that at a certain concentration and ratio (5×10^{-8} mol/L E2 and 1×10^{-8} mol/L T) E2 and T have an optimal effect on cardiomyocyte such as downregulation of the expression levels of cleaved caspase 3, Bax, Caspase 8 and upregulation of the expression level of Bcl-2. Previous studies have indicated that apoptosis is associated with upregulation of proapoptotic Bax and downregulation of antiapoptotic Bcl-2 [35]. The results of the present study also demonstrated that levels of Bcl-2 were attenuated and Bax were increased in response to IL-6-induced apoptosis in H9c2 cells. These changes in levels of Bcl-2 family members may have a direct effect on mitochondrial membrane pore formation and consequent activation of caspase-3. These results show that there exists an appropriate E2/T ratio, which can protect cardiomyocyte through antiapoptotic mechanisms.

After defining the appropriate E2/T ratio, we compared the antiapoptotic effect of estradiol alone, testosterone alone and the defined appropriate E2/T ratio on cardiomyocyte. Our results show that the defined appropriate E2/T ratio has a stronger anti-apoptotic effect than either sex hormone (estradiol or testosterone) alone. We also explored the related antiapoptotic mechanisms.

To our knowledge, estradiol prevented TAC-Induced myocardial remodeling inhibiting the release and activation TGF-β1 in Mast Cell [12], and TGF-β1 down-regulates the level of E2 secretion in return. Wei QI et al [36] found that activation or repression of TGF-β1 expression by androgens is mediated through positive or negative AREs in its promoter, and both the positive and negative AREs are functional in the androgen-regulated transcription of the TGF-β1 promoter, which imply that androgen signaling may positively or negatively regulate TGF-β1 expression in response to various signals or under different environmental conditions. Therefore, it is essential to determine whether TGF-β1 is involved in resisting cardiomyocyte apoptosis. In the present study, we found that E2 combined with T was able to downregulate TGF-β1 expression level. It has also been postulated that the antiapoptotic effects of E2 combined with T may due to its

ability to suppress IL-6-induced apoptosis mediated by TGF-β1.

Furthermore, to determine whether E2 and T treatment restored cell apoptosis through the TGF-β1 pathway, we used TGF-β1 signaling inhibitor (SB431542) [37]. We found that after culture with the inhibitor, the cleaved caspase 3, Bax, Caspase 8 expression level decreased and the expression level of Bcl-2 increased in the IL-6 treated group. But the expression level did not return to baseline levels even when treated with the E2/T balance point. This data indicated that estradiol combined with testosterone were able to partially restore cardiomyocytes apoptotic through the TGF-β1 pathway.

To better understand the role that TGF-β1 plays in the cardioprotective effect of estradiol combined with testosterone, we transfected H9c2 cells with si-r-TGF-β1 and p-EGFP-TGF-β1. This data indicated that down-regulation of TGF-β1 through transfecting si-r-TGF-β1 contributed to several apoptosis associated proteins expression level were changed including decreased expression of cleaved-caspase 3, Bax and caspase 8 and increased expression of Bcl 2. These evidences demonstrated that apoptosis inhibition of H9c2 induced by IL-6 was realized via decreasing expression of TGF-β1 protein, at least in part. What's more, our study revealed that the pro-apoptoticeffects were dependent on the up-regulation of TGF-β1 protein expression which further suppressedby estradiol combined with testosterone in H9c2 cells and in primary cardiomyocytes exposed to IL-6.

Future studies are required to clarify further how E2 combined with T play a synergistic effect on anti-apoptosis. T mayimprove the cardioprotective effect of estrogen through activating estrogen receptors [38]. In addition, although the canonical SMAD pathway is regarded as the main pathway of TGFβ-signaling, there exist also several non-canonical pathways, so which pathway TGF-β1 involved in the protective effects of E2 combined with T on cardiomyocytes, the specific mechanism remains to be studied.

In conclusion, TGF-β1 plays an important role in antiapoptotic effects of estradiol combined with testosterone. Studies on the effect of estradiol combined with testosterone in subjects with heart failure are needed to determine whether estradiol combined with testosterone is an effective cardioprotective agent in humans with heart failure.

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Disclosure of conflict of interest

None.

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References

[1] Mathers CD, Loncar D. Projections of global mortality and burden of disease from 2002 to 2030. PLoS Med 2006; 3: e442.

[2] Braunwald E. The war against heart failure: the lancet lecture. Lancet 2015; 385: 812-824.

[3] Gurtl B, Kratky D, Guelly C, Zhang L, Gorkiewicz G, Das SK, Tamilarasan KP, Hoefler G. Apoptosis and fibrosis are early features of heart failure in an animal model of metabolic cardiomyopathy. Int J Exp Pathol 2009; 90: 338-346.

[4] Komamura K. Similarities and differences between the pathogenesis and pathophysiology of diastolic and systolic heart failure. Cardiol Res Pract 2013; 2013: 824135.

[5] Harvey PJ, O'Donnell E, Picton P, Morris BL, Notarius CF, Floras JS. After-exercise heart rate variability is attenuated in postmenopausal women and unaffected by estrogen therapy. Menopause 2016; 23: 390-5.

[6] Jankowska EA, Rozentryt P, Ponikowska B, Hartmann O, Kustrzycka-Kratochwil D, Reczuch K, Nowak J, Borodulin-Nadzieja L, Polonski L, Banasiak W, Poole-Wilson PA, Anker SD, Ponikowski P. Circulating estradiol and mortality in men with systolic chronic heart failure. JAMA 2009; 301: 1892-901.

[7] Malkin CJ, Pugh PJ, West JN, van Beek EJ, Jones TH, Channer KS. Testosterone therapy in men with moderate severity heart failure_ a double-blind placebo controlled trial. Eur Heart J 2006; 27: 57-64.

[8] Vasconsuelo A, Pronsato L, Ronda AC, Boland R, Milanesi L. Role of 17beta-estradiol and testosterone in apoptosis. Steroids 2011; 76: 1223-1231.

[9] Low YL, Li Y, Humphreys K, Thalamuthu A, Li Y, Darabi H, Wedrén S, Bonnard C, Czene K, Iles

MM, Heikkinen T, Aittomäki K, Blomqvist C, Nevanlinna H, Hall P, Liu ET, Liu J. Multi-variant pathway association analysis reveals the importance of genetic determinants of estrogen metabolism in breast and endometrial cancer susceptibility. PLoS Genet 2010; 6: e1001012.

[10] Xiao FY, Nheu L, Komesaroff P, Ling S. Testosterone protects cardiac myocytes from superoxide injury via NF-κB signalling pathways. Life Sci 2015; 133: 45-52.

[11] Stevenson S, Nelson LD, Sharpe DT, Thornton MJ. 17beta-estradiol regulates the secretion of TGF-beta by cultured human dermal fibroblasts. J Biomater Sci Polym Ed 2008; 19: 1097-1109.

[12] Li J, Jubair S, Janicki JS. Estrogen inhibits mast cell chymase release to prevent pressure overload-induced adverse cardiac remodeling. Hypertension 2015; 65: 328-334.

[13] Song K, Wang H, Krebs TL, Kim SJ, Danielpour D. Androgenic control of transforming growth factor-beta signaling in prostate epithelial cells through transcriptional suppression of transforming growth factor-beta receptor II. Cancer Res 2008; 68: 8173-8182.

[14] Edgley AJ, Krum H, Kelly DJ. Targeting fibrosis for the treatment of heart failure: a role for transforming growth factor-β. Cardiovasc Ther 2012; 30: e30-e40.

[15] Okada H, Takemura G, Kosai K, Li Y, Takahashi T, Esaki M, Yuge K, Miyata S, Maruyama R, Mikami A, Minatoguchi S, Fujiwara T, Fujiwara H. Postinfarction gene therapy against transforming growth factor-beta signal modulates infarct tissue dynamics and attenuates left ventricular remodeling and heart failure. Circulation 2005; 111: 2430-2437.

[16] Schneiders D, Heger J, Best P, Michael Piper H, Taimor G. SMAD proteins are involved in apoptosis induction in ventricular cardiomyocytes. Cardiovasc Res 2005; 67: 87-96.

[17] Huntgeburth M, Tiemann K, Shahverdyan R, Schlüter KD, Schreckenberg R, Gross ML, Mödersheim S, Caglayan E, Müller-Ehmsen J, Ghanem A, Vantler M, Zimmermann WH, Böhm M, Rosenkranz S. Transforming growth factor β1 oppositely regulates the hypertrophic and contractile response to β-adrenergic stimulation in the heart. PLoS One 2011; 6: e26628.

[18] Dai W, Li Y, Zheng H. Estradiol/Testosterone imbalance: impact on coronary heart disease risk factors in postmenopausal women. Cardiology 2012; 121: 249-254.

[19] Briasoulis A, Androulakis E, Christophides T, Tousoulis D. The role of inflammation and cell death in the pathogenesis, progression and treatment of heart failure. Heart Fail Rev 2016; 21: 169-176.

[20] Zhao L, Cheng G, Jin R, Afzal MR, Samanta A, Xuan YT, Girgis M, Elias HK, Zhu Y, Davani A, Yang Y, Chen X, Ye S, Wang OL, Chen L, Hauptman J, Vincent RJ, Dawn B. Deletion of interleukin-6 attenuates pressure overload-induced left ventricular hypertrophy and dysfunction. Circ Res 2016; 118: 1918-1929.

[21] Nasir GA, Mohsin S, Khan M, Shams S, Ali G, Khan SN, Riazuddin S. Mesenchymal stem cells and Interleukin-6_attenuate liver fibrosis in mice. J Transl Med 2013; 11: 78.

[22] Dobaczewski M, Chen W, Frangogiannis NG. Transforming growth factor (TGF)-beta signaling in cardiac remodeling. J Mol Cell Cardiol 2011; 51: 600-606.

[23] Aihara K, Ikeda Y, Yagi S, Akaike M, Matsumoto T. Transforming growth factor-beta1 as a common target molecule for development of cardiovascular diseases, renal insufficiency and metabolic syndrome. Cardiol Res Pract 2010; 2011: 175381.

[24] Schultz JE, Witt SA, Glascock BJ, Nieman ML, Reiser PJ, Nix SL, Kimball TR, Doetschman T. TGF-β1 mediates the hypertrophic cardiomyocyte growth induced by angiotensin II. J Clin Invest 2002; 109: 787-796.

[25] Leask A. Potential therapeutic targets for cardiac fibrosis: TGFbeta, angiotensin, endothelin, CCN2, and PDGF, partners in fibroblast activation. Circ Res 2010; 106: 1675-1680.

[26] Brooks WW, Conrad CH. Myocardial fibrosis in transforming growth factor beta (1) heterozygous mice. J Mol Cell Cardiol 2000; 32: 187-195.

[27] Wang J, Huang W, Xu R, Nie Y, Cao X, Meng J, Xu X, Hu S, Zheng Z. MicroRNA-24 regulates cardiac fibrosis after myocardial infarction. J Cell Mol Med 2012; 16: 2150-2160.

[28] Aukrust P, Ueland T, Lien E, Bendtzen K, Müller F, Andreassen AK, Nordøy I, Aass H, Espevik T, Simonsen S, Frøland SS, Gullestad L. Cytokine network in congestive heart failure secondary to ischemic or idiopathic dilated cardiomyopathy. Am J Cardiol 1999; 83: 376-82.

[29] Rydlewska A, Maj J, Katkowski B, Biel B, Ponikowska B, Banasiak W, Ponikowski P, Jankowska EA. Circulating testosterone and estradiol, autonomic balance and baroreflex sensitivity in middle-aged and elderly men with heart failure. Aging Male 2013; 16: 58-66.

[30] Abel EL, Kruger MM, Pandya K. Sopranos but not tenors live longer. Aging Male 2012; 15: 109-110.

[31] Liu H, Pedram A, Kim JK. Oestrogen prevents cardiomyocyte apoptosis by suppressing p38alpha-mediated activation of p53 and by down-regulating p53 inhibition on p38beta. Cardiovasc Res 2011; 89: 119-128.

[32] Liu A, Gao L, Kang S, Liu Y, Xu C, Sun H, Li D, Yan C. Testosterone enhances estradiol's cardioprotection in ovariectomized rats. J Endocrinol 2012; 212: 61-69.

[33] Doublier S, Lupia E, Catanuto P, Periera-Simon S, Xia X, Korach K, Berho M, Elliot SJ, Karl M. Testosterone and 17beta-estradiol have opposite effects on podocyte apoptosis that precedes glomerulosclerosis in female estrogen receptor knockout mice. Kidney Int 2011; 79: 404-413.

[34] Dai W, Ming W, Li Y, Zheng HY, Wei CD, Rui Z, Yan C. Synergistic effect of a physiological ratio of estradiol and testosterone in the treatment of early-stage atherosclerosis. Arch Med Res 2015; 46: 619-29.

[35] Shimabukuro M, Wang MY, Zhou YT, Newgard CB, Unger RH. Protection against lipoapoptosis of beta cells through leptin-dependent maintenance of Bcl-2 expression. Proc Natl Acad Sci U S A 1998; 95: 9558-61.

[36] Qi W, Gao S, Wang Z. Transcriptional regulation of the TGF-beta1 promoter by androgen receptor. Biochem J 2008; 416: 453-462.

[37] Davies MR, Liu X, Lee L, Laron D, Ning AY, Kim HT, Feeley BT. TGF-beta small molecule inhibitor SB431542 reduces rotator cuff muscle fibrosis and fatty infiltration by promoting fibro/adipogenic progenitor apoptosis. PLoS One 2016; 11: e0155486.

[38] Ballantyne T, Du Q, Jovanovic S, Neemo A, Holmes R, Sinha S, Jovanović A. Testosterone protects female embryonic heart H9c2 cells against severe metabolic stress by activating estrogen receptors and up-regulating IES SUR2B. Int J Biochem Cell Biol 2013; 45: 283-291.