Am J Transl Res 2014;6(6):809-819
www.ajtr.org /ISSN:1943-8141/AJTR0002268

Original Article

Effects of low-dose spironolactone combined with irbesartan on cardiac hypertrophy induced by pressure overload in rats

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Received September 2, 2014; Accepted October 15, 2014; Epub November 22, 2014; Published November 30, 2014

Abstract: Background: The mineralocorticoid receptor (MR) blockade in the heart is an attractive therapeutic option for the treatment of heart failure. However, the use of MR antagonist is limited by an increased incidence of hyperkalemia owing to MR blockade in the kidney. This study was designed to evaluate and compare the effectiveness of a low, non-pressure-lowering dose of spironolactone (Sp) with that of a conventional blood pressure-lowering dose combined with irbesartan on pathological cardiac remodeling as well as serum potassium level in pressure-overload rats. Methods: The pressure-overloaded myocardial remodelling was produced by partial abdominal aortic constriction (PAAC) in rats. Four weeks after PAAC, animals were respectively treated with vehicle, irbesartan (15 mg/kg) alone, low-dose Sp (1 mg/kg) or conventional-dose of Sp (20 mg/kg) in combination with irbesartan for consecutive four weeks. Results: The result demonstrated that compared to irbesartan monotherapy, the combination of irbesartan and spironolactone both in low- and conventional-dose exhibited additional cardioprotection against PAAC-induced cardiac remodelling. Low-dose spironolactone was as effective in inhibiting cardiac hypertrophy, fibrosis and in improving diastolic function as high dose. Low-dose spironolactone did not lead to a rise in potassium serum levels, but high dose did. Conclusions: This study suggests that combined low dose of spironolactone and irbesartan may be an effective and safety therapeutic strategy for cardiac hypertrophy and heart failure.

Keywords: Spironolactone, irbesartan, myocardial remodelling, aldosterone

Introduction

Aldosterone is a steroid hormone and downstream effector of angiotensin II in the renin-angiotensin-aldosterone system (RAAS). An increasing number of studies have demonstrated that aldosterone, through the activation of the mineralocorticoid receptor (MR) is involved in processes associated with cardiac hypertrophy and heart failure [1-3]. The Randomized Aldactone Evaluation Study (RALES) has demonstrated that spironolactone (Sp), an MR antagonist, added to an angiotensin-converting enzyme inhibitor (ACEI), significantly reduces morbidity and mortality in patients with severe congestive heart failure [4]. Similarly, the EPHESUS study has shown that another MR antagonist, eplerenone, combined with ACEI or angiotensin AT_1 receptor blocker (ARB) decreases cardiovascular events and death in patients with left ventricular dysfunction following myocardial infarction [5]. Therefore, clinical practice guidelines for the treatment of heart failure recommend the use of MR antagonist as a first-line drug therapy for heart failure patients [6]. However, MR blockade carry an increased risk of complications, especially hyperkalemia may cause serious arrhythmic events [7-9]. Accordingly, in clinical practice, the benefits of MR antagonists may be reduced and they are used significantly less frequently compared with the other guideline-recommended medications [10, 11]. In particular, the use of MR antagonists for treatment in cases of hypertension and early cardiac hypertrophic remodeling remains controversial [12].

In recent years, many studies have used "low dose" of MR antagonists [6, 13, 14]. The generally accepted concept for low dose is a dose that does not affect blood pressure, although the doses of MR antagonists vary among different studies. Prior investigations have demonstrated that in transgenic rats that overexpress RAAS, low-dose (1 mg/kg) Sp provides comparable cardioprotective effects to a conventional-dose (30 mg/kg) [15]. A low-dose of Sp, added to ACEI, significantly lowers blood pressure and urinary albumin creatinine ratio in obese patients with hypertensive target organ damage [16]. These data suggest a notion that MR antagonist, at dosages below conventional natriuretic and diuretic doses, has beneficial effects on organ damage, through mechanisms independent of hemodynamic changes. In this case, using low dose of MR antagonists may avoid an elevated risk of hyperkalemia. To verify this notion, this study was designed to evaluate the effectiveness of a low, non-pressure-lowering dose of Sp with that of a conventional blood pressure-lowering dose combined with an ARB irbesartan on pathological cardiac remodeling in a pressure-overloaded cardiac hypertrophy rat model. Serum concentrations of K^+ were determined to assess the risks of hyperkalemia.

Materials and methods

Animals and treatments

Adult male Sprague-Dawley rats were provided by the Experimental Animal Center of Hebei Medical University (Shijiazhuang, China). Animal experiments were performed in adherence with the US National Institutes of Health Guidelines on the Use of Laboratory Animals and were approved by Hebei Medical University Institutional Animal Care and Use Committee. The animals were kept under standard laboratory conditions (12 h light: 12 h dark and 24 ± 3°C). Feed and water were provided ad libitum. The pressure-overloaded myocardial remodelling was produced by partial abdominal aortic constriction (PAAC) as previously described [17]. Rats were anesthetized with thiopentone sodium (35 mg/kg i.p.) and a midline incision of 1-2 cm was made in the abdomen to expose the aorta between the diaphragm and celiac artery. The 4-0 silk suture was passed beneath the abdominal aorta and was tied along with a blunted 8 gauge needle. The needle was withdrawn to leave the abdominal aorta partially constricted. The incision was sutured in layers and neosporin antibiotic powder was applied locally. Sham-operated animals underwent the same surgical procedures without PAAC. Rats were allowed to recover and were kept under observation for 8 weeks. At 4 weeks after surgery, PAAC-caused cardiac hypertrophy was confirmed by echocardiographic measurements. Then, all of rats with PAAC were divided into four groups: vehicle control, irbesartan (15 mg/kg daily) alone, low-dose Sp (1 mg/kg daily) or conventional-dose of Sp (20 mg/kg daily) in combination with irbesartan. Irbesartan and Sp were suspended in 0.5% carboxymethylcellulose (CMC) and were orally administered for 4 consecutive weeks. Control animals were treated with 0.5% CMC. The low dose of Sp was chosen based a previous report [15]. To confirm that it was a non-pressure-lowering dose, a monotherapy group with low-dose Sp alone was designed to test its effects on hemodynamic and echocardiographic parameters. At the end of drug treatment, the left and right ventricular weights were separately measured and normalized by body weight.

Echocardiographic measurements

At 4 weeks after surgery and the end of treatment, rats were subjected to transthoracic echocardiography. Rats were anesthetized with a mixture of ketamine HCl (50 mg/kg, i.p.) and xylazine HCl (5 mg/kg, i.p.). Transthoracic echocardiographic measurement was performed using a commercially available echocardiograph (Sequoia 512 with 15 MHz transducer; Acuson, New York, USA). Left ventricular end-systolic and end-diastolic dimensions as well as systolic and diastolic wall thickness were measured from the M-mode tracings at mid-papillary level. For each M-mode measurement, at least 4 consecutive cardiac cycles were sampled. Left ventricular fractional shortening were calculated as described previously [18]. Studies and analysis were performed by investigators in a blinded fashion.

Hemodynamic measurements

Animals were anesthetized with the sodium pentobarbitone solution (60 mg/kg i.p.) to obtain hemodynamic data. The right common

Table 1. Effects of low-dose spironolactone (LSp) or conventional-dose spironolactone (CSp) combined with irbesartan (Ir) on ventricular mass. Means ± SEM, n = 6 for each group

Parameters	Sham	PAAC			
		Control	Ir	CSp + Ir	LSp + Ir
LV, mg	812 ± 30	997 ± 50**	922 ± 30	912 ± 63	907 ± 40
LV/BW, mg/g	2.26 ± 0.05	3.06 ± 0.10**	2.90 ± 0.10#	2.60 ± 0.07##,$	2.61 ± 0.05##,$
RV, mg	272 ± 19	338 ± 10**	259 ± 24	325 ± 12	300 ± 12
RV/BW, mg/g	0.76 ± 0.06	1.04 ± 0.05*	0.84 ± 0.05#	0.95 ± 0.04	0.87 ± 0.04#

LV, left ventricular; BW, body weight; RV, right ventricular; PAAC, partial abdominal aortic constriction; *p < 0.05, **p < 0.01 vs Sham; #p < 0.05, ##p < 0.01 vs Control; $p < 0.05 vs Ir.

Table 2. Echocardiographic parameters at 4 weeks after partial abdominal aortic constriction

Group	n	IVs, mm	LVPW, mm	LVs, mm	LVd, mm	LVEF, %	LVFS, %
Sham	10	1.72 ± 0.07	1.73 ± 0.17	3.95 ± 0.07	6.02 ± 0.02	58.4 ± 2.3	34.0 ± 4.1
PAAC	40	2.05 ± 0.07**	2.02 ± 0.13*	4.08 ± 0.07	5.50 ± 0.13*	48.4 ± 4.2	30 ± 5.9

IVs, ventricular septal thickness; LVPW, left ventricular posterior wall thickness; LVs, left ventricular end systolic dimension; LVd, left ventricular end diastolic dimension; LVEF, left ventricular ejection fraction; LVFS, left ventricular fractional shortening. *p < 0.05, **p < 0.01 vs Sham.

carotid artery was catheterized with a fluid-filled polyethylene catheter connected to a pressure transducer (TSD 104A, BioPAC, USA) and a digital system (MP100, BioPAC, USA). The systolic (SBP) and diastolic (DBP) blood pressure were initially measured in the aorta. The catheter was then advanced into the left ventricular cavity to record systolic (LVSP) and end-diastolic (LVEDP) pressures. The maximum rate of pressure rise and fall (dP/dt_{max} and dP/dt_{min}, respectively) was obtained electronically, and all records were obtained at 2 kHz. According to a literature [19], the normalized parameter dP/dt_{min}/LVSP was used since dP/dt_{min} was related to LVSP.

Morphological assessments

Part of heart was maintained in a fixative solution (4% paraformaldehyde). Tissue samples were excised from similar sections of the lateral wall of the left ventricular and processed for light microscopy. Specimens were embedded in paraffin wax, serially sectioned (5 μm thickness), and stained with hematoxylin and eosin to analyze cardiomyocyte diameter. Masson staining allowed evaluation of interstitial fibrosis. Images were acquired using an Olympus BX50 microscope. To quantify cardiomyocyte size, digital images were captured on cardiomyocytes in cross-section, and the cross-sectional area (CSA) was determined with the aid of image-analysis software (Image-Pro® Plus, Media Cybernetics, Inc. USA). Twenty cells from two distinct sites were measured per sample, and only cardiomyocytes with a well-defined cellular membrane and visible nucleus were measured. The average size of all measured cardiomyocytes within a sample was determined and expressed in units of cross-sectional area (in μm^2). The quantification of fibrosis was analyzed as a percentage of fibrotic area to total area using image-analysis software.

Reverse transcriptase PCR

Total RNA was prepared using TRIzol reagent (Qiangen, Hilden, Germany) from LV tissue. Complementary DNAs were synthesizes by standard techniques using a QuantiTect Reverse Transcription Kit (Qiangen, Hilden, Germany). Real-time PCR was performed, recorded, and analyzed using a Line-Gene K Real Time PCR System (Bioer Techology CO., LTD, Hangzhou, China) with SYBR Green I detection. The cDNA was amplified using a TransStart Top Green qPCR SuperMix kit (TransGen Biotech, Beijing, China) with specific primers (ANP, forward: 5'-GGCTCCTTCTCCATCACCAA-3'; reverse: 5'-CGAGAGCACCTCCATCTCTC-3'; β-MHC: forward: 5'-ATGCTGGCACCGTGGACT-3'; reverse: 5'-TTAGGAGCTTGAGGGAGGACTT-3'; transforming growth factor-β1 (TGF-β1), forward: 5'-AAGAAGTCACCCGCGTGCTA-3; reverse: 5'-TGTGTGATGTCTTTGGTTTTGTCA-3; procollagen I, forward: 5'-TGCCGTGACCTCAAGATGTG-3'; rever-

Table 3. Effects of low-dose spironolactone (LSp) or conventional-dose spironolactone (CSp) combined with irbesartan (Ir) on echocardiographic parameters. Means ± SEM, n = 6 for each group

Parameters	Sham	PAAC			
		Control	Ir	CSp + Ir	LSp + Ir
IVs, mm	1.75 ± 0.03	2.07 ± 0.05**	1.88 ± 0.06	1.78 ± 0.07#	1.85 ± 0.07#
LVPW, mm	1.75 ± 0.05	2.08 ± 0.08*	1.95 ± 0.06	1.76 ± 0.07#	1.78 ± 0.04#
LVs, mm	4.35 ± 0.20	4.08 ± 0.20	4.37 ± 0.20	4.04 ± 0.20	4.22 ± 0.20
LVd, mm	6.33 ± 0.16	5.53 ± 0.14**	5.86 ± 0.15	6.23 ± 0.17##	6.29 ± 0.16##
LVEF, %	59.4 ± 3.1	47.9 ± 7.0	48.0 ± 3.7	54.6 ± 3.0	53.9 ± 4.2
LVFS, %	36.0 ± 2.3	34.6 ± 4.7	30.0 ± 2.9	35.7 ± 2.5	33.5 ± 2.7

IVs, ventricular septal thickness; LVPW, left ventricular posterior wall thickness; LVs, left ventricular end systolic dimension; LVd, left ventricular end diastolic dimension; LVEF, left ventricular ejection fraction; LVFS, left ventricular fractional shortening. *$p < 0.05$, **$p < 0.01$ vs Sham; #$p < 0.05$, ##$p < 0.01$ vs Control.

se: 5'-CACAAGCGTGCTGTAGGTGA-3'; β-actin: forward: 5'-GAGGCTCTCTTCCAGCCTTC-3'; reverse: 5'-AGGGTGTAAAACGCAGCTCA-3'). To confirm the specificity of the PCR, products from each primer pair were subjected to a melting curve analysis.

Serum Na^+ and K^+ analysis

A blood sample was collected from the right carotid artery after hemodynamic measurements. The blood was centrifuged at 3000g for 15 min to separate the serum that were kept at -80°C for Na^+ and K^+ analyses. Serum Na^+ and K^+ concentrations were determined according to standard methods using diagnostic kits from BioSystems S.A. (Barcelona, Spain) by CHEMIX-180 automatic biochemistry analyzer (Sysmex).

Statistical analysis

All results were expressed as mean ± SEM. The data obtained from various groups were statistically analyzed using one-way ANOVA followed by Tukey or Dunnett's T3 test as appropriate. A p value < 0.05 was considered to be statistically significant.

Results

The effects of low- or conventional-dose Sp in combination with irbesartan on ventricular weight

As shown in **Table 1**, compared with the sham operation group, crude left and right ventricular weights, and normalized weights with body weight (LV/BW and RV/BW) were significantly higher in the control animals ($p < 0.01$), reflecting the presence of cardiac hypertrophy in PAAC rats. Irbesartan monotherapy resulted in a significant decrease both in LV/BW and RV/BW ($p < 0.05$). The administration of conventional-dose Sp in combination with irbesartan significantly decreased LV/BW ($p < 0.01$). The low-dose Sp had similar effect to conventional-dose. Moreover, the combination therapy both in low- and conventional-dose Sp induced more decrease in LV/BW than irbesartan monotherapy.

The effects of low- or conventional-dose Sp in combination with irbesartan on the echocardiographic parameters

At 4 weeks after abdominal aortic stenosis surgery, animals exhibited significantly enhancement both in interventricular septal thickness (IVs) and left ventricular posterior wall thickness (LVPW), and reduction in left ventricular internal diameter (LVd) ($p < 0.05$ or $p < 0.01$), without significant changes in left ventricular ejection fraction (LVEF) and left ventricular fractional shortening (LVFS) (**Table 2**). These findings indicated that partial abdominal aortic constriction succeeded in causing cardiac hypertrophy, although systolic function remains normal in the compensatory stage. After 4 weeks of drug treatment, all of rats in different groups exhibited similar LVEF and LVFS values (all $p > 0.05$), suggesting that the overall systolic functions of the heart remained normal (**Table 3**). Low-dose Sp alone did not affect echocardiographic parameters (Supplementary Table 1). Whereas, irbesartan monotherapy showed a trend to improve parameters including IVs, LVPW, and LVd. The administration of low- or conventional-dose Sp in combination

Table 4. Effects of low-dose spironolactone (LSp) or conventional-dose spironolactone (CSp) combined with irbesartan (Ir) on hemodynamic parameters. Means ± SEM, n = 6 for each group

Parameters	Sham	PAAC			
		Control	Ir	CSp + Ir	LSp + Ir
SBP, mmHg	134 ± 7	183 ± 3**	165 ± 10	144 ± 5$^{\#\#,\$\$}$	148 ± 10$^{\#}$
DBP, mmHg	88 ± 6	123 ± 6**	105 ± 6	92 ± 6$^{\#}$	94 ± 6$^{\#}$
LVSP, mmHg	148 ± 5	180 ± 6**	169 ± 10	149 ± 5$^{\#\#,\$}$	152 ± 3$^{\#\#}$
LVEDP, mmHg	3.55 ± 0.49	9.99 ± 0.61**	8.02 ± 0.36$^{\#}$	4.43 ± 0.52$^{\#\#,\$}$	5.98 ± 0.67$^{\#\#}$
dP/dt_{max}, mmHg/s	8625 ± 652	8452 ± 746	7097 ± 836	7883 ± 211	7889 ± 685
dP/dt_{min}, mmHg/s	6970 ± 548	4479 ± 565*	6077 ± 520	6881 ± 680$^{\#}$	6129 ± 236$^{\#}$
dP/dt_{min} /LVSP, s^{-1}	48 ± 3	25 ± 3**	36 ± 4	42 ± 3$^{\#\#}$	45 ± 3$^{\#\#}$

SBP, systolic blood pressure; DBP, diastolic blood pressure; LVSP, left ventricular systolic pressure, LVEDP, left ventricular end diastolic pressure; dP/dt_{max} and dP/dt_{min}, the maximum rate of pressure rise and fall. $^{*}p < 0.05$, $^{**}p < 0.01$ vs Sham; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs Control; $^{\$}p < 0.05$ vs Ir.

with irbesartan could decrease IVs and LVPW and increase LVd ($p < 0.05$) (**Table 3**). There was no significant difference between these two combination regimens. These findings suggested that both low- and conventional-dose Sp in combination with irbesartan attenuated PAAC-induced cardiac hypertrophy.

The effects of low- or conventional-dose Sp in combination with irbesartan on the hemodynamic parameters

As shown in **Table 4**, compared with the sham operation group, the vehicle control animals had higher SBP, DBP, LVSP, and LVEDP, but lower dP/dt_{min}/LVSP (all $p < 0.05$), indicating of PAAC-induced high blood pressure and left ventricular diastolic dysfunction. The parameter dP/dt_{max} was no changes between the control and sham operation animals, reflecting systolic function was maintained in this animal model. These findings were consistent with the aforementioned echocardiography results. Low-dose Sp alone did not affect hemodynamic parameters (Supplementary Table 2). Irbesartan monotherapy displayed a tendency to lower SBP DBP and LVSP, and significantly decreased LVEDP ($p < 0.05$), indicating that irbesartan could improve the cardiac diastolic function. The combination of irbesartan with Sp, either at low- or conventional-dose remarkably lowered SBP, DBP and LVSP, ameliorated PAAC-induced increases in LVEDP, and decrease dP/dt_{min}/LVSP. In combination regimens, the low-dose Sp produced similar effects to conventional-dose in lowering blood pressure and improving left ventricular diastolic function.

The effects of low- or conventional-dose Sp in combination with irbesartan on myocardial pathological changes

Hematoxylin and eosin (HE) staining showed a higher cardiomyocyte cross-sectional area ($p < 0.01$) in control animals, compared with sham operation rats. The administration of irbesartan alone tended to decrease cardiomyocyte cross-sectional area. Whereas, the combination of irbesartan with low- or conventional-dose Sp significantly decreased the area (both $p < 0.05$) (**Figure 1**), indicating that the combination treatment alleviated cardiomyocyte hypertrophy. In this regard, low- and conventional-dose Sp achieved similar effects.

Masson staining demonstrated that PAAC induced a robust myocardial interstitial fibrosis with increased amounts of surrounding matrix. Irbesartan alone reduced myocardial fibrotic area. The administration of irbesartan in combination with low- or conventional-dose Sp produced further improvement in myocardial interstitial fibrosis compared to irbesartan monotherapy ($p < 0.05$) (**Figure 2**).

The effects of low- or conventional-dose Sp in combination with irbesartan on cardiac hypertrophy and fibrosis-related biomarkers

As shown in **Figure 3**, the mRNA expressions of natriuretic peptide (ANP), β-myosin heavy chain (β-MHC), and procollagen I and transforming growth factor-β1 (TGF-β1) were significantly elevated in rat hearts at 8 weeks after abdominal aortic stenosis surgery (all $p < 0.01$). Irbesartan monotherapy tended to reduce all

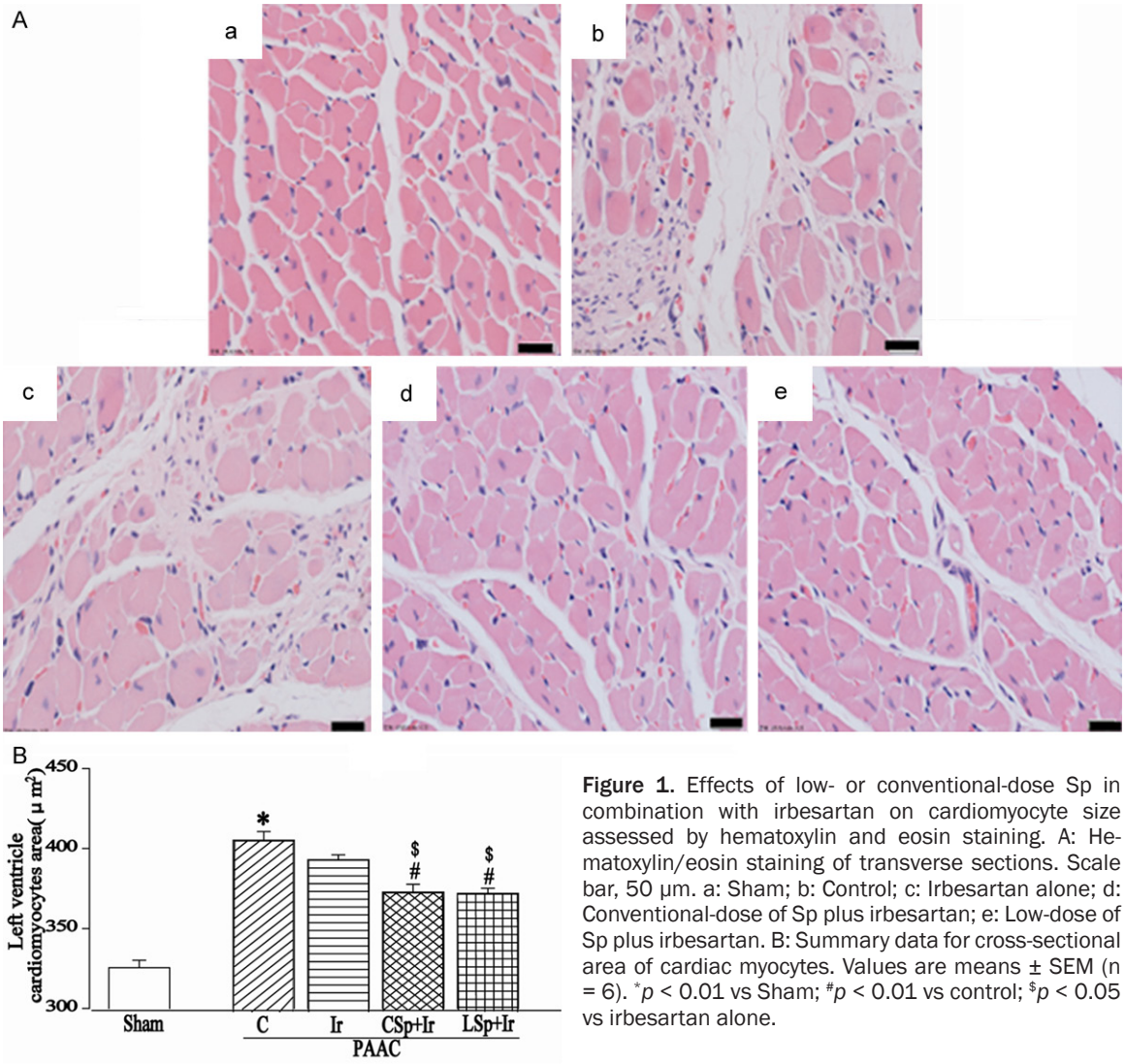

Figure 1. Effects of low- or conventional-dose Sp in combination with irbesartan on cardiomyocyte size assessed by hematoxylin and eosin staining. A: Hematoxylin/eosin staining of transverse sections. Scale bar, 50 μm. a: Sham; b: Control; c: Irbesartan alone; d: Conventional-dose of Sp plus irbesartan; e: Low-dose of Sp plus irbesartan. B: Summary data for cross-sectional area of cardiac myocytes. Values are means ± SEM (n = 6). $^{*}p < 0.01$ vs Sham; $^{\#}p < 0.01$ vs control; $^{\$}p < 0.05$ vs irbesartan alone.

these parameters, but the difference did not reach statistical significance. The administration of irbesartan in combination with low- or conventional-dose Sp significantly decreased all of mRNA expressions (both $p < 0.05$) (**Figure 3**). Low-dose of Sp had similar efficacy as conventional-dose.

The effects of low- or conventional-dose Sp in combination with irbesartan on serum potassium and sodium levels

The most important and common adverse reaction to the use of aldosterone antagonists is hyperkalemia. To compare the effects of regimens involving low- or conventional-dose Sp in combination with irbesartan, we further observed the effects of treatment on the serum potassium and sodium levels. As shown in **Table 5**, conventional-dose Sp in combination with irbesartan significantly increased serum potassium concentrations, whereas, low-dose Sp added to irbesartan exhibited no significant change in serum potassium concentrations. Neither of the combination regimen groups significantly differed from the control group with respect to serum sodium levels.

Discussion

Cardiac hypertrophy is regarded as an adaptive response to increased workload. However, clinical and experimental observations have shown that the degree of cardiac hypertrophy is not proportional to workload [20]. Over activation of the renin-angiotensin system plays a pivotal role in hypertrophic remodeling by modulating the cardiovascular growth response. Recent

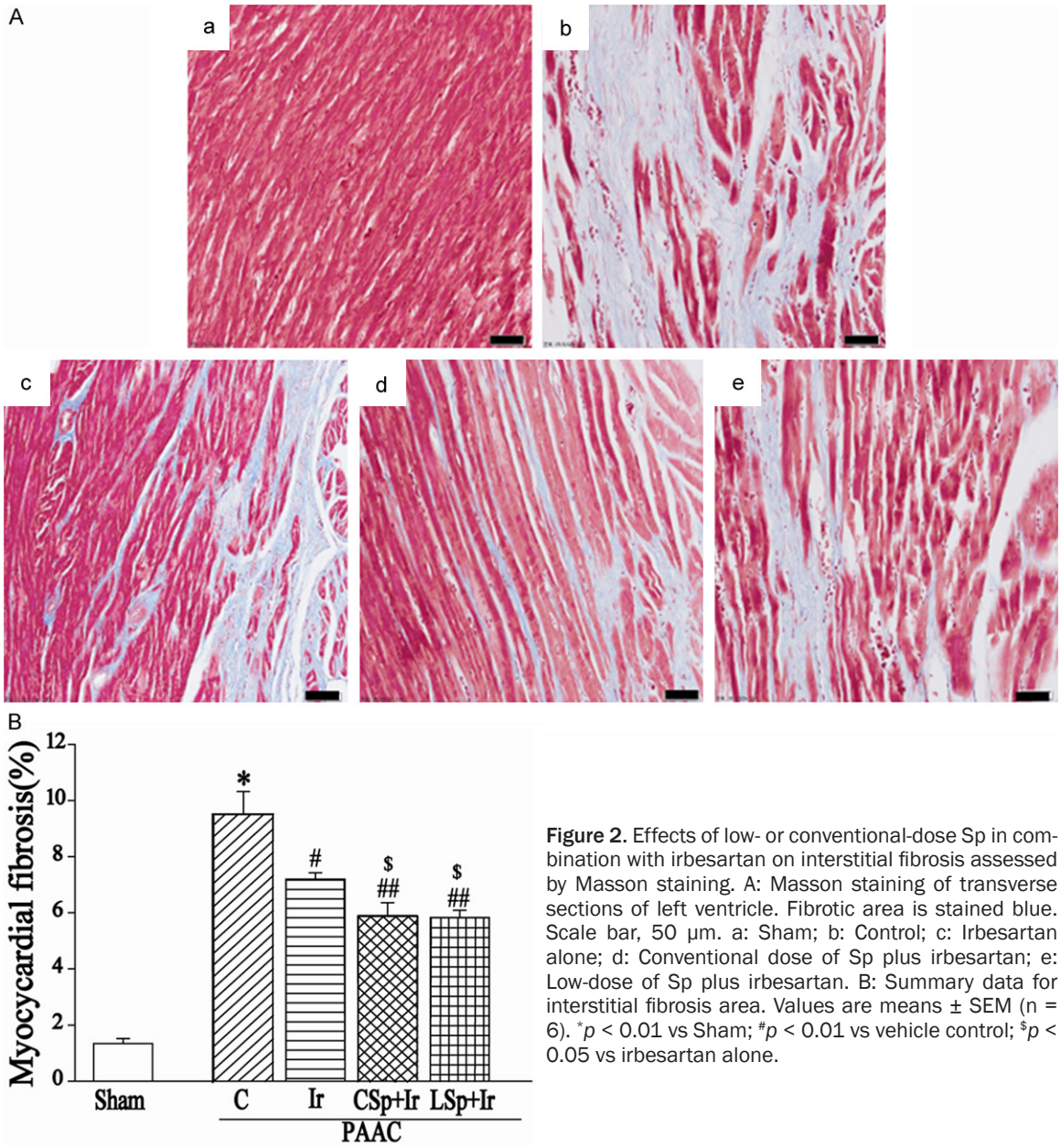

Figure 2. Effects of low- or conventional-dose Sp in combination with irbesartan on interstitial fibrosis assessed by Masson staining. A: Masson staining of transverse sections of left ventricle. Fibrotic area is stained blue. Scale bar, 50 μm. a: Sham; b: Control; c: Irbesartan alone; d: Conventional dose of Sp plus irbesartan; e: Low-dose of Sp plus irbesartan. B: Summary data for interstitial fibrosis area. Values are means ± SEM (n = 6). $^{*}p < 0.01$ vs Sham; $^{\#}p < 0.01$ vs vehicle control; $^{\$}p < 0.05$ vs irbesartan alone.

years, emerging experimental evidence suggests that cardiomyocyte MR signaling also contributes to the development of cardiac hypertrophy and fibrosis [21-23]. The study demonstrated that PAAC produced significant cardiac hypertrophy and fibrosis in rats along with left ventricular diastolic dysfunction. Consistent with previous studies [24-26], AT_1 blocker irbesartan monotherapy showed an anti-hypertrophic effect, which manifested by significant decrease in normalized left and right ventricular weights, left ventricular end diastolic pressure, and myocardial fibrotic area. Moreover, the combination of irbesartan and MR antagonist spironolactone exhibited marked protective effects on PAAC-induced cardiac remodeling, which was evidenced by a decrease in normalized left ventricular weight, left ventricular wall and septal thickness, cardiomyocyte cross-sectional area, and reduced myocardial fibrotic area. This was further supported by the improvement of hemodynamic function and the reduction in the mRNA expressions of cardiac hypertrophy- and fibrosis-associated biomarkers including natriuretic peptide, β-myosin heavy chain, procollagen I and transforming

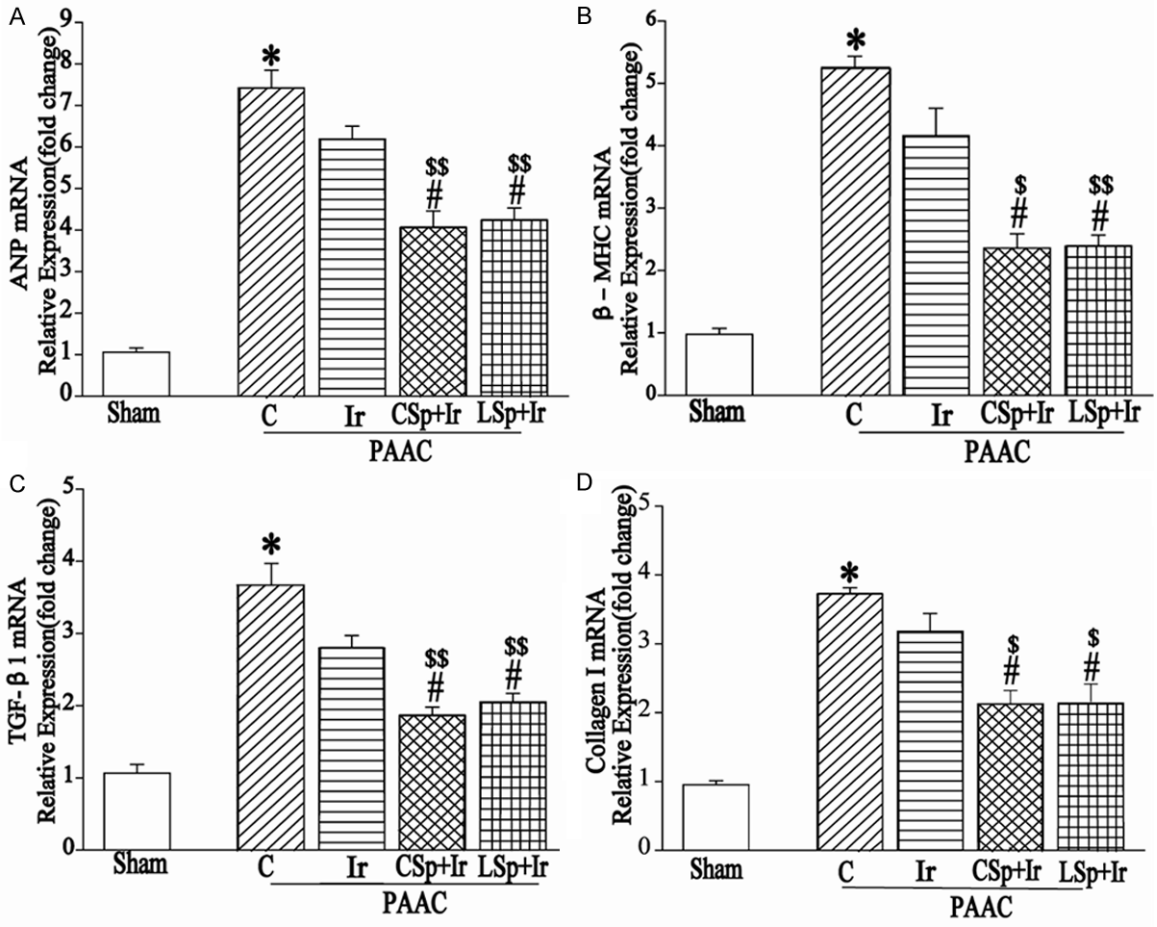

Figure 3. Effects of low- or conventional-dose Sp in combination with irbesartan on cardiac hypertrophy and fibrosis-related biomarkers. The mRNA level was quantified using RT-PCR and normalized to β-actin housekeeping gene. Triplicate reactions were performed for each experiment, and the values presented are the means of 4 independent experiments. Values are means ± SEM. $^{*}p < 0.01$ vs Sham; $^{\#}p < 0.01$ vs control; $^{\$}p < 0.05$, $^{\$\$}p < 0.01$ vs irbesartan alone.

growth factor-β1. The findings indicated that spironolactone combined with ARB produced additional cardioprotection against PAAC-induced cardiac remodelling.

More importantly, our results revealed that spironolactone at a very low dose (1 mg/kg) also exhibited potentiated action with a comparable efficacy to a conventional dose (20 mg/kg), which was manifested by similar changes in various measurements and histological parameters. Low-dose spironolactone itself had no significant effect on sodium and water reabsorption in kidney and thus didn't affect blood pressure [15]. The result suggested that the cardioprotection produced by the low-dose spironolactone was independent of hemodynamic changes. This was supported by the result that low-dose spironolactone monotherapy did not affect hemodynamic and echocardiographic parameters (Supplementary data). However, we did find that low-dose spironolactone potentiated the antihypertensive effects of irbesartan. A previous report has demonstrated that eplerenone, at low dose without affecting renal sodium or potassium excretion effectively improve vascular endothelial function in hypertensive rats [27]. Therefore, the effect on blood pressure by low-dose spironolactone may result from its blockage on vascular endothelial MR. Increasing evidence has shown that elevated plasma aldosterone levels are found in cardiovascular diseases [28, 29]. For example, aldosterone in patients with HF may reach many-fold higher than those measured in normal subjects [30]. In addition to plasma aldosterone synthesized in the adrenal cortex, there is a possibility that aldosterone

Table 5. Effects of low-dose spironolactone (LSp) or conventional-dose spironolactone (CSp) combined with irbesartan (Ir) on the concentration of plasma K+ and Na+. Means ± SEM, n = 6 for each group.

	Sham	PAAC			
		Control	Ir	CSp + Ir	Sp + Ir
K, mmol/L	4.39 ± 0.09	4.38 ± 0.11	4.41 ± 0.10	4.89 ± 0.16*	4.36 ± 0.11
Na, mmol/L	138.9 ± 0.8	140.2 ± 0.5	142.1 ± 0.6	140.5 ± 0.6	141.2 ± 0.4

*$p < 0.05$ vs Sham.

may be synthesized in the heart under certain pathologic conditions [31]. A report has demonstrated that rats subjected to myocardial infarction exhibit an increase in both cardiac aldosterone synthase and aldosterone levels [32]. Therefore, the blockage on over-activation of MR under pathologic conditions contributes to beneficial effects of low-dose spironolactone on cardiac hypertrophic remodeling.

At present, hyperkalemia is probably considered the most concerning adverse effect for MR antagonist therapy with a rate ranging from 2%-12% [33]. In this study, combination therapy of irbesartan and conventional dose spironolactone resulted in a significant increase in the concentration of serum potassium. On the contrary, low-dose spironolactone hardly affected serum potassium levels. The results support the notion that low dose spironolactone exert anti-hypertrophic effect and meanwhile, minimize the risk of hyperkalemia. The target dose for spironolactone is 20 mg/day in the combination therapy for HF in guideline [34]. Although the dose is much lower than the commonly used diuretic dose (40-200 mg/day), some clinical trials have shown that most frequent adverse event is still hyperkalemia at this dose [7]. If the low dose of spironolactone (1 mg/kg) in rats is converted to human equivalent dose based on body surface area [35], it will be about 10 mg/d for an adult. Our result suggest that 10 mg/d maybe an effective and safety dose for spironolactone in the combination therapy for HF. This notion is supported by a clinical trial, in which serum potassium levels are unchanged by low-dose spironolactone (12.5 mg/d) [16] and spironolactone at this dose is clinically effective in combination therapy for essential hypertension [6]. However, whether 10 mg/d of spironolactone is effectiveness and safety need to be further tested in clinical trials.

In conclusion, our data indicated that the addition of low-dose spironolactone to irbesartan possessed comparable effectiveness to conventional dose in blunting the progression of heart hypertrophy and ventricular dysfunction in rats. Concomitantly, low-dose spironolactone did not affect serum potassium levels. This study suggests that combined low dose of spironolactone and irbesartan may be an effective and safety therapeutic strategy for cardiac hypertrophy and heart failure.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (312-71210) and Program for University Innovation Team Leading Talent of Hebei Province (LJRC019).

Disclosure of conflict of interest

None.

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Supplementary Table 1. Effects of low-dose spironolactone (LSp) on echocardiographic parameters. Means ± SEM, n = 6 for each group

Parameters	Sham	PAAC	
		Control	LSp
IVs, mm	1.75 ± 0.03	2.07 ± 0.05**	2.04 ± 0.08
LVPW, mm	1.75 ± 0.05	2.08 ± 0.08*	2.07 ± 0.06
LVs, mm	4.35 ± 0.20	4.08 ± 0.20	4.07 ± 0.26
LVd, mm	6.33 ± 0.16	5.53 ± 0.14**	5.51 ± 0.12
LVEF, %	59.4 ± 3.1	47.9 ± 7.0	42.6 ± 7.1
LVFS, %	36.0 ± 2.3	34.6 ± 4.7	32.2 ± 5.2

*$p < 0.05$, **$p < 0.01$ *vs* Sham.

Supplementary Table 2. Effects of low-dose spironolactone (LSp) on hemodynamic parameters. Means ± SEM, n = 6 for each group

Parameters	Sham	PAAC	
		Control	LSp
SBP, mmHg	134 ± 7	183 ± 3**	179.2 ± 11
DBP, mmHg	88 ± 6	123 ± 6**	120.2 ± 9
LVSP, mmHg	148 ± 5	180 ± 6**	180 ± 3
LVEDP, mmHg	3.55 ± 0.49	9.99 ± 0.61**	8.8 ± 0.9
$+dP/dt_{max}$, mmHg/s	8625 ± 652	8452 ± 746	7786 ± 240
$-dP/dt_{min}$, mmHg/s	6970 ± 548	4479 ± 565*	5137 ± 415
dP/dt_{min}/LVSP, s^{-1}	48 ± 3	25 ± 3**	29 ± 3

SBP, systolic blood pressure; DBP, diastolic blood pressure; LVSP, left ventricular systolic pressure, LVEDP, left ventricular end diastolic pressure; $\pm dP/dt_{max}$ and min, the maximum rate of pressure rise and fall. *$p < 0.05$, **$p < 0.01$ *vs* Sham.