Original Paper

Anti-Fibrosis Effect of Relaxin and Spironolactone Combined on Isoprenaline-Induced Myocardial Fibrosis in Rats via Inhibition of Endothelial–Mesenchymal Transition

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Key Words

Relaxin • Spironolactone • Cardiac fibrosis • Transforming growth factor β • Endothelial-mesenchymal transition

Abstract

Background: The effect of relaxin and spironolactone combined on myocardial fibrosis has not been reported. Thus, we investigated the effect of the combined therapy on isoprenaline-induced myocardial fibrosis and the mechanism. **Methods:** Rats were injected subcutaneously with isoprenaline to induce myocardial fibrosis and underwent subcutaneous injection with relaxin ($2 \mu\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$) and given a gavage of spironolactone ($30 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$) alone or combined for 14 days. *In vitro*, the endothelial–mesenchymal transition was induced with transforming growth factor β (TGF- β) in human umbilical vein endothelial cells (HUVECs) pretreated with relaxin, 200 ng/ml, and/or spironolactone, 1 μM . **Results:** Relaxin and spironolactone used alone or combined improved cardiac function and decreased cardiac weight indices; reduced fibrous tissue proliferation; reduced levels of type I and III collagen; decreased the expression of α -smooth muscle actin (α -SMA) and transforming growth factor- β 1 (TGF- β 1), and increased the expression of cluster of differentiation-31 (CD31) in rats with isoprenaline-induced myocardial fibrosis. *In vitro*, compared with TGF- β treatment, relaxin and spironolactone used alone or combined with TGF- β decreased cell mobility, α -SMA and vimentin levels but increased vascular endothelial cadherin (VE-cadherin) and endothelial CD31 levels. Especially, combined therapy had more remarkable effect than relaxin and spironolactone used alone both *in vitro* and *in vivo*. **Conclusion:** Relaxin and spironolactone combined affected isoprenaline-induced myocardial fibrosis in rats that the mechanism might be inhibition of the cardiac endothelial–mesenchymal transition.

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Introduction

Myocardial fibrosis (MF) is the terminal pathological manifestation of a variety of cardiovascular diseases, and its pathological process is myocardial interstitial cell hyperplasia and extracellular matrix accumulation, which is related closely to chronic heart failure. How to stop the progression of myocardial fibrosis is crucial in the treatment of chronic heart failure.

Relaxin, formerly known for its effects on reproduction and pregnancy [1], has been recently found to have an anti-fibrotic effect. Male but not female relaxin-null mice showed increased left-ventricular (LV) collagen content and collagen type I expression with aging, which was reversed by exogenous relaxin treatment [2, 3]. Relaxin treatment of primary rat atrial and ventricular fibroblasts decreased the content of collagen type I and III, the fibroblast-myofibroblast transition and cell proliferation and increased matrix metalloproteinase secretion [3]. As a novel anti-fibrotic agent, the mechanism that how Relaxin inhibits the cardiac fibrosis remains unclear.

Relaxin had a dose-dependent anti-fibrosis effect on isoprenaline (Iso)-induced myocardial fibrosis in rats. At 2.0 µg/kg/d, relaxin had obvious anti-fibrosis activity [4]. However, we still found fibrous tissue among the myocardial cells. High-dose relaxin is expensive, so a drug combination needs to be found.

The renin-angiotensin-aldosterone system (RAAS) is associated with adverse structural remodeling of the right and left ventricles. Rats showed fibroblast proliferation on days 2 - 4 after angiotensin II treatment and increased collagen volume fraction on day 14 in both ventricles [5]. In contrast, fibroblast proliferation during aldosterone infusion appeared in both ventricles at week 3 and was associated with reparative and reactive fibrosis as early as 4 weeks. Therefore, angiotensin II and aldosterone can induce myocardial fibrosis, with separate pathogenic mechanisms. Accumulating evidence shows an aldosterone escape phenomenon in up to 40% of patients with heart failure on angiotensin II treatment [6]. Spironolactone, an aldosterone receptor antagonist, may be important in blocking the RAAS. Previous research found that spironolactone could decrease myocardial fibrosis, and with its diuretic effect and low price, it now is widely used for treating chronic heart failure. However, the combined effect of relaxin and spironolactone has not been reported.

In the endothelial-mesenchymal transition (EndMT), endothelial cells lose their endothelial phenotype and gain myofibroblastic properties, as sources for fibroblasts. Fibroblasts, the activated form as muscle fibroblasts, can synthesize multiple extracellular matrix components. The EndMT plays a role in fibrosis in various tissues, including the lungs, kidneys, and the mesenteric and coronary arteries. Moreover, Zeisberg et al. [7] recently reported on the EndMT in heart fibrosis. Transforming growth factor β1 (TGF-β1), a fibrosis factor, is the strongest inducer of EndMT and is used for inducing EndMT frequently *in vivo* and *in vitro*.

We hypothesized that the molecular mechanism of relaxin and spironolactone combined could inhibit the EndMT in myocardial fibrosis.

We investigated whether relaxin and spironolactone combined could reduce myocardial fibrosis and examined the effects of the combination on EndMT in an isoprenaline-induced myocardial fibrosis rat model and in TGF-beta-treated human umbilical vein endothelial cells.

Materials and Methods

Animals and treatments

We obtained 50 male Sprague-Dawley rats (200-220 g; approximately 6 weeks old) from the Wenzhou Medical University Laboratory Animal Centre (Zhejiang province, Wenzhou City, China). The study was approved by the institutional research ethics committee of Wenzhou Medical University and in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Rats were randomly

divided into 5 groups (n=10 per group) for treatment: (1) control; (2) myocardial fibrosis model (Iso treatment); (3) Iso+spironolactone; (4) Iso+relaxin; and (5) Iso+relaxin+spironolactone. On day 1-7, rats were subcutaneously injected with Iso (I6504) (5 mg·kg body mass⁻¹, 7 days, Sigma, St. Louis, MO, USA) to induce experimental myocardial fibrosis, and rats in the control group were subcutaneously injected with normal saline. From days 1 to 14, rats were subcutaneously injected with relaxin (130-15) (2 µg·kg⁻¹·d⁻¹, Peprotech, Rocky Hill, NJ, USA) and/or given a gavage of spironolactone (H33020070) (30 mg·kg⁻¹·d⁻¹, minsheng pharma, hangzhou city, China) once a day.

Cardiac functional measurements

Rats were anaesthetised by intraperitoneal injection of 1% pentobarbital sodium (40 mg·kg⁻¹), then the left ventricle was catheterized to monitor the changes in LV mean systolic pressure (LVSP), LV end diastolic pressure (LVEDP), and the maximum rate of change in LV pressure (+dp/dtmax, -dp/dtmax) with use of a PowerLab polygraph recorder (AD Instruments, Castle Hill, Australia) through the right common carotid artery.

Left and right ventricular weight index (LVWI and RVWI, respectively)

The rats were humanely euthanized and infused with precooled saline (4°C) into the left ventricle until the heart and kidney paled. The heart was rapidly excised, rinsed and weighed, then separated into left and right ventricles and weighed. The LV and RV weight indices (LVWI and RVWI) were calculated as the LV and RV free wall mass (mg) / by body mass (g), respectively.

Haematoxylin and eosin staining of LV myocardium

Cardiac apex samples of the left ventricle were embedded in paraffin and cut into slices. Paraffin sections (4-µm thick) were stained with haematoxylin and eosin (Beyotime Institute of Biotechnology, Shanghai, China). The sections were examined by light microscopy (Nikon Corporation, Tokyo, Japan) and photomicrographs were taken at ×200 magnification.

Masson trichrome staining of the LV myocardium

Paraffin sections were stained with Masson trichrome (GenMed Scientifics Inc., Boston, MA, USA). Cardiomyocytes were stained in red and fibrous tissue in blue. The sections were examined by light microscopy and photographed at ×40 and ×200 magnification. Five non-repeating visual fields of one section, magnification ×200, were randomly selected, and myocardial collagen areas were measured by use of Image-Pro Plus (Media Cybernetics, Inc., Bethesda, MD, USA) and averaged.

Enzyme-linked immunoassay (ELISA) for type I and III collagen

A piece of the LV myocardium (100 mg) was cut into pieces, added to 1 ml phosphate buffered saline (PBS) (pH 7.4) and vortexed on ice. The homogenate was centrifuged at 3000 rpm for 20 min and the supernatant was separated. The content of type I and III collagen was measured by ELISA (Shanghai Boyun Biotech, Shanghai, China).

Cell culture and treatment

Endothelial cells isolated from human umbilical vein endothelial cells (HUVECs) were cultured in Dulbecco's modified Eagle's medium (DMEM, Lonza, USA) supplemented with 10% fetal bovine serum (FBS, Gibco BRL, USA), penicillin (100 U/ml, Gibco BRL, USA) and streptomycin (100 U/ml, Gibco BRL, USA) at 37°C in a humidified atmosphere of 5% CO₂. Cells were randomly divided into 5 groups for treatment: (1) blank control; (2) TGF-β; (3) TGF-β+spironolactone, 1µM; (4) TGF-β+relaxin, 200 ng/ml; (5) TGF-β+relaxin, 200 ng/ml, +spironolactone, 1µM. Spironolactone (1619006) was from Sigma and recombinant human TGF-β (100-21) was from Roche.

Cell Invasion detected by Transwell assay

Cell chemotaxis assay was measured by Transwell chamber assay (Corning, NY, USA) with EGM-2 containing 10% fetal bovine serum added to the lower chamber and cell (5×10⁴ cells/well) suspension with starved for 12 h and drug treatment for 24 hr added to the upper chamber for incubation at 37°C for 24 hr, then the lower chamber was observed on inverted microscopy (Nikon Corporation, Tokyo, Japan).

Incubation was terminated when cells passed into lower chamber. The inside of the upper chamber was cleaned with use of a cotton swab. The lower chamber was immersed and washed with PBS, fixed with 4% paraformaldehyde, stained with 0.1% crystal violet, washed 3 times with water, then photographed, and cell numbers were counted.

Immunofluorescence assay

For single immunofluorescence staining, HUVECs were incubated with primary antibodies (anti-CD31, ab134168; anti-VE-cadherin, ab33168; anti- α -SMA, ab7817; anti-vimentin, ab92547) (all from Abcam, 1:100) respectively at 4 °C overnight. After a washing with PBS (3 washes, 5 min per wash), sections were incubated with DyLight 488 antibodies (111-515-045) (EarthOX, San Francisco, CA, USA) for 1 hr, then incubated with 1 μ g/ml Hoechst 33342 (C1025) (Sigma, St. Louis, MO, USA) for 20 min to stain nuclei. Sections incubated with the appropriate isotype control primary antibodies and fluorescently labeled secondary antibodies were negative controls.

For dual immunofluorescence staining, sections of myocardial tissue were incubated with primary mixed anti-CD31 (sc-1505-R) (Santa Cruz Biotechnology, Inc., SantaCruz, CA, USA, 1:30) and anti- α -smooth muscle actin (α -SMA, BM0002) (Wuhanboshide, Wuhan, China, 1:100) antibodies. After a washing, a mixture of secondary antibodies (DyLight 594 AffiniPure goat anti-rabbit IgG [H+L], 111-515-045, DyLight 488 AffiniPure goat anti-mouse IgG [H+L], 107-485-142, EarthOX, San Francisco, CA, USA, 1:300) and DAPI staining solution (C1002) (Beijing Leagene Biotechnology, Beijing, China) was added and washed sequentially. Photographs were taken at $\times 200$ magnification. The results were analyzed by fluorescence microscopy (Nikon Corporation, Tokyo, Japan) and processed with Image-Pro Plus (Media Cybernetics, Inc., Bethesda, MD, USA). Cells were immunostained with antibodies against CD31 (red) and α -SMA (green) and nuclei were labelled with DAPI dihydrochloride (blue).

Western blot analysis

LV myocardium and HUVECs were homogenized in an appropriate volume of ice-cold lysis buffer respectively. After centrifugation at 12,000 g for 10 min at 4°C, the supernatant was collected. Equal amounts of samples (50 μ g) were loaded on gels for SDS-PAGE and transferred to polyvinylidene fluoride membranes (Beyotime Institute of Biotechnology, Shanghai, China), which were blocked with 5% skim milk for 1 hr and incubated with primary antibodies (anti-TGF- β 1, BS1361, Bioworld Technology, Nanjing, China, 1:1000; anti-CD31, ab134168, anti-VE-cadherin, ab33168, anti- α -SMA, ab7817, anti-vimentin, ab92547, all from Abcam, Cambridge, 1:1000) overnight at 4°C. Immunoreactive bands were detected by use of chemiluminescent horseradish peroxidase substrate (Applygen Technologies, Beijing, China), and scans were obtained by the Bio-Rad gel image analysis system (BioRad, Hercules, CA, USA) and processed with Image-Pro Plus (Media Cybernetics, Inc., Bethesda, MD, USA).

Statistical analysis

Data are expressed as mean $\pm$ SD. Statistical analysis involved use of SPSS 16.0 (SPSS Inc., Chicago, IL, USA) with Student *t* test for comparing 2 groups or one-way ANOVA. *P* < 0.05 was considered statistically significant.

Results

Effect of relaxin and spironolactone combination on cardiac function in rats

Rats subcutaneously injected with Iso to induce myocardial fibrosis showed a deterioration of cardiac functional as compared with controls (LVSP, 108.5 $\pm$ 11.7 vs. 139.8 $\pm$ 12.8 mmHg; +dp/dtmax, 2498.5 $\pm$ 733.0 vs. 9030.5 $\pm$ 1459.8 mmHg/s; -dp/dtmax, -2132.8 $\pm$ 764.4 vs. -7774.1 $\pm$ 1506.5 mmHg/s; LVEDP, 17.7 $\pm$ 7.0 vs. -5.7 $\pm$ 2.6 mmHg). Relaxin (2 μ g $\cdot$ kg $^{-1}\cdot$ d $^{-1}$) and spironolactone (30 mg $\cdot$ kg $^{-1}\cdot$ d $^{-1}$) used alone or combined for 14 days attenuated these Iso-induced changes in ventricular function, for significant increases in LVSP, +dp/dtmax and -dp/dtmax and decreased LVEDP compared to Iso treatment. Combined therapy had more remarkable effect than relaxin and spironolactone used alone (Table 1).

Table 1. Effect of relaxin (RLX) and spironolactone (SP) combined on cardiac functional index in an isoprenaline (Iso)-induced myocardial fibrosis rat model. **P<0.01 vs. control; #P<0.05, ## P<0.01 vs. Iso; +P<0.05, ++ P<0.01 vs. Iso+SP; &P<0.05, &&P<0.01 vs. Iso+RLX

Group	No.	LVSP /mmHg	LVEDP /mmHg	+dp/dtmax /mmHg/s	-dp/dtmax /mmHg/s
Control	8	139.8±12.8	-5.7±2.6	9030.5±1459.8	-7774.1±1506.5
Iso	8	108.5±11.7**	17.7±7.0**	2498.5±733.0**	-2132.8±764.4**
Iso+SP	8	125.9±7.6###	10.6±1.9##	4013.1±749.5#	-3697.2±682.4###
Iso+RLX	7	126.0±8.2##	10.2±2.3##	4383.6±1004.2#	-3827.1±609.2##
Iso+SP+RLX	7	138.0±8.7###&	3.8±3.1###&&	8608.4±2412.8###&&	-5209.1±1161.9###&&

Table 2. Effect of RLX and spironolactone combined on left and right ventricular weight in an Iso-induced myocardial fibrosis rat model. Data are mean±SD. (n=10). **P<0.01 vs control; #P<0.05, ## P<0.01 vs. Iso; ++ P<0.01 vs. Iso+SP; &P<0.05 vs. Iso+RLX

Group	LVWI /mg.g ⁻¹	RVWI /mg.g ⁻¹
Control	2.43±0.16	0.61±0.05
Iso	3.08±0.31**	0.86±0.12**
Iso+SP	2.84±0.19#	0.75±0.09##
Iso+RLX	2.76±0.21##	0.73±0.09##
Iso+SP+RLX	2.56±0.16###&	0.64±0.09###&&

Effect of relaxin and spironolactone combined on LVWI and RVWI in rats

LVWI and RVWI were significantly higher with Iso than control treatment (LVWI, 3.08±0.31 vs. 2.43±0.16 mg.g⁻¹; RVWI, 0.86±0.12 vs. 0.61±0.05 mg.g⁻¹); treatment with relaxin and spironolactone alone or combined decreased LVWI and RVWI as compared with Iso treatment. Combined therapy had lower values of LVWI and RVWI than relaxin and spironolactone used alone (Table 2).

Histopathological observations of the rat myocardium

Heart tissues from Iso-treated rats showed an irregular morphology, and were disorganized with considerable fibrous tissue hyperplasia and leukocyte infiltration compared with controls. Compared with Iso treatment, administration with relaxin and spironolactone alone or combined produced relatively neat myocardial cells, and the degree of fibrous hyperplasia was significantly reduced, and these changes were most obvious in the combined group (Fig. 1).

Effect of relaxin and spironolactone combined on myocardial collagen area in rats

Stained myocardia from Iso-treated rats showed widespread blue fibrous tissue as compared with controls. Treatment with relaxin and spironolactone alone or combined decreased fibrous tissue proliferation as compared with Iso treatment. The fibrous tissue in combined group was less than that in relaxin and spironolactone groups (P < 0.05) (Fig. 2A, B and C).

Effect of relaxin and spironolactone combined on types I and III collagen content in the rat heart

Content of type I and III collagen in cardiac tissue homogenate was higher with Iso treatment than control treatment (Types I collagen, 7.60±1.58 vs. 2.33±0.90 ng.ml⁻¹; Type III, collagen 5.64±1.26 vs. 2.74±0.86 ng.ml⁻¹. P < 0.01, respectively). Treatment with

Fig. 1. Haematoxylin and eosin staining of the left ventricular myocardium of rats (n=8). Magnification x200. Iso, isoprenaline; SP, spironolactone; RLX, relaxin.

Fig. 2. Effect of relaxin and spironolactone combined on myocardial collagen area and types I/III collagen content in rats (n=8). A) Masson trichrome staining, Magnification x40. B) Masson trichrome staining, Magnification x200. Cardiomyocytes were stained red, fibrous tissues were stained blue. C) Statistical analysis of myocardial collagen areas. D) Statistical analysis of types I/III collagen content. **P<0.01 vs. Control; #P<0.05, ##P<0.01 vs. Iso; ++P<0.01 vs. Iso+spironolactone; &P<0.05, &&P<0.01 vs. Iso+RLX.

relaxin and spironolactone alone or combined decreased the content as compared with Iso treatment (Types I collagen, 5.23 ± 1.72 , 6.20 ± 1.70 and 3.40 ± 1.39 vs. 7.60 ± 1.58 ng.ml⁻¹; Type III collagen, 3.89 ± 1.02 , 4.51 ± 0.89 and 2.85 ± 0.81 vs. 5.64 ± 1.26 ng.ml⁻¹). The content of type I and III collagen was less with relaxin and spironolactone treatment combined than these two drugs treatment alone (P < 0.05) (Fig. 2D).

Effect of relaxin and spironolactone combination on cell invasion stimulated by TGF- β in HUVECs

Cell migration ability was analyzed by Transwell chamber assay 24 h after TGF- β , relaxin or spironolactone treatment. TGF- β increased the number of migrating cells as compared with controls (503 ± 22 vs. 117 ± 24 , P < 0.01). Relaxin and spironolactone used alone or combined decreased the number of migrating cells as compared with TGF- β group (316 ± 21 , 331 ± 24 and 277 ± 13 vs. 503 ± 22). And the number of migrating cells was lower with TGF- β +relaxin+spironolactone than TGF- β +relaxin and TGF- β +spironolactone treatment

Fig. 3. Effect of RLX and spironolactone combined on cell migration ability stimulated by transforming growth factor β (TGF- β) in human umbilical vein endothelial cells (HUVECs). Magnification x200, n=8. ££P<0.01 vs. negative control; \$\$ P<0.01 vs. TGF- β ; ¥¥P<0.01 vs. TGF- β +spironolactone; \$\$P<0.01 vs. TGF- β +RLX.

(P < 0.05) (Fig. 3). Thus, treatment with relaxin and spironolactone combined could significantly inhibit HUVEC migration stimulated by TGF- β .

Effect of relaxin and spironolactone combined on EndMT in vitro and in vivo

CD31 and VE-cadherin protein were used as endothelial phenotypes and α -SMA and vimentin protein were used as myofibroblast phenotypes. The protein levels of α -SMA and vimentin were higher with TGF- β than negative control treatment, but the expressions of CD31 and VE-cadherin protein were lower (P<0.01). The levels of CD31 and VE-cadherin protein were higher but that of α -SMA and vimentin protein were lower with relaxin and spironolactone used alone or combined as compared with TGF- β treatment (P<0.05), and that, The levels of CD31 and VE-cadherin protein in combined group were higher but that of α -SMA and vimentin protein were lower than groups relaxin and spironolactone used alone (P<0.05) (Fig. 4). On dual immunofluorescence staining and western blot analyses of rat cardiac tissue, Iso decreased CD31 protein level and increased α -SMA protein level as compared with the control (P<0.01). Relaxin and spironolactone used alone or combined increased the protein level of CD31 protein and decreased that of α -SMA as compared with Iso treatment (P<0.05), and the level of CD31 protein of combined treatment was higher but that of α -SMA protein was lower than Iso+relaxin and Iso+spironolactone treatment (P<0.05) (Fig. 5).

Effect of relaxin and spironolactone combined on expression of TGF- β 1 protein in vivo

The expression of endogenous TGF- β 1 was significantly higher with Iso than control treatment (0.89 ± 0.08 vs. 0.46 ± 0.11 , P < 0.01); treatment with relaxin and spironolactone alone or combined reduced the level of TGF- β 1 as compared with Iso treatment (0.69 ± 0.16 , 0.72 ± 0.18 and 0.57 ± 0.10 vs. 0.89 ± 0.08). Similarly, the expression of TGF- β 1 in combined group was less than groups relaxin and spironolactone used alone (P < 0.05) (Fig. 5D).

Discussion

Myocardial fibrosis plays a major role in the occurrence and development of chronic heart failure, caused by overdeposition of myocardial interstitial collagen and disordered

Fig. 4. Effect of RLX and spironolactone combined on EndMT *in vitro* (n=8). A) Single immunofluorescence staining in HUVECs *in vitro*. CD31, VE-cadherin, Vimentin and α -SMA proteins are stained in green. Nuclei are stained in blue. Magnification x200. B) Effect of RLX and spironolactone combined on expression of CD31, VE-cadherin, α -SMA, and Vimentin proteins *in vitro*. $\text{£P} < 0.01$ vs. negative control; $\text{P} < 0.05$, $\text{P} < 0.01$ vs. TGF- β ; $\text{¥P} < 0.05$, $\text{¥P} < 0.01$ vs. TGF- β +spironolactone; $\text{SP} < 0.05$, $\text{SP} < 0.01$ vs. TGF- β +RLX group. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

content of all kinds of collagen, thereby altering heart function from compensated to decompensated. Thus, antifibrotic therapy is needed for treating chronic heart failure.

Relaxin (RLX) was discovered firstly by Frederick L. Hisaw in 1926 [1]. RLX is produced in the reproductive tract of many mammals during pregnancy, but recently, it has been proved that RLX exerts cardiovascular protection [8]. Male relaxin-null mice showed increased left-ventricular (LV) collagen content and collagen type I expression with aging, which was reversed by exogenous relaxin treatment [2, 3]. H2-RLX treatment of diabetic rats led to significant decreases in interstitial and total LV collagen deposition, resulting in decreased myocardial stiffness and improved LV diastolic function in streptozotocin-treated transgenic mRen-2 rats [9]. Relaxin treatment of primary rat atrial and ventricular fibroblasts decreased the content of collagen type I and III, the fibroblast-myofibroblast transition and cell proliferation and increased matrix metalloproteinase secretion [3].

Fig. 5. Effect of RLX and spironolactone combined on EndMT *in vivo* (n=8). A) Effect of RLX and spironolactone combined on single immunofluorescence staining in an Iso-induced myocardial fibrosis rat model. B) Effect of RLX and spironolactone combined on dual immunofluorescence staining. α -SMA protein is stained in green, CD31 protein is stained in red. Magnification x200. C) Statistical analysis of fluorescence signal. D) Effect of relaxin and spironolactone combined on expression of TGF- β 1 protein *in vivo*. **P<0.01 vs. Control; # P<0.05, ## P<0.01 vs. Iso; +P<0.05, ++P<0.01 vs. Iso+spironolactone; &P<0.05, &&P<0.01 vs. Iso+RLX.

Spironolactone (SP) is the most common aldosterone receptor antagonist used to pharmacologically block actions of aldosterone and for treatment of chronic heart failure. Blockade of aldosterone receptors by spironolactone, in addition to standard therapy, substantially reduces the risk of both morbidity and death among patients with severe heart failure [10]. Studies showed that spironolactone treatment reversed the increased deposition of perivascular and interstitial collagen [11, 12].

In our research, we found altered cardiac function, increased LVWI and RVWI, impaired cardiac structure and increased fibrous tissue proliferation and content of type I and III collagen in Iso-treated rats as compared with controls. Relaxin or spironolactone treatment alleviated these changes as compared with Iso group.

Our results are consistent with Zhang J et al. [4] that relaxin has obvious anti-fibrosis activity on Iso-induced myocardial fibrosis in rats at the dose of 2.0 μ g/kg/d. It has been proved that the anti-fibrosis effect of relaxin is dose-dependent [4]. The therapeutic dose of relaxin used in animal experiments ranges from a few tenths to several hundred microgram per kilogram a day. The dosage of 2.0 μ g/kg/d is a small dose with a weaker anti-fibrosis effect compared to high-dose. And we still found fibrous tissue among the myocardial cells.

Small doses of spironolactone can inhibit myocardial fibrosis and left ventricular hypertrophy without disrupt patient's electrolyte and water-retention balance [13]. Considering with the experimental method and dosage in other researches, a gavage of spironolactone ($30 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$) was given to Iso-induced rat models. Spironolactone treatment also decreased myocardial fibrosis in our experiments.

However, combined treatment with relaxin and spironolactone significantly alleviated cardiac function and interstitial fibrosis as compared with Iso and/or relaxin or spironolactone alone, which supports the better anti-fibrosis effect of the drug combination. In consideration of the vast expense of relaxin, together with the widespread use of spironolactone as a common therapeutic drug of chronic heart failure for its diuretic effect and low price, we deem the combined treatment with relaxin and spironolactone not only has clinical significance but also makes economic sense.

Cardiac fibroblasts are of crucial importance in myocardial fibrosis [14], because they can transform into myofibroblasts to secrete extracellular matrix components such as collagen, fibronectin, and laminin, thereby promoting the development of fibrosis. During the EndMT, endothelial cells lose their endothelial phenotypes and gain myofibroblastic properties, as sources for fibroblasts. Specifically, the phenotypes of endothelial cells, such as CD31 and VE-cadherin expression, are lost during the EndMT but specific properties of mesenchymal cells such as vimentin and α -SMA expression are gained [15]. TGF- β signaling pathway is closely related to cardiac fibrosis [16-20]. Now that TGF- β is generally believed as the strongest inducer of EndMT which is frequently used for inducing EndMT *in vivo* and *in vitro*. Zeisberg et al. [7] showed endothelial cells converted into fibroblasts via the EndMT in a mouse model of pressure overload and chronic allograft rejection, and *in vitro* with TGF- β 1 induction. And that, about 27%~35% of myofibroblasts were of endothelial origin through EndMT. Moreover, the expression of bone morphogenic protein 7, an inhibitor of the TGF- β signaling cascade, preserved the endothelial phenotype, significantly inhibiting the EndMT and the progression of cardiac fibrosis.

In this study, we found that vascular endothelial cells from Iso-treated rats showed decreased expression of CD31 and increased expression of α -SMA as compared with control rats; combined treatment with relaxin and spironolactone attenuated these effect. *In vitro*, TGF- β was used to induce the EndMT in HUVECS. Transwell cell migration was used to evaluate cell proliferation and migration. Compared with TGF- β and/or relaxin or spironolactone alone, relaxin and spironolactone combined decreased cell mobility. TGF- β decreased the expressions of CD31 and VE-cadherin, and induced the expressions of α -SMA and vimentin in HUVECS. However, TGF- β +spironolactone+relaxin treatment increased the expressions of α -SMA and vimentin, and reduced that of CD31 and VE-cadherin as compared with TGF- β , TGF- β +spironolactone and TGF- β +relaxin treatment. Our results suggest that relaxin and spironolactone combined enhances myofibroblastic properties of HUVECS, and increases the expression of phenotypes of endothelial cells and decreases that of mesenchymal cell both *in vivo* and *in vitro*. Thus, we speculate that relaxin and spironolactone combined improves myocardial fibrosis by inhibiting the EndMT.

In vivo, the expression of TGF- β 1 was significantly higher with Iso than control treatment; Treatment with drugs reduced the expression of TGF- β 1 as compared with Iso. Interestingly, the increase of the expression of TGF- β 1 in relaxin and spironolactone combined group was much more obviously. TGF- β is a important mediator that increases the synthesis of matrix proteins and decreases the production of matrix metalloproteinase during fibrosis [21]. Our results on the one hand indirectly support the better anti-fibrosis effect of the drug combination, on the other hand, considering TGF- β was one of the strongest inducer of EndMT, prompt TGF- β may play a pivotal role in inhibiting the EndMT of the combination therapy of relaxin and spironolactone. In recent research, relaxin was found to down-regulate TGF- β 1-induced α -smooth muscle actin and type I collagen expression. The addition of RLX to TGF- β 1-stimulated cells caused a significant decrease in Smad3 phosphorylation, a typical downstream event of TGF- β 1 receptor activation, while the treatment with a prevented this effect [22]. Studies also have suggested that spironolactone improved cardiac remodeling

by reducing proinflammatory cytokine levels and inhibiting TGF- β and R-Smads expression [23], without affecting basal collagen expression [24]. However, further studies should be done to assess the anti-fibrosis effect of diverse matching schemes and the security of the combined treatment, and to go into more detail on how TGF- β mediate the effects of relaxin and spironolactone on EndMT.

In conclusion, this study demonstrates the cardioprotective effect of relaxin and spironolactone combined on Iso-induced myocardial fibrosis in rats. The mechanism might be inhibition of the cardiac endothelial-mesenchymal transition. These 2 drugs might be used in combination in clinical practice.

Abbreviations

MF, (Myocardial fibrosis); LV, (left-ventricular); Iso, (Isoprenaline); RAAS, (renin-angiotensin-aldosterone system); EndMT, (Endothelial-mesenchymal transition); TGF- β , (transforming growth factor- β); LVSP, (Left Ventricular Systolic Pressure); LVEDP, (Left Ventricular End Diastolic Pressure); LVWI, (Left ventricular weight index); RVWI, (Right ventricular weight index); $\pm dp/dt_{max}$, (the maximum rate of change in left ventricular pressure); PBS, (phosphate buffered saline); HUVECs, (human umbilical vein endothelial cells); FBS, (fetal bovine serum); CD31, (cluster of differentiation-31); α -sma, (α -smooth muscle actin); VE-cadherin, (vascular endothelial cadherin); RLX, (relaxin); SP, (spironolactone).

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Disclosure Statement

No potential conflicts of interest were disclosed.

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