Experimental Mild Renal Insufficiency Mediates Early Cardiac Apoptosis, Fibrosis and Diastolic Dysfunction: A Kidney – Cardiac Connection

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ABSTRACT

Background: Impaired renal function with loss of nephron number in chronic renal disease (CKD) is associated with increased cardiovascular morbidity and mortality. However, the structural and functional cardiac response to early and mild reduction in renal mass is poorly defined. We hypothesized that mild renal impairment produced by unilateral nephrectomy (UNX) would result in early cardiac fibrosis and impaired diastolic function which would progress to a more global left ventricular (LV) dysfunction.

Methods and Results: Cardiorenal function and structure were assessed in rats at 4 and 16 weeks following UNX or sham operation (Sham); (n=10 per group). At 4 weeks, blood pressure (BP), aldosterone, glomerular filtration rate (GFR), proteinuria, and plasma B-type natriuretic peptide (BNP) were not altered by UNX representing a model of mild early CKD. However, UNX was associated with significantly greater LV myocardial fibrosis compared to Sham. Importantly, TUNEL staining revealed increased apoptosis in the LV myocardium. Further, diastolic dysfunction, assessed by strain echocardiography, but with preserved LVEF was observed. Changes in genes related to the TGF-β and apoptosis pathways in the LV myocardium were also observed. At 16 weeks after UNX, we observed persistent LV fibrosis and impairment in LV diastolic function. In addition, LV mass significantly increased, as did LVEDd while there was a reduction in LVEF. Aldosterone, BNP, and proteinuria were increased while GFR was decreased. The myocardial structural and functional alterations were associated with persistent changes in the TGF-β pathway and even
more widespread changes in the LV apoptotic pathway.

Conclusions: These studies demonstrate that mild renal insufficiency in the rat results in early cardiac fibrosis and impaired diastolic function which progresses to more global LV remodeling and dysfunction. Thus, these studies importantly advance the concept of a kidney – heart connection in the control of myocardial structure and function.

Keywords: kidney – heart failure - fibrosis – remodeling – nephrectomy – natriuretic peptides
INTRODUCTION

Increasing evidence from human studies underscore an interaction between the kidney and heart where impairment of one organ contributes to progressive and combined failure of both organs. This concept is supported by both experimental and clinical investigations where even mild renal impairment contributes to increased cardiovascular risk.(4, 9, 15, 21, 25) Furthermore, in recent human studies using magnetic resonance imaging, very early renal disease characterized by mild increases in serum Cystatin C are associated with increased myocardial mass.(23) This later study raises the possibility that the heart may remodel early during the natural history of chronic kidney disease (CKD).

In the current study we sought to define LV structure and function in a model of mild renal insufficiency in rats 4 and 16 weeks after removal of one kidney (UNX) to reduce nephron number. Specifically, we assessed myocardial structure with a special focus on fibrosis and employed microarray gene analysis of two key molecular pathways involved with cardiac fibrosis. As microarray analysis identified widespread changes in apoptotic pathway genes we also assessed the presence of LV apoptosis. Finally, myocardial function was assessed by echocardiography. Our overall working hypothesis is that mild renal insufficiency results in early cardiac apoptosis and fibrosis with mild impairment in diastolic function and preserved early systolic function which progresses to more global LV dysfunction and remodeling.

METHODS

Animals
Male Wistar rats (Charles River Laboratories - Wilmington, MA) 150-250g (6 to 8 weeks) were used (n=10 per group). The experimental design was approved by the Institutional Animal Care and Use Committee of the Mayo Clinic, Rochester, MN. Groups were as follows: Group 1: Sham-4 weeks, Group 2: UNX-4 weeks (Groups 1 and 2 had acute studies performed 4 weeks after sham or UNX surgery), Group 3: Sham-16 weeks, and Group 4: UNX-16 weeks (Groups 3 and 4 had acute studies performed 16 weeks after sham or UNX surgery). On day one animals were randomly assigned to the four different groups. The rats in the UNX group underwent a surgical procedure to remove the whole right kidney. The left kidney was undisturbed. The rats in the Sham group underwent the same procedure but no kidney was removed.

**Surgical Procedure:** Laparotomy was performed under gas anesthesia (Isoflurane 2%). The right kidney was carefully separated from the adrenal gland and the surrounding tissue.(24) The renal artery and vein, as well as the ureter, were ligated with a 4.0 silk suture followed by removal of the right kidney.

**Acute Studies:** On week 4 and 16 the rats underwent an acute study using the same anesthesia. Conventional and 2D speckle tracking echocardiography was performed prior to acute experiments to assess LV chamber and myocardial function and geometry. After echocardiography, PE-50 tubing was placed into the carotid artery for blood pressure monitoring and blood sampling. The bladder was cannulated for urine collection. Inulin (2%) (Sigma, St. Louis, MO) in normal saline and p-Aminohippuric acid (PAH) (1%) (Sigma, St. Louis, MO) were then continuously infused into the jugular vein. After equilibration (60 min), a clearance study was performed. A 60-min urine
collection was obtained with blood sampling at the end to calculate glomerular filtration rate (GFR) and renal plasma flow from the clearance of inulin and PAH.

Blood for hormone analysis was placed in heparin or EDTA tubes on ice. It was analyzed for electrolytes, inulin and hormones (BNP and aldosterone). After centrifugation at 2,500 rpm at 4°C for 10 minutes, plasma was separated and stored at –80°C until assay. Plasma BNP and aldosterone were determined by radioimmunoassay. Plasma and urine concentrations for electrolytes were determined by flame photometer (Model 1L943 Instrumentation Lab, Lexington, MA). Plasma and urine concentrations for inulin were measured by anthrone method for calculation of GFR. On the day prior to the acute experiment rats were housed in a metabolic cage for 24 hour measurement of urine and assessment of proteinuria.

In two additional subgroups of animals Sham (n=5) and UNX (n=5) we assessed continuous blood pressure using a continuous telemetry device (Data Sciences, Inc., St. Paul, MN, USA) a week before randomizing the animals to the two different groups.

**Echocardiography Studies:** *Conventional Echocardiography:* Ultrasonic scans were performed in all rats using a Vivid 7 system (GE Healthcare, Milwaukee, WI) equipped with a 10S ultrasound probe (11.5 MHz) with ECG monitoring. M-mode images and gray scale 2D images (300-350 frames/sec) of parasternal long axis and mid-LV were recorded for off-line analysis. LV end-diastolic and end-systolic dimensions, septal diastolic and posterior wall diastolic and systolic thicknesses were measured from M-mode images. LV mass was calculated according to uncorrected cube assumptions as LV mass = 1.055 x [(LVDd+SWTd+PWTd)³-(LVDd)³]. End-systolic (ESV), end-diastolic and stroke volumes (SV), and ejection fraction (EF) were calculated using the Teichholz
formula: \( LV \) volume = \( 7 \times \frac{[(LVDd)^3]}{2.4+LVDd} \). Relative wall thickness (RWT) was calculated as \( RWT = \frac{(SWTd + PWTd)}{LVDd} \). All parameters represent the average of 3 beats.

**Two-Dimensional Speckle-Derived Strain Echocardiography (2DSE)** Using EchoPAC software (EchoPAC PC – 2D strain, BTO 6.0.0, GE Healthcare, Milwaukee, WI), endocardial border was carefully manually traced at end-systole in LV short-axis views and ideal width of circular region of interest was chosen in order to include the entire myocardial wall. Speckle tracking was performed by the software and global strain and strain rates parameters were measured. (18, 19) The analysis included peak circumferential contraction strains \( Cs-C \) and strain rates \( Csr-C \) for evaluation of myocardial systolic function and peak early (\( Csr-E \)) and late relaxation (i.e. atrial contraction, \( Csr-A \)) circumferential strain rates and their ratio for evaluation of myocardial diastolic function. All parameters represent the average of 3 beats.

**Histological Analysis:** Hearts and kidneys were harvested after the acute experiment. Sections of the left ventricle, renal cortex and medulla were immersed in formalin for later histological analysis. The remaining tissue was snap frozen in liquid nitrogen for molecular analysis and microarray studies. Picrosirius red staining was utilized to assess collagen content. Glomerular volume measurements were performed in order to evaluate glomerular hypertrophy. For this purpose, the Weibel formula was used.(13) An Axioplan II KS 400 microscope (Carl Zeiss, Inc., Germany) and KS 400 software were utilized to analyze the histological slides and to calculate the percentage of picrosirius red stain as well as to determine glomerular volumes. TUNEL stain was performed with the CardioTACS™™in situ kit (Trevigen, Gaithersburg, MD).
**Microarray Studies:** Total RNA was isolated from snap frozen left ventricle, kidney cortex and kidney medulla using the Trizol method. Purified cDNA was used as a template for in vitro transcription reaction for the synthesis of biotinylated complementary RNA (cRNA) using an RNA transcript labeling reagent (Affymetrix, Santa Clara, CA). Labeled cRNAs were then fragmented and hybridized onto the Affymetrix Rat GeneChip oligonucleotide array (Affymetrix GeneChip® Rat Genome 230 2.0). Expression of genes in the UNX group samples was compared to the Sham group controls using GeneSifter® (Seattle, WA) software. Microarray-based expression differences were confirmed with real-time RT-PCR. The genes utilized for this purpose were ubiquitin conjugating enzyme (microarray yielded a 4.62 fold decrease and qPCR a 4.96 fold decrease) and indolmethylamine N-methyltransferase (microarray yielded a 2.3 fold increase and qPCR a 3.4 fold increase). First-strand cDNA was generated using the iScript cDNA synthesis kit (Bio-Rad). qPCR was performed using the Lightcycler (Roche) and expression was normalized versus GAPDH.

**Statistical Analysis**

All data are expressed as mean ± SEM. The comparison between each measurement was performed by t-test. Significant difference was accepted at p<0.05. For gene microarray chip analysis the software Genesifter® was utilized. For overall analysis a p<0.05 and a 1.5 fold change were used. For pathway analysis a Z score greater than 2 was used.

**RESULTS**
Four Weeks Following Uninephrectomy

**Renal and Neurohumoral Function:** Four weeks after UNX, an increase in glomerular volume was observed consistent with glomerular hypertrophy (Sham: 1±0.1, UNX: 1.6±0.1 %, p<0.0001). Renal fibrosis was observed in both cortex (Sham: 1.06±0.2, UNX: 4.24±0.7 %, p<0.01) and medulla (Sham: 1.13±0.3, UNX: 6.89±2.1%, p<0.05) with a trend toward a reduction in GFR (p=0.06). There was no change in proteinuria or alterations in sodium or water excretion (Table 1). Compared to sham controls, we observed no differences in plasma BNP or aldosterone system in rats 4 weeks following UNX (Table 1). Further, arterial pressure was not different between groups (Table 1 and Figure 1).

**LV Structure:** We next assessed structural changes in the LV following UNX. To assess cardiac fibrosis, we utilized Picrosirius red staining to quantitate collagen content in the LV (Figure 2). Here we observed a significant increase in interstitial and perivascular collagen in the UNX group compared to Sham. Consistent with increased LV fibrosis, microarray analysis of the LV myocardium demonstrated significant alterations in the apoptosis and TGF-β pathways where 47 and 5 genes in each respective pathway were altered (1.5 fold, p<0.05). Figure 3 summarizes the 52 genes in these two pathways which were significantly altered at 4 weeks after UNX. Consistent with alterations in the apoptosis pathways, specific staining for apoptosis using the TUNEL assay in the LV myocardium demonstrated increased apoptosis following UNX. (Sham: 0.2±0.1, UNX: 36.3 ± 14.7 %, p<0.05). (Figure 4).

**Echocardiography:** Functional and structural LV assessment was performed using conventional and speckle strain echocardiographic data (Table 2). Diastolic
septal wall thickness (SWTd) as well as diastolic posterior wall thickness (PWTd) were significantly lower (p<0.05) in UNX resulting in lower (p<0.05) relative wall thickness (RWT). LV systolic chamber function as assessed with LVEF, systolic myocardial function as measured by peak circumferential contraction strain (Cs-C) and strain rate (Csr-C) as well as LV mass were not different between the groups. Early diastolic strain rate (Csr-E) was significantly (p<0.05) lower in the UNX group while late relaxation (atrial contraction) circumferential strain rates (Csr-A) increased resulting in a decreased Csr-E/A ratio consistent with mild myocardial diastolic impairment.

**Sixteen Weeks Following Uninephrectomy**

*Renal and Neurohumoral Function:* Sixteen weeks after UNX, renal cortical and medullary fibrosis was similar between the sham group and the UNX group, although there was a strong trend for greater collagen content in the medulla in the UNX group. GFR was however reduced compared to sham (Sham: 3.3±0.5, UNX: 1.6±0.3 mL/min, p<0.01) as well as a trend for RBF to decrease (Sham: 6.55±1.5, UNX: 5.26±0.8 ml/min, NS; would report p value) (Table 3). Moreover, at 16 weeks we now observed a significant increase in proteinuria compared to sham (Sham: 16.2±3.8, UNX: 95.9±33.8 mg/24hr, p<0.05) with no alterations in sodium or water excretion (Table 3). Compared to sham controls, there was an increase in plasma BNP (Sham: 8.04±0.3, UNX: 10.13±0.7 pg/mL, p<0.01) and aldosterone (Sham: 20.93±3.2, UNX: 44.2±7 ng/dL, p<0.01) in rats 16 weeks following UNX. Arterial pressure was not different between groups. (Sham: 87.7±3.4, UNX: 80.7±4.0 %, NS) (Table 3).

*LV Structure:* Picrosirius red staining revealed that the cardiac fibrosis seen at 4 weeks persisted at 16 weeks (Sham: 2.1±0.1, UNX: 4.4±0.4 %, p<0.01) (Figure 5). This
fibrosis was interstitial as well as perivascular in the UNX group as compared to Sham. Microarray analysis of the apoptotic and TGF-β pathways of the LV myocardium 16 weeks after UNX revealed that 96 and 7 genes in each respective pathway were changed (1.5 fold, p<0.05). Figure 3 reports these 103 genes, which were changed in these two pathways at 16 weeks between groups. Of note the alterations in the apoptosis pathway observed at 4 weeks post UNX was more widespread at 16 weeks. Also, at 16 weeks, some other important genes showed increased activation compared to UNX at 4 weeks (p<0.05): IL-6 (marker for hypertrophic cardiomyopathy) (UNX 4wk: 8.4±0.2 vs UNX 16wk: 10.2±0.1), transforming growth factor beta 2 (TGF-β2) (UNX 4wk: 4.5±0.2 vs UNX 16wk: 6±0.3) and connective tissue growth factor (UNX 4wk: 9.9±0.1 vs UNX 16wk: 11.2±0.3) (both involved in increased collagen deposition and alteration of cardiac contraction).

**Echocardiography:** Table 4 reports echocardiographic data. At 16 weeks there was no difference in SWTd while PWTd was significantly higher in UNX 16 weeks group as was LVEDd. Importantly, and in contrast to 4 weeks following UNX, at 16 weeks following UNX LVEF was decreased compared to Sham. Cs-C and Csr-C were significantly lower in UNX group, and in contrast to 4 weeks post UNX, LV mass was now significantly higher. Thus, at 16 weeks there is reduction in systolic function associated with ventricular hypertrophy which was not present at 4 weeks post UNX. As observed at 4 weeks, early diastolic strain rate (Csr-E) was also significantly (p<0.05) lower in the UNX group while late relaxation (atrial contraction) circumferential strain rates (Csr-A) increased resulting in a decreased Csr-E/A ratio consistent with mild myocardial diastolic impairment.
DISCUSSION

The major finding of the current study is that mild CKD, produced by UNX, results in early cardiac fibrosis with mild diastolic impairment and preserved systolic function at four weeks after UNX. These findings seem to be independent of significant increase in BP, sodium or water retention, or activation of aldosterone. Importantly, cardiac hypertrophy and impairment of heart function progresses overt time (16 weeks). While structural changes in the heart are not associated with activation of aldosterone at 4 weeks they are characterized by a late (16 weeks) activation of aldosterone. This kidney-heart connection in mild early CKD may involve, at least, two important gene pathways as evidenced in our study by alterations in the TGF-β and apoptotic pathways. Indeed, at 4 weeks after UNX, the observed fibrotic response of the heart is associated with evidence for increased myocardial apoptosis. All together these findings indicate that reduction of renal mass is characterized by early and late structural and functional modifications accompanied by gene pathways alterations and hormonal pro-fibrotic factor activation, such as aldosterone at 16 weeks.

There are likely multiple mechanisms by which UNX may mediate early myocardial fibrosis. While it is increasingly noted that aldosterone mediates cardiac fibrosis (1, 2, 14) in the current study there is no activation of aldosterone at 4 weeks, which is, however, elevated at 16 weeks. Other possible mechanisms that may contribute to the early fibrosis observed in this study, can be related to the TGF-β and apoptosis pathways.(17, 27) Therefore, we sought to determine the extent of perturbation of these two pathways by assessing changes in their related genes by
microarray analysis in the LV myocardium. After 4 weeks from UNX, when mild cardiac fibrosis and impairment in diastolic function, as assessed by echo-strain analysis, are present, both TGF-β and apoptosis pathways exhibited numerous gene changes. Furthermore, these apoptotic gene alteration, are associated with TUNEL positive staining demonstrating a significant increase in apoptosis after 4 weeks of UNX. While our goal was not to identify the specific mechanisms, our study provides direction for further investigations to understand the myocardial response to mild early renal insufficiency. We would speculate that the remaining kidney, which itself is undergoing remodeling as demonstrated by the increase in glomerular volume and fibrosis, may release humoral factors, yet to be defined, that induce TGF-β and apoptotic pathways in the heart. Alternatively, a systemic inflammatory process could occur, which may also contribute to myocardial fibrosis and myocyte death.

Of note, even though LV fibrosis does not progress form 4 to 16 weeks, there is a worsening of cardiac function as well as an increase of LV mass at 16 UNX vs 4 UNX. Although, we cannot account for an increase in fibrosis to explain the progressive worsening of cardiac function at 16 weeks, we observed an alteration of other important genes associated with myocardial dysfunction that are not yet activated at 4 weeks. These genes are IL-6, a known marker of hypertrophic cardiomyopathy, and TGF-β2 and connective tissue growth factor (both involved in increased collagen deposition and alteration of cardiac contraction). These genes could possibly be relevant in understanding the mechanisms of progression of cardiac dysfunction and hypertrophy of this model in time.
At 16 weeks, LV mass increases and LVEF modestly but significantly decreases compared to Sham. Further, there is a modest but significant increase in LVEDd together with an increase in plasma BNP consistent with both LVH and myocardial stretch. In addition to continued diastolic impairment, strain analysis demonstrates systolic impairment. Specifically, Cs-C and Csr-C are both reduced 16 weeks after UNX. This progressive cardiac remodeling paralleled progressive renal impairment. GFR is significantly reduced, although moderately (50% lower than Sham), and proteinuria is also present. There are, however, no signs of sodium and water retention as sodium and water excretion are not different in the UNX as compared to the Sham group.

Although we do not observe activation at 4 weeks, aldosterone is activated 16 weeks post UNX. The current study was designed to detect the presence of possible cardiac structural and functional changes at 4 and 6 weeks post UNX, thus we cannot exclude that an early, yet transient, activation of aldosterone occurs immediately after surgery returning to normal range by week 4. Indeed an early and transient activation of aldosterone could also contribute to the presence of fibrosis at 4 weeks. Further studies are needed to investigate possible early activation of aldosterone. The activation of aldosterone at 16 weeks without marked sodium and water retention is consistent with the phenomenon of mineralocorticoid escape.(3, 8) Specifically, the kidney escaped from the sodium retaining properties of aldosterone excess as we previously demonstrated in a canine model of mild LV dysfunction.(8) Relevant to this pathological role of aldosterone in this model of mild CKD is the study by Edwards et al that have recently demonstrated using magnetic resonance imaging that spironolactone, in
patients with early mild CKD, resulted in a reduced LVH in association with a reduction in proteinuria.(12) With the report too of a mortality benefit in the recently reported findings from the EMPHASIS-trial (29) in which Epleronone was given to humans with mild HF, an important cardiorenal protective role for aldosterone antagonism warrants further investigation in the setting of early CKD although the problem of hyperkalemia could be a limiting factor.

Although we investigated several possible pathways of the kidney-heart connection (aldosterone, BNP, gene pathways), the mechanisms of such interaction remain not completely understood. A limitation of the current study is that we did not assess LV end diastolic pressure to more invasively assess LV pressure and pressure/volume relationships which should be considered in future studies. Also, this study shows a very minor increase in BNP levels at 16 weeks in the UNX group as compared to Sham. The lack of activation of BNP in this study is consistent to the findings by Cataliotti et al.(6). Indeed, in this study the authors showed the lack of activation of BNP in patient with end stage renal disease (ESRD). Further studies measuring ANP in this setting are needed to determine if this is a better marker than BNP for cardiac volume overload. Indeed, a possible elevation of ANP could explain the activation of cGMP and the lack of progression of cardiac fibrosis at 16 weeks.

Our findings are consistent with the clinical observations of Edwards et al, that reported that patients with mild renal insufficiency have cardiovascular adaptations similar to patients with heart failure with preserved ejection fraction.(10, 12) Other possible mechanisms involved in the reno-cardiac connection are starting to emerge. One of such mechanisms was recently reported by Cappola et al. (5) who described a
genetic variant in the CLCNKa chloride channel (present only in the kidney), that was implicated in increased risk for heart failure. Even though in the present study we did not find a significant change in that gene between groups, this is clear evidence that a kidney-heart connection also exists in the human.

Perspectives and Significance

Our findings have important clinical implications, which are supported by recent and ongoing epidemiological investigations.(7, 11, 12, 23) In a community based cohort, we observed that the prevalence of mild chronic renal insufficiency (i.e., calculated GFR < 60 ml/min) was 23%. (7) Importantly, those with reduced GFR were characterized by a greater prevalence of HF and greater structural remodeling of the heart compared to subjects with normal GFR. Indeed, in a further study involving normal subjects as well as subjects with hypertension and diastolic dysfunction, GFR was the strongest predictor for concentric hypertrophy independent of BP.(20) In the seminal report from the Dallas Heart Study, subjects with an elevated serum cystatin C, reflecting a reduction in renal function, had demonstrable BP independent changes in myocardial structure with increased LV mass .(23) Such clinical observations support the speculation that impaired kidney function is associated with release of factors (humoral and/or cellular) from the kidney that contribute to changes in myocardial function and structure. Cardiac remodeling and hypertrophy is known to be an important risk factor in humans with ESRD (30-32), thus our findings have important clinical implication because of the possible consequences the development of cardiac remodeling and hypertrophy could have in patients with reduced renal mass or mild
renal disease over time. Of relevance, and because of the possible clinical implication this model may have for kidney donors, we need to emphasize that there is relevant literature regarding safety in these population. Indeed, in a retrospective study, Ibrahim et al (16) reported that kidney donors’ risk of CKD is similar to that of the general population and that quality of life and kidney function was preserved. It should be noted however, that other studies have reported that renal donation does result in a slight reduction in GFR of approximately 20 to 40 percent, and the presence of proteinuria has been observed.(28) It remains, however, unknown whether such small reduction in renal function and the increase in proteinuria are associated with cardiac impairments in these otherwise normal individuals. Indeed, to date, detailed examination of the heart in kidney donors has not occurred and we have no data to understand the myocardial response in the setting of a reduction in renal mass by UNX. Cardiac surveillance studies should be considered in kidney donors to assess the myocardial response to UNX and the possible role of other cardiovascular risks in this population.

In summary, our study indicates that structural, functional and genetic changes occur in the LV myocardium after UNX. Future studies are necessary to address the precise mechanisms of this kidney-heart connection and possible clinical implications in the setting of mild renal disease and uninephrectomy.
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References


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Figure and Table Legends

Figure 1. MAP (mean arterial blood pressure) assessed continuously by telemetry. Measurements were continuously and averaged at: a week before performing UNX (Pre) and at Weeks (Wk) 1, 2, 3 and 4. All data are expressed as mean ± SEM. The comparison between the two groups was done by two-way ANOVA and each time point was compared between groups with t-test. There was no statistical difference between groups.

Figure 2. Left ventricular fibrosis at 4 weeks. (%) Representative graphic and histology (10x). Picrosirius Red stain. Sham on left, UNX on right.

Figure 3. Left ventricular gene microarray of Sham and UNX. Apoptosis and TGF-β related genes altered at 4 and 16 weeks after UNX. (Genesifter® software, Geospiza, Inc., Seattle, WA) Red color means increased expression of the gene and green color decreased expression as compared to Sham. Apoptosis related genes (4 weeks): genes 1 to 22 have increased its expression and genes 23 to 47 have decreased its expression in the LV of the UNX group compared to Sham. TGF-β related genes (4 weeks): Genes 1 to 3 have increased its expression and genes 4 and 5 have decreased its expression in the LV of the UNX group compared to Sham. Apoptosis related genes (16 weeks): Genes 1 to 41 have increased its expression and genes 42 to 96 have decreased its expression in the LV of the UNX group compared to Sham. TGF-β related genes (16 weeks): Gene 1 has increased its expression and genes 2 to 7 have decreased its expression in the LV of the UNX group compared to Sham.

Figure 4. Left ventricular apoptosis at 4 weeks. (TUNEL, Trevigen, Gaithersburg, MD). Sham and UNX. Blue color reveals TUNEL positive nuclei. Sham on left, UNX on right.

Figure 5. Left ventricular fibrosis at 16 weeks. (%) Representative graphic and histology (10x). Picrosirius Red stain. Sham on left, UNX on right.

Table 1. Cardiorenal and humoral data at 4 weeks. MBP (mean blood pressure), GFR (glomerular filtration rate), RBF (renal blood flow), U Vol (urinary volume excretion), UNaV (urinary sodium excretion), U Prot (poretnuria), BNP (brain natriuretic peptide), cGMP (cyclic guanosine monophosphate), PRA (plasma renin activity). All data are expressed as mean ± SEM. The comparison between each measurement was performed by t-test. Significant difference was accepted at p<0.05. *(significantly different versus sham).

Table 2. Echocardiographic profile at 4 weeks. HR (heart rate), SWTd (diastolic septal wall thickness), PWTd (diastolic posterior wall thickness), LVEDd (left ventricular
end diastolic diameter), RWT (relative wall thickness), EF (ejection fraction), LV mass (left ventricular mass), Cs-C (peak circumferential contraction strain), Csr-C (peak circumferential contraction strains rate), Csr-E (early diastolic strain rate), Csr-A (late relaxation circumferential strain rate), Csr-E/A (early diastolic strain rate/late relaxation circumferential strain rate ratio). All data are expressed as mean ± SEM. The comparison between each measurement was performed by t-test. Significant difference was accepted at p<0.05. *(significantly different versus sham).

Table 3. Cardiorenal and humoral data at 16 weeks. MBP (mean blood pressure), GFR (glomerular filtration rate), RBF (renal blood flow), U Vol (urinary volume excretion), UNaV (urinary sodium excretion), U Prot (proteinuria), BNP (brain natriuretic peptide), cGMP (cyclic guanosine monophosphate), PRA (plasma renin activity). All data are expressed as mean ± SEM. The comparison between each measurement was performed by t-test. Significant difference was accepted at p<0.05. *(significantly different versus sham).

Table 4. Echocardiographic profile at 16 weeks. HR (heart rate), SWTd (diastolic septal wall thickness), PWTd (diastolic posterior wall thickness), LVEDd (left ventricular end diastolic diameter), RWT (relative wall thickness), EF (ejection fraction), LV mass (left ventricular mass), Cs-C (peak circumferential contraction strain), Csr-C (peak circumferential contraction strains rate), Csr-E (early diastolic strain rate), Csr-A (late relaxation circumferential strain rate), Csr-E/A (early diastolic strain rate/late relaxation circumferential strain rate ratio). All data are expressed as mean ± SEM. The comparison between each measurement was performed by t-test. Significant difference was accepted at p<0.05. *(significantly different versus sham).
Figure 2

- **Picrosirius red staining (%)**
  - **Sham**: 2%
  - **UNX**: 4%, P<0.01

Images showing the staining patterns for Sham and UNX treatment groups.
Fig 3

Apoptosis Pathway

4 weeks

- V-sea murine thymoma viral oncogene homolog 2
- Voltage-dependent anion channel 1
- Seven in absentia 1A
- TNFRSF1A-associated via death domain
- Mitochondrial ubiquitin ligase activator of NFκB 1
- Splicing factor 1
- TGF-β-associated cell death containing protein kinase 1
- Son of sevenless homolog 1 (Drosophila)
- Wilms tumor 1
- Activin A receptor, type I
- Calcineurin 10
- Eph receptor A2
- Activin A receptor, type II
- Splicing factor 1
- V-rel reticuloendotheliosis viral oncogene homolog A (Avian)
- Humicrine receptor FG2, ligand-gated ion channel 1
- B-cell CLL/Lymphoma 6
- Akt2 cardiac myocytes
- TAF9B RNA polymerase II, TATA box binding protein (TBP)-associated factor
- Serine/threonine kinase 4
- Protein disulphide isomerase family A, member 2
- Angiopoietin 4
- MRT (mesothelioma region Y)-box 1
- Calcium channel, voltage-dependent, alpha 1A subunit
- Polycomb (DNA-directed), beta
- Epithelial cell transforming sequence 1 oncogene
- Similar to transcription factor 7-like 2, T-cell specific, HMGB-box
- Hes1 derived growth factor receptor, beta polypeptide
- Neurophin 3
- Epidermal growth factor receptor
- ApoC-Ga synthetase bubblegum family member 1
- Bcl2l1a IAP repeat-containing 3
- Unc-5 homolog B (C. elegans)
- DIP10 nuclear body protein
- Serine (or cysteine) peptidase inhibitor, clade D, member 9
- Unc-5 homolog B (C. elegans)
- ATP-binding cassette, sub-family C (CFTR/MRP), member 12
- Cell death inducing DFFA-like effector c
- Tumor necrosis factor receptor superfamily, member 11b
- Intra-liker growth factor binding protein 3
- Forkhead box O1
- Egr-1 kinase (TRKA) activating factor 13
- Triple functional domain (PTPRF interacting)
- Myocyte enhancer factor 2a
- Heat shock factor 2a
- Ee5d enhancer factor type B
- B-cell translocation gene 2, anti-proliferative

16 weeks

- X-ray repair complementing defective repair in Chinese hamster cells 4
- Interleukin 1 beta
- Ghrilin/obestatin propeptide
- Kalirin, RhogEF kinase
- BCL-2-related ovarian killer
- Keratin 18
- Inhibin alpha
- Tumor necrosis factor receptor superfamily, member 8
- B-cell CLL/Lymphoma 6
- Cannabinoid receptor 1 (brain)
- Matrix metalloproteinase 2
- Aryl hydrocarbon receptor
- Glucocorticoid receptor, oleosin-related 4
- Ring finger and FYVE like domain containing protein
- Calcium channel, voltage-dependent, alpha 1A subunit
- Mat, T-cell differentiation protein
- SMAO family member 6
- UOP-Gal:betaGalNAc beta 1, 4-galactosyltransferase, polypeptide 1
- Smooth muscle alpha-actin
- Paired box 4
- Albumin
- Tnsd 4
- Glutamate receptor, ionotropic, N-methyl D-aspartate 2A
- Interleukin 1 receptor antagonist
- Sequestosome 1
- Heart and neural crest derivatives expressed 2
- Catalase
- T-cell lymphoma invasion and metastasis 1
- Insulin-like 3
- Mib homolog 1
- Bcl2l1a IAP repeat-containing 5
- UOP-Gal:betaGalNAc beta 1, 4-galactosyltransferase, polypeptide 1
- Heat shock factor 1, polypeptide homolog (mouse)
- Catalase
- 15-lipoxygenase
- Eosinophilic granulocyte 2
- T-cell receptor alpha
- Ubiquitin specific peptidase 7-like (herpes virus-associated)
- Siah binding protein 1; FIP interacting repressor, pyrimidine tract binding spl...
- V-rel reticuloendotheliosis viral oncogene homolog A (Avian)

TGF-β Pathway

4 weeks

- Activin A receptor type I-like 1
- Activin A receptor, type I
- Activin A receptor, type II
- Hes1 derived growth factor receptor, beta polypeptide
- Nemo like kinase
- Smad family member 6
- Mthr serine peptidase 2
- Heat shock protein 5
- C-Jun
- C-Jun
- C-Jun
- C-Jun

16 weeks

- Smad family member 6
- Mthr serine peptidase 2
- Heat shock protein 5
- C-Jun
- C-Jun
- C-Jun
- C-Jun
- Interferon, alpha-inducible protein 27 like 1
- Death-associated protein kinase 2
- Phosphatase 2 (formerly 2A), regulatory subunit B (PR 52), beta isoform
Figure 5

Picrosirius red staining (%)

- Sham
- UNX

P = 0.0013
# Cardiorenal and Humoral Data at 4 weeks

<table>
<thead>
<tr>
<th></th>
<th>SHAM</th>
<th>UNX</th>
</tr>
</thead>
<tbody>
<tr>
<td>MBP (mm Hg)</td>
<td>89.8±2.5</td>
<td>92.5±2.6</td>
</tr>
<tr>
<td>GFR (ml/min)</td>
<td>2.9±0.3</td>
<td>2.1±0.2</td>
</tr>
<tr>
<td>RBF (ml/min)</td>
<td>8.0±0.7</td>
<td>4.7±0.6*</td>
</tr>
<tr>
<td>U Vol (ml/24hr)</td>
<td>22.5±2.8</td>
<td>21.6±2.2</td>
</tr>
<tr>
<td>UNaV (mEq/24hr)</td>
<td>1.3±0.2</td>
<td>1.4±0.2</td>
</tr>
<tr>
<td>U Prot (mg/24hr)</td>
<td>14.3±2.8</td>
<td>11.2±1.8</td>
</tr>
<tr>
<td>BNP (pg/ml)</td>
<td>17.1±0.8</td>
<td>16.0±1.9</td>
</tr>
<tr>
<td>cGMP (pg/ml)</td>
<td>5.5±0.6</td>
<td>6.5±0.6</td>
</tr>
<tr>
<td>Aldosterone (ng/dl)</td>
<td>16.7±3.2</td>
<td>30.4±6.1</td>
</tr>
<tr>
<td>PRA (ng/ml/hr)</td>
<td>30.2±2.0</td>
<td>32.5±1.1</td>
</tr>
</tbody>
</table>
Table 2

### Echocardiographic Profile at 4 weeks

<table>
<thead>
<tr>
<th></th>
<th>SHAM</th>
<th>UNX</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (bpm)</td>
<td>377±21</td>
<td>395±41</td>
</tr>
<tr>
<td>SWTd (mm)</td>
<td>1.7±0.1</td>
<td>1.5±0.1*</td>
</tr>
<tr>
<td>PWTd (mm)</td>
<td>1.7±0.1</td>
<td>1.5±0.1*</td>
</tr>
<tr>
<td>LVEDd (mm)</td>
<td>7.3±0.5</td>
<td>7.6±0.3</td>
</tr>
<tr>
<td>RWT</td>
<td>0.46±0.04</td>
<td>0.40±0.02*</td>
</tr>
<tr>
<td>EF (%)</td>
<td>77±4</td>
<td>77±2</td>
</tr>
<tr>
<td>LV mass (g)</td>
<td>0.86±0.14</td>
<td>0.79±0.08</td>
</tr>
<tr>
<td>Cs-C (%)</td>
<td>17.3±1.9</td>
<td>16.5±2.0</td>
</tr>
<tr>
<td>Csr-C (s⁻¹)</td>
<td>4.4±0.7</td>
<td>4.2±0.5</td>
</tr>
<tr>
<td>Csr-E (s⁻¹)</td>
<td>5.4±0.4</td>
<td>4.1±1.1*</td>
</tr>
<tr>
<td>Csr-A (s⁻¹)</td>
<td>3.0±0.8</td>
<td>4.0±1.1*</td>
</tr>
<tr>
<td>Csr-E/A</td>
<td>2.0±0.8</td>
<td>1.1±0.4*</td>
</tr>
<tr>
<td></td>
<td>SHAM</td>
<td>UNX</td>
</tr>
<tr>
<td>----------------------</td>
<td>------------</td>
<td>-----------</td>
</tr>
<tr>
<td><strong>MBP (mm Hg)</strong></td>
<td>87.7±3.4</td>
<td>80.7±4.0</td>
</tr>
<tr>
<td><strong>GFR (ml/min)</strong></td>
<td>3.3±0.5</td>
<td>1.6±0.3 *</td>
</tr>
<tr>
<td><strong>RBF (ml/min)</strong></td>
<td>6.5±1.5</td>
<td>5.3±0.8</td>
</tr>
<tr>
<td><strong>U Vol (ml/24hr)</strong></td>
<td>18.1±1.2</td>
<td>25.6±6.7</td>
</tr>
<tr>
<td><strong>UNaV (mEq/24hr)</strong></td>
<td>1.1±0.2</td>
<td>1.2±0.2</td>
</tr>
<tr>
<td><strong>U Prot (mg/24hr)</strong></td>
<td>16.2±3.8</td>
<td>95.9±33.8</td>
</tr>
<tr>
<td><strong>BNP (pg/ml)</strong></td>
<td>8.04±0.3</td>
<td>10.1±0.7 *</td>
</tr>
<tr>
<td><strong>cGMP (pg/ml)</strong></td>
<td>6.9±1</td>
<td>12.2±1.3 *</td>
</tr>
<tr>
<td>**Aldosterone (ng/dl)</td>
<td>20.9±3.2</td>
<td>44.2±7 *</td>
</tr>
<tr>
<td><strong>PRA (ng/ml/hr)</strong></td>
<td>17.7±0.8</td>
<td>19.0±0.3</td>
</tr>
</tbody>
</table>
**Table 4**

**Echocardiographic Profile at 16 weeks**

<table>
<thead>
<tr>
<th>Metric</th>
<th>Sham</th>
<th>UNX</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR (bpm)</td>
<td>377±26</td>
<td>382±22</td>
</tr>
<tr>
<td>SWTd (mm)</td>
<td>1.8±0.1</td>
<td>1.8±0.1</td>
</tr>
<tr>
<td>PWTd (mm)</td>
<td>1.7±0.1</td>
<td>1.9±0.1*</td>
</tr>
<tr>
<td>LVEDd (mm)</td>
<td>7.8±0.5</td>
<td>8.7±0.7*</td>
</tr>
<tr>
<td>RWT</td>
<td>0.45±0.04</td>
<td>0.43±0.04</td>
</tr>
<tr>
<td>EF (%)</td>
<td>79±4</td>
<td>74±2*</td>
</tr>
<tr>
<td>LV mass (g)</td>
<td>1.03±0.11</td>
<td>1.32±0.19*</td>
</tr>
<tr>
<td>Cs-C (%)</td>
<td>18.1±1.9</td>
<td>13.6±1.6*</td>
</tr>
<tr>
<td>Csr-C (s⁻¹)</td>
<td>5.1±0.4</td>
<td>3.5±0.4*</td>
</tr>
<tr>
<td>Csr-E (s⁻¹)</td>
<td>4.9±0.9</td>
<td>3.5±0.6*</td>
</tr>
<tr>
<td>Csr-A (s⁻¹)</td>
<td>3.4±0.8</td>
<td>3.5±0.7</td>
</tr>
<tr>
<td>Csr-E/A</td>
<td>1.5±0.5</td>
<td>1.0±0.3*</td>
</tr>
</tbody>
</table>