

Title: Effects of spironolactone treatment on an experimental model of chronic aortic valve regurgitation

Short title: Spironolactone treatment for aortic valve regurgitation

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Abstract

Background: Aortic regurgitation (AR) is a disease for which there is currently no effective medical treatment. We have previously shown in an experimental model of AR that the renin-angiotensin-aldosterone system (RAAS) plays a major role and that medications blocking RAAS are effective to protect against left ventricular hypertrophy and also help maintain a normal systolic function. The role of aldosterone receptor blockers in this disease has never been evaluated. We therefore studied the effects of the aldosterone receptor blocking agent spironolactone in a model of chronic aortic regurgitation in rats.

Methods and Results: The effects of a 6-month treatment with spironolactone were evaluated in adult Wistar rats with severe AR compared to Sham-operated and untreated AR animals. Spironolactone treatment decreased total heart weight. The left ventricular expression of ANP mRNA was decreased by spironolactone treatment as well as the expression of collagen 1 and LOX1 mRNAs. Left ventricular fibrosis was decreased by spironolactone treatment.

Conclusions: Spironolactone protected against volume-overload cardiomyopathy in this model of aortic valve regurgitation. The predominant protective effect was a decrease in myocardial fibrosis.

Key words:

aortic valve regurgitation

volume overload

left ventricular hypertrophy

aldosterone receptor blockers

Introduction:

Chronic aortic valve regurgitation (AR) affects the left ventricle (LV) slowly over the years causing progressively a severe dilatation, eccentric hypertrophy (LVH) and eventually diastolic and systolic heart failure [1]. The management of patients with chronic AR is currently limited to surgical aortic valve replacement when specific clinical or echocardiographic criteria are reached [2]. Drug therapy of AR remains controversial and no drug has yet been shown to slow the progressive evolution towards left ventricular dysfunction in this disease [2].

Many small clinical trials have been designed over the past decades to search for an effective pharmacological treatment for AR but these trials have unfortunately been inconclusive or contradictory probably due to methodological differences or small sample sizes[3]. Using an animal model of chronic AR, our team has demonstrated that the renin-angiotensin-aldosterone system (RAAS) is affected by this disease and that angiotensin converting enzyme inhibitors (ACEI) or angiotensin 2 receptor blockers (ARB) protect against LVH [4–8]. The role of the downstream effector of the RAAS, aldosterone, has never been studied in chronic AR. Aldosterone not only acts as a major modulator of renal sodium-potassium homeostasis but is also a potent pro-hypertrophic and pro-fibrotic agent [9–12]. We therefore designed the present study to evaluate the effects of the aldosterone-receptor blocking agent spironolactone in a rat model of pure severe chronic aortic valve regurgitation. We hypothesized that it would improve LV remodeling in rats with chronic severe AR.

Material and Methods

Twenty-four male Wistar rats (300-350g, Charles River, Qc, Canada) had severe AR induced by retrograde puncture of the aortic valve leaflets as previously described [13,14] and randomly divided in 2 groups (n=12/gr): (1) AR untreated and (2) AR treated with spironolactone 100 mg/kg/day orally. Twenty sham operated rats were used as controls and randomly assigned to the following groups: (3) sham untreated and (4) sham treated with 100 mg/kg/day of spironolactone orally. AR was considered severe by echocardiography by the presence of all of the following criteria at the time of surgery: color-Doppler ratio of regurgitant jet width to LVOT diameter >50%, retrograde holo-diastolic flow in proximal descending aorta with end-diastolic velocity >18 cm/s, ratio of time-velocity integral of reversed diastolic flow to forward systolic flow in descending thoracic aorta >60% and acute increase in LV diastolic dimension during the surgical procedure. Echocardiographic criteria of AR severity had to be accompanied by an acute drop of aortic diastolic pressure of at least 30% to qualify. Animals not meeting the echographic and hemodynamic criteria were not included in the study. Drug treatment was started 2 weeks after the surgical procedure to allow recovery from the acute phase and continued for 6 months. Animals were clinically evaluated daily by experienced animal laboratory technicians for the presence of signs of heart failure (increased respiratory rate/distress and/or peripheral edema) and were weighed weekly. At the end of the protocol, surviving animals were sacrificed, hearts were quickly dissected and all cardiac chambers were weighed. LVs were snap-frozen in liquid nitrogen and kept at -80°C for further analysis. This protocol was approved by the Laval University Animal Protection Committee according to the recommendations of the Canadian Council on Laboratory Animal Care.

Echocardiography

A complete M-Mode, 2D and Doppler echocardiogram was performed on the animals under 1.5% inhaled isoflurane anesthesia using a 12 MHz probe with a Sonos 5500 echograph (Philips Medical Imaging, Andover, MA) immediately before and during surgery and after 6 months. An echocardiogram was also performed after 2 weeks to double-check AR severity and confirm that some LV dilatation had taken place before starting drug treatment to make sure all animals still met the entry criteria. Left ventricular dimensions, wall thickness, ejection fraction, diastolic function, cardiac output (ejection volume in the left ventricular outflow tract X heart rate) were evaluated as previously reported [7,15]. AR was semi-quantified at each time-point as described in the previous section. Animals had to meet all the criteria of severe AR by semi-quantization at each time-point to remain included in the protocol.

Hemodynamic measurements

Left ventricular end-diastolic pressures (LVEDP) and dP/dt (positive and negative) were measured invasively using a dedicated a 2F impedance catheter (Millar Instruments, Houston, TX) under 1.5% isoflurane anesthesia after 6 months (n=5-8/gr).

Analysis of mRNA accumulation by quantitative RT-PCR

Tissues stored frozen in RNAlater (Ambion, Austin, TX) were homogenized in Trizol (Invitrogen, Burlington, ON, Canada) and quantitative RT-PCR was conducted on the appropriate tissue samples [6,16]. QuantiTech Primers (Qiagen, Mississauga, ON, Canada) used for this study are listed in Table 1. Cyclophilin A was used as a control.

The quantification of gene expression was based on the $-2\Delta\Delta C_t$ method. Results are expressed relative to the sham group mRNA levels which were arbitrarily fixed at 1.

Staining for capillary density measurement

Sections of 8- μ m thickness were cut from the frozen left ventricle and were stained with isolectin B4 from *Bandeiraea simplicifolia* coupled with horseradish peroxidase (Sigma, Mississauga, ON, Canada), and capillary density was analyzed in the subendocardial region of the LV myocardium (inner third). Pictures of three different LV fields of 8 animals per group were taken at 200X magnification. The number of capillaries per field was measured for each 3 fields and reported as a mean for each animal. The observer was blinded for the groups during the analysis [17].

Fibrosis quantification:

The amount of LV fibrosis was evaluated semi-quantitatively by visual evaluation of a blinded observer on LV tissue samples stained for fibrosis by Trichrome-Masson. The amount of fibrosis was evaluated on a scale from 0 to 4 (where 0 = no fibrosis and 4 = severe fibrosis). Reproducibility of the method was tested by a second evaluation of the tissue samples by another blinded observer.

Statistical analysis

Results are presented as mean \pm SEM. Intergroup comparisons were done using 2-way ANOVA followed by Bonferroni post-test if interaction between disease (AR) and treatment (Spiro) was significant. Statistical significance was set at p values <0.05 . Data

and statistical analysis were performed using Graph Pad Prism version 5.02 for Windows, Graph Pad Software (San Diego CA).

Results

Clinical data: Spironolactone treatment was well tolerated. There were no deaths after 6 months in both sham groups (treated or not). 4 animals died in the AR untreated group whereas 1 animal died in the AR-spironolactone group. Due to the too small sample size, survival could not be analyzed statistically since the protocol was not designed to assess survival. Deaths were sudden, occurred during the night in the awake/active cycle of the rats and were not preceded by signs of heart failure on clinical evaluation. There were no differences in tibial length (similar growth) between all groups after 6 months.

Treatment effect on cardiac hypertrophy (table 2):

Hearts were explanted at the end of the protocol and cardiac chambers were weighed individually. Spironolactone did not affect heart weight in the sham control animals. Animal with AR (treated or not) developed as expected a severe cardiac hypertrophy as shown by their increased total heart, left ventricular, right ventricular and left atrial mass. Spironolactone treatment strongly tended to reduce total heart weight and significantly reduced right ventricular and left atrial mass. Statistical analysis confirms a significant treatment and disease-treatment interaction in favor of spironolactone on right ventricular and left atrial masses.

Echocardiographic data (table 3): The echocardiographic data obtained after 6 months are summarized in table 3. AR severity was similar in both AR groups. As expected, AR resulted in significant end-diastolic and end-systolic dilatation as well as

eccentric remodeling (decreased relative wall thickness (RWT)). Ejection fraction remained in the normal range in all AR animals although it was slightly lower than in the 2 sham groups (significant disease effect) confirming that the animals were in a compensated phase of the disease regarding their systolic function. Spironolactone had no statistically significant impact on LV dimensions, LV wall thickness, macroscopic remodeling (RWT) or ejection fraction.

Hemodynamic data (table 4):

Hemodynamic data obtained in 5-8 animals/group after 6 months are summarized in table 4. As expected, AR resulted in a higher stroke volume and cardiac output due to severe volume overload of the LV. Systolic blood pressure remained unchanged whereas diastolic blood pressure was lower in both AR groups. Maximal and minimal Dp/dts were lower in the AR groups compared to sham groups suggesting a mild decrease in systolic and diastolic functions. Left ventricular end-diastolic filling pressures were increased in the AR groups as expected. Spironolactone had no significant effect on any of those hemodynamic measurements.

mRNA expression of markers of heart hypertrophy (figures 1 and 2)

The relative expression of ANP and BNP were measured after 6 months in specifically preserved LV tissues. Results are reported in figure 1. All AR groups displayed a significant increase in ANP and BNP mRNA expression as shown in figure 1. Both over-expressions were significantly decreased by spironolactone treatment. A similar pattern was observed for Fstl1 (follistatin-like protein 1) as shown in Figure 2.

Fibrosis-related and extracellular matrix remodeling gene expression (figures 3-5)

Results for the mRNA relative expression of collagen I, collagen III, fibronectin in LV tissue are shown in Figure 3. The expression of collagen 1 and collagen III was increased in the AR groups whereas fibronectin expression remained unchanged. Spironolactone decreased collagen 1 expression but had no effect on collagen III. Spironolactone decreased fibronectin expression both in sham and AR animals.

The levels of mRNA expression of other components of the ECM regulation (MMP2, TIMP1, TGF β 1, TGF β 2, LOX1) are summarized in figures 4 and 5. There were no significant impacts of AR or spironolactone treatment on the expression of MMP2. TIMP1 expression was increased by AR but spironolactone did not reduce this over-expression. The expression of TGF β 1 was unaffected by disease or treatment status. TGF β 2 expression was strongly increased by AR but unaffected by treatment. LOX1 expression was also strongly increased and spironolactone treatment effectively decreased this over-expression.

Capillary density and myocardial fibrosis

Results of left ventricular myocardial capillary density are shown in figure 6. AR strongly decreased capillary density in the LV. Statistical analysis revealed a significant treatment-disease interaction in favor of spironolactone to increase capillary density. AR increased the amount of myocardial fibrosis whereas spironolactone significantly reduced the accumulation of fibrosis in the left ventricular myocardium in both normal and AR groups (Figure 7).

Discussion

The problem of finding the best treatment for chronic aortic valve regurgitation remains unresolved [2]. Vasodilators have been investigated in many small clinical trials but results have been inconsistent. The most recent treatment guidelines do not support anymore the use of vasodilators to treat chronic AR with normal ejection fraction. Drugs targeting the RAAS have shown promising results in experimental models as well as in some clinical trials but those results need to be reproduced in other carefully designed large scale prospective trial [5–8,17-19].

Aldosterone receptor blockers have been shown to be an effective treatment in various animal models as well as in humans with heart failure or hypertension [19,20]. This class of drug seems to be mainly anti-hypertrophic and anti-fibrotic in combination with its renal diuretic effect.

Our results show protective effects of spironolactone on the heart submitted to chronic volume overload caused by severe aortic valve regurgitation. Rats with chronic severe AR treated with spironolactone had smaller hearts and less LV fibrosis than the untreated animals. The fact that the reduction of cardiac mass was predominant in the right ventricle and left atrium suggests that spironolactone acted mainly by improving diastolic parameters and filling pressures. The reduction of ANP expression points in the same direction. Invasive intra-cardiac pressure monitoring did not confirm a statistically significant difference in end-diastolic pressure probably due to the small sample size. We did not uncover any significant difference in cardiac output, chamber dilatation, ejection fraction or systemic pressure (systolic or diastolic). Aldosterone therefore did not act by improving the hemodynamic status of the AR animals.

Interestingly, aldosterone treatment resulted in significant reductions in the expression of collagen I and Lox 1. This corroborated well with the decrease in LV myocardial fibrosis. We can therefore suggest that the decrease in fibrosis in the spironolactone-treated group improved diastolic filling reflected by a decrease in ANP expression and a decrease in LA and RV mass. Larger groups will need to be tested to better understand the mechanisms involved and evaluate the long term effects of these findings.

The mechanisms by which aldosterone exert its effects on the heart are still debated [21–23]. Considering that our animals were not in a hypovolemic state or in systolic heart failure or in low cardiac output, it is highly improbable that adrenal aldosterone production was stimulated from the kidneys. It has been suggested that receptors are not very receptive to aldosterone *per se* in the LV cardiomyocytes where their ligand is suspected to be mostly circulating cortisol (or corticosterone in rodents) due to the lack of co-localization of 11 β -hydroxysteroid dehydrogenase 2 [24,25]. However, a pro-fibrotic effect of aldosterone mediated through myocardial fibroblasts could have been prevented by spironolactone [26]. This, in turn, would improve diastolic performance, myocardial stiffness and filling pressures. Whether this protective effect of spironolactone would translate in a delay in symptom occurrence or survival benefits remain to be evaluated. Non-genomic actions of aldosterone such as a direct vasoconstrictive effect have also been reported [27–29]. Spironolactone could have blocked the vasoconstrictive action of aldosterone in our AR animals and thereby could have decreased the afterload. However, we have not been able to demonstrate any significant difference in hemodynamics in the aldosterone-treated group arguing against a significant hemodynamic effect of aldosterone. Aldosterone has also been shown to

induce oxidative stress in cardiac tissues by increasing NADPH oxidase activity [30]. This increase in oxidative stress can result in an increase in fibrosis and apoptosis. This mechanism of action was not explored in this protocol and deserves further investigation.

Conclusions:

We have shown for the first time that an aldosterone receptor blocker (spironolactone) can be beneficial in an animal model of chronic aortic valve regurgitation mainly by reducing myocardial fibrosis. Long term effects need to be evaluated in longer protocols.

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Conflict of interest disclosures:

None to declare for all the co-authors.

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Figure legends

Figure 1:

Gene expression of atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) in LV. White columns: untreated sham and AR control (ctrl) groups; black columns: spironolactone-treated (spiro) groups. Results are expressed as mean \pm standard error of the mean (SEM) of 8-11 individual determinations. P values from 2-way ANOVA and Bonferroni post testing when applicable to evaluate disease, treatment or disease-treatment interactions (D X T) are shown below each panel.

Figure 2:

Gene expression of follistatin-like 1 protein (Fstl1) in LV. White columns: untreated sham and AR control (ctrl) groups; black columns: spironolactone-treated (spiro) groups. Results are expressed as mean \pm standard error of the mean (SEM) of 8-11 individual determinations. P values from 2-way ANOVA and Bonferroni post testing when applicable to evaluate disease, treatment or disease-treatment interactions (D X T) are shown below each panel.

Figure 3:

Gene expression of collagen type 1 (Col I), collagen type III (Col III), fibronectin (Fn) and follistatin-1 (Fstl1) in LV. White columns: untreated sham and AR control (ctrl) groups; black columns: spironolactone-treated (spiro) groups. Results are expressed as mean \pm standard error of the mean (SEM) of 8-11 individual determinations. P values from 2-

way ANOVA and Bonferroni post testing when applicable to evaluate disease, treatment or disease-treatment interactions (D X T) are shown below each panel.

Figure 4:

Gene expression of matrix metalloprotease 2 (MMP2) and tissue inhibitor of metalloprotease 1 (TIMP1) in LV. White columns: untreated sham and AR control (ctrl) groups; black columns: spironolactone-treated (spiro) groups. Results are expressed as mean \pm standard error of the mean (SEM) of 8-11 individual determinations. P values from 2-way ANOVA and Bonferroni post testing when applicable to evaluate disease, treatment or disease-treatment interactions (D X T) are shown below each panel.

Figure 5:

Gene expression of TGF β 1, TGF β 2 and Lox1 in LV. White columns: untreated sham and AR control (ctrl) groups; black columns: spironolactone-treated (spiro) groups. Results are expressed as mean \pm standard error of the mean (SEM) of 8-11 individual determinations. P values from 2-way ANOVA and Bonferroni post testing when applicable to evaluate disease, treatment or disease-treatment interactions (D X T) are shown below each panel. **: $p < 0.01$ vs. corresponding untreated group.

Figure 6:

Myocardial capillary density quantification. Typical examples of tissue stainings are shown in the upper sections for normal sham, AR untreated (nt) and AR treated with spironolactone (spiro). Bottom section: graph displaying the quantification of myocardial

capillary density per field. White columns: untreated sham and AR control (ctrl) groups; black columns: spironolactone-treated (spiro) groups. P values from 2-way ANOVA and Bonferroni post testing when applicable to evaluate disease, treatment or disease-treatment interactions (D X T) are shown to the right of the bottom panel.

Figure 7:

Myocardial fibrosis quantification. Typical examples of tissue stainings are shown in the upper sections for normal sham, AR untreated (nt) and AR treated with spironolactone (spiro). Bottom section: graph displaying the semi-quantification of myocardial capillary density per field ranging from 0 (no visible fibrosis) to 4 (severe fibrosis). White columns: untreated sham and AR control (ctrl) groups; black columns: spironolactone-treated (spiro) groups. P values from 2-way ANOVA and Bonferroni post testing when applicable to evaluate disease, treatment or disease-treatment interactions (D X T) are shown to the right of the bottom panel.

Table 1. QuantiTect[®] Primer Assays used in Q-PCR analysis of gene expression.

mRNA	Symbol	Genbank Acc. No.	Amplicon size(bp)
Natriuretic peptide precursor type A	ANP	NM_012612	107
Natriuretic peptide precursor type B	BNP	NM_031545	94
Pro-collagen-1 alpha-1	Col1a1	NM_053304	92
Pro-collagen-3 alpha-1	Col3a1	NM_032085	111
Fibronectin	Fn	NM_019143	92
Matrix metalloprotease 2	Mmp2	NM_031054	103
Follistatin-like protein 1	Fstl1	NM_024369	131
Tissue inhibitor of metalloprotease 1	Timp1	NM_053819	113
Lysyl oxidase (LOX)	Lox	NM_017061	148
Transforming growth factor beta 1	Tgfb1	NM_021578	145
Transforming growth factor beta 2	Tgfb2	NM_031131	139
Cyclophilin A	Ppia	NM_017101	106

Table 2. Data at sacrifice

Parameters	Sham (10/10)	Sham Spiro (10/10)	AR (8/12)	AR Spiro (11/12)	Disease	Treatment	D x T
Tibial length, mm	65.8 ± 0.30	64.8 ± 0.29	63.7 ± 1.06	64.9 ± 0.40	0.059	0.842	0.053
Heart, mg	1476 ± 20.5	1499 ± 29.8	2661 ± 107.3	2328 ± 96.3*	<0.0001	0.064	0.036
LV, mg	1023 ± 19.7	1020 ± 28.8	1881 ± 72.4	1692 ± 76.8	<0.0001	0.129	0.141
RV, mg	301 ± 5.7	291 ± 9.1	459 ± 23.5	394 ± 15.0	<0.0001	0.024	0.089
LA, mg	33 ± 2.4	36 ± 1.4	93 ± 10.6	62 ± 4.0**	<0.0001	0.022	0.0052
Lungs, mg	2.6 ± 0.14	2.6 ± 0.20	3.4 ± 0.31	3.0 ± 0.28	0.24	0.15	0.76

Values are mean ± SEM. P values from 2-way ANOVA and Bonferroni post testing when applicable to evaluate disease, treatment or disease-treatment interactions (D X T) are shown to the right of each panel. *: p<0.05 and **: p<0.01 vs. AR group. LV: left ventricle, RV: right ventricle and LA: left atrium.

Table 3. Echocardiography data

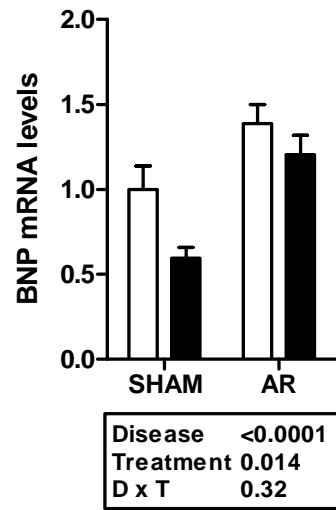
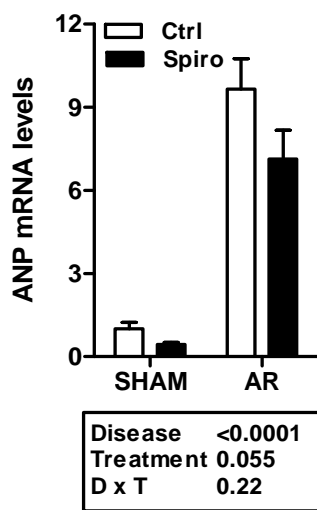
Parameters	Sham	Sham Spiro	AR	AR Spiro	Disease	Treatment	D x T
EDD, mm	9.2 ± 0.09	9.1 ± 0.10	12.4 ± 0.26	11.9 ± 0.22	<0.0001	0.14	0.36
ESD, mm	4.4 ± 0.10	4.4 ± 0.09	7.0 ± 0.14	6.6 ± 0.25	<0.0001	0.12	0.21
SW, mm	1.5 ± 0.03	1.5 ± 0.02	1.8 ± 0.02	1,7 ± 0.03	<0.0001	0.23	0.07
PW, mm	1.6 ± 0.04	1.5 ± 0.02	1.8 ± 0.03	1,8 ± 0.02	<0.0001	0.76	0.92
RWT	0.34 ± 0.008	0.34 ± 0.004	0.29 ± 0.008	0,30 ± 0.007	<0.0001	0.57	0.99
EF, %	77 ± 0.8	77 ± 0.9	66 ± 0.5	69 ± 1,4	<0.0001	0.31	0.23
MPI	0.42 ± 0.018	0.43 ± 0.020	0.52 ± 0.032	0,52 ± 0.020	0.0009	0.89	0.87
AR (%reg.)	na	na	78 ± 4.0	78 ± 4.0	na	na	na

Values are mean ± SEM. P values from 2-way ANOVA and Bonferroni post testing when applicable to evaluate disease, treatment or disease-treatment interactions (D X T) are shown to the right of each panel. EDD: end-diastolic diameter, ESD: end-systolic diameter, SW: septal wall thickness, PW: posterior wall thickness, RWT: relative wall thickness ((SW+PW)/EDD), EF: ejection fraction, MPI: myocardial performance index, AR: AR severity by echocardiographic semi-quantification. na: non applicable.

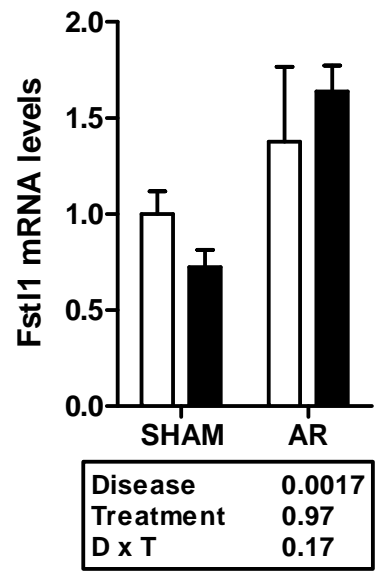
Table 4. Hemodynamics

Parameters	Sham	Sham Spiro	AR	AR Spiro	Disease	Treatment	D x T
HR (bpm)	361 ± 10.2	352 ± 3.6	352 ± 11.5	368 ± 9.9	0.67	0.69	0.20
SV (µl)	231 ± 4.7	240 ± 5.0	421 ± 16.7	370 ± 14.0*	<0.0001	0.17	0.022
CO (ml/min)	83 ± 2.4	85 ± 1.9	147 ± 5.4	137 ± 5.8	<0.0001	0.40	0.19
SBP (mm Hg)	116 ± 2.2	131 ± 6.1	127 ± 4.5	125 ± 1.6	0.67	0.35	0.17
DBP (mm Hg)	88 ± 2.3	93 ± 4.5	63 ± 3.7	65 ± 1,8	<0.0001	0.46	0.74
dP/dt max	8580 ± 141.9	8188 ± 189.5	7047 ± 339.0	6549 ± 187.4	<0.0001	0.18	0.87
dP/dt min	7231 ± 287.7	7061 ± 245.0	4627 ± 183.1	4703 ± 170.1	<0.0001	0.88	0.71
EDP (mm Hg)	9 ± 0.3	8 ± 0.9	15 ± 1.0	12 ± 0.6	0.0003	0.25	0.64

Values are mean ± SEM. P values from 2-way ANOVA and Bonferroni post testing when applicable to evaluate disease, treatment or disease-treatment interactions (D X T) are shown to the right of each panel. *: p<0.05 vs. AR group. HR: heart rate; SV: stroke volume in left ventricular outflow tract by pulsed Doppler; CO: cardiac output (SV X HR); SBP: systolic blood pressure; DBP: diastolic blood pressure. EDP: end diastolic pressure.



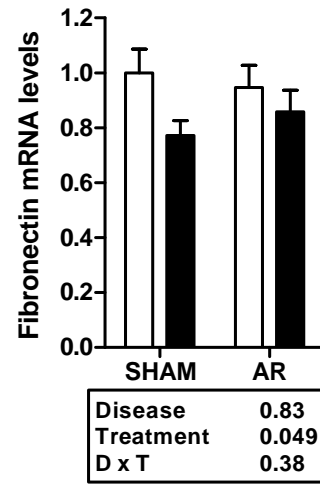
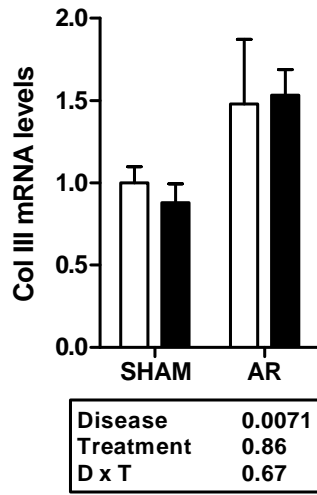
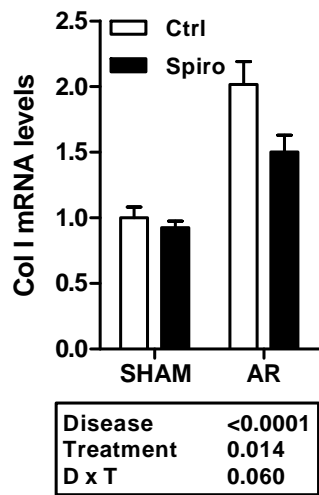
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Figure 1



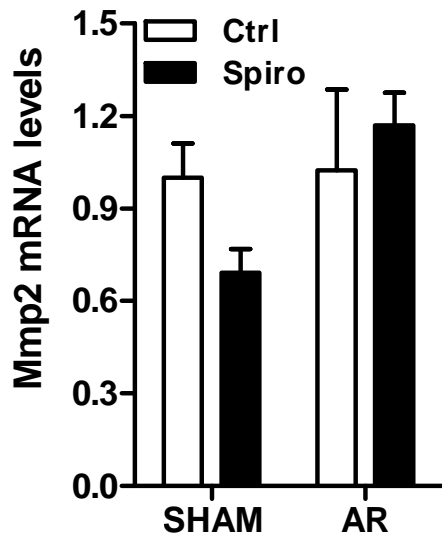
Disease	0.0017
Treatment	0.97
D x T	0.17

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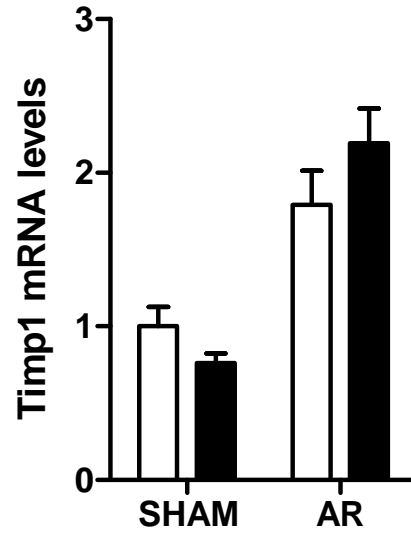
Figure 2



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Figure. 3

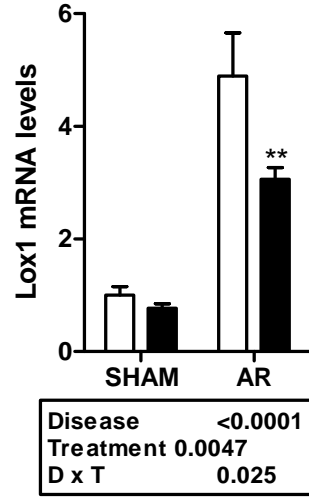
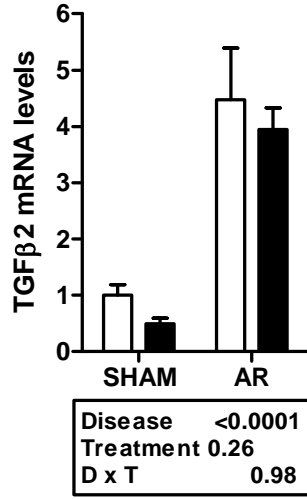
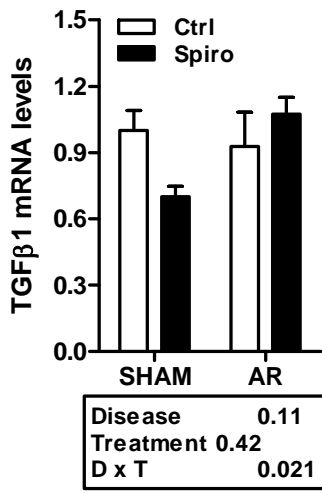


Disease	0.083
Treatment	0.57
D x T	0.12

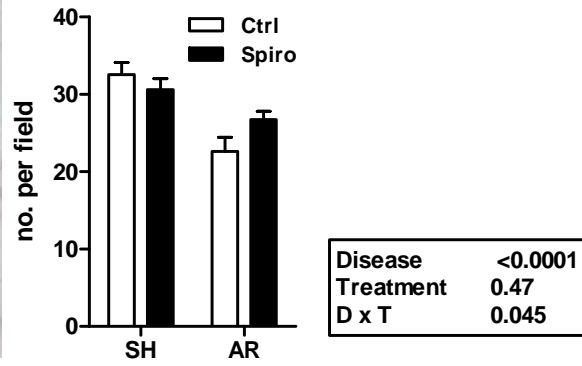
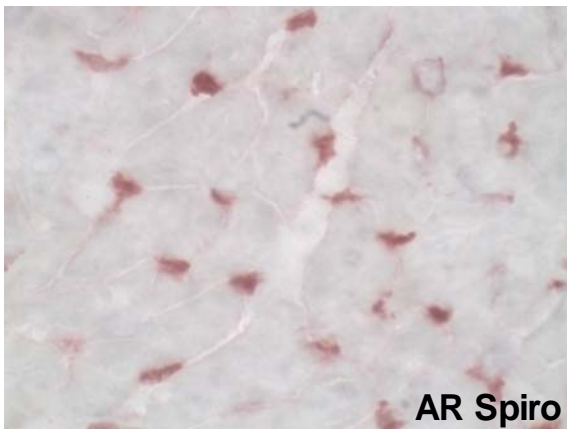
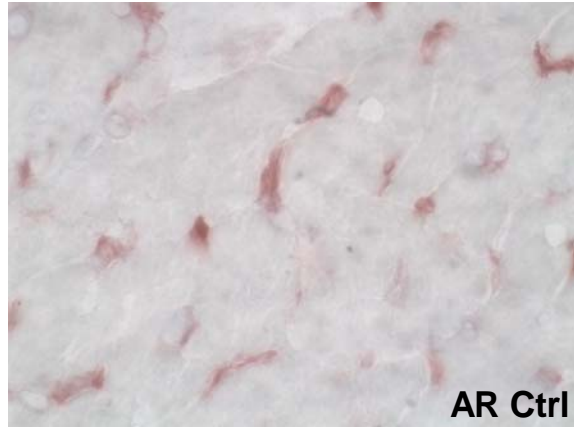


Disease	<0.0001
Treatment	0.64
D x T	0.073

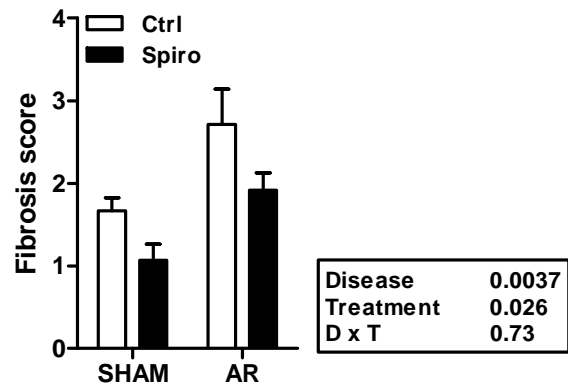
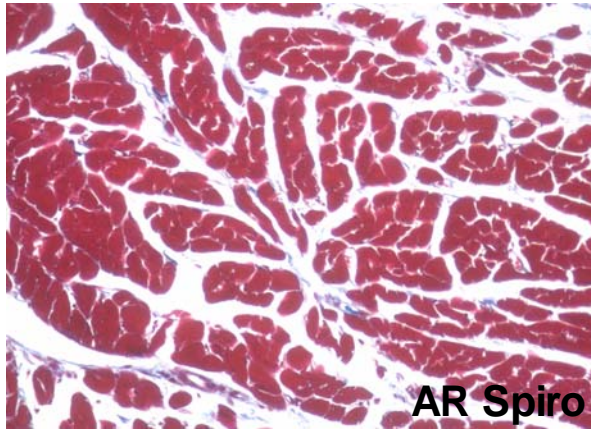
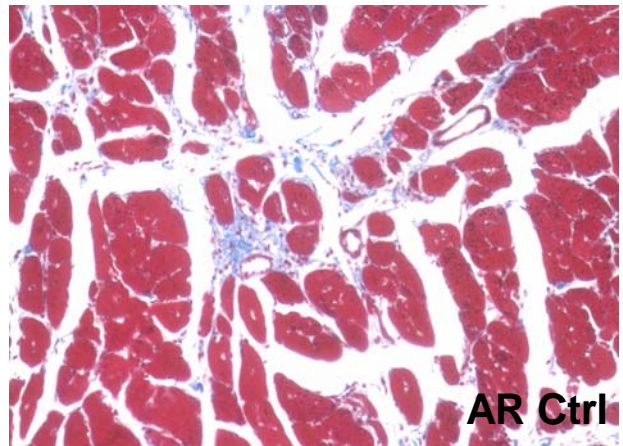
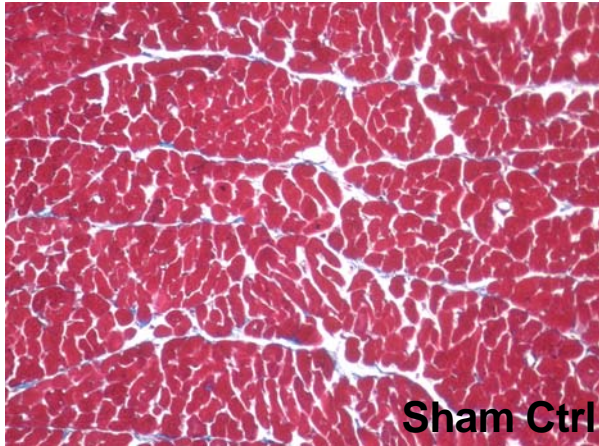
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Figure. 4



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Figure. 5



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Figure.6



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Figure.7