Cardiac Hypertrophy and Cardiac Cell Death in Chronic Kidney Disease

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ABSTRACT: **Background:** Chronic kidney disease (CKD) is a prevalent clinical condition affecting 15% of the general population. Cardiorenal syndrome (CRS) type 4 is characterized by an underlying CKD condition leading to impairment of cardiac function and increased risk for major cardiovascular events. To date, the mechanisms leading from CKD to CRS are not completely understood. In particular, it is unclear whether the pathological changes that occur in the heart in the setting of CKD involve enhanced cell death of cardiac cells.

Objectives: To assess whether CKD may mediate loss of cardiac cells by apoptosis.

Methods: We established rat models for CKD, acute myocardial infarction (acute MI), left ventricular dysfunction (LVD), and sham. We measured the cardiac-to-body weight as well as kidney-to-body weight ratios to validate that renal and cardiac hypertrophy occur as part of disease progression to CRS. Cardiac cells were then isolated and the percent of cell death was determined by flow cytometry following staining with annexin-FITC and propidium iodide. In addition, the levels of caspase-3-dependent apoptosis were determined by Western blot analysis using an anti-cleaved caspase-3 antibody.

Results: CKD, as well as acute MI and LVD, resulted in significant cardiac hypertrophy. Nevertheless, unlike the increased levels of cell death observed in the acute MI group, in the CKD group, cardiac hypertrophy was not associated with induction of cell death of cardiac cells. Caspase-3 activity was even slightly reduced compared to sham-operated controls.

Conclusions: Our data show that while CKD induces pathological changes in the heart, it does not induce cardiac cell death.

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KEY WORDS: cardiorenal syndrome (CRS), chronic kidney disease (CKD), cleaved caspase-3, cardiac cell death, left ventricular hypertrophy

Cardiorenal syndrome (CRS) can generally be defined as a pathophysiological disorder of the heart and kidneys where acute or chronic failure in one organ affects the other [1]. The current classification of CRS includes five subtypes, according to the different pathogenesis whose etymology reflects the primary and secondary pathologies.

The rat model used in the current work represents type IV CRS, which is characterized by primary chronic kidney disease (CKD) leading to an impairment of cardiac function, left ventricular hypertrophy (LVH), diastolic dysfunction, and/or increased risk of adverse cardiovascular events [2]. Recently it became apparent that CKD is prevalent in 15% of the general population. Moreover, it has been established that cardiovascular involvement occurs at each stage of CKD and major cardiac events cause almost 50% of deaths in CKD patients [3]. Several studies, including ours, have recently shown that CRS type IV leads to fibrosis, tubular cell apoptosis and further diminishing of renal function [4,5]. Likewise, systolic dysfunction associated with heart failure and cardiomyopathy is known to involve myocyte apopotosis [6-9]. Indeed, cardiomyocyte apoptosis has been demonstrated in animal models of cardiac injury as well as in patients with congestive heart failure or acute myocardial infarction. Therefore, apoptosis has been proposed as an important process in cardiac remodeling and progression of heart failure. However, the mechanisms underlying cardiac apoptosis are poorly understood.

In the current study we wished to assess whether CKD may mediate loss of cardiac cells through apoptosis. Apoptosis refers to cell death that occurs in a programmed manner, characterized by cellular condensation [8,10]. After a cell receives a stimulus, it undergoes organized degradation of cellular organelles by activated proteolytic caspases.

The caspases are a family of proteins representing one of the main executors of the apoptotic process. Induction of apoptosis via death receptors typically results in the activation of an initiator caspase such as caspase-8 or caspase-10. These caspases can then activate other caspases in a cascade. The cascade eventually leads to the activation of the effector caspases, such as caspase-3 and caspase-6 which mediate the cleavage of critical cellular substrates, including poly(ADP-ribose) polymerase and

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lamins, leading to the typical morphological changes observed in cells undergoing apoptosis [11].

Caspase-3 is an excellent accepted marker for detection of apoptosis in various cell types [9]. The caspase-related apoptosis pathway has been shown to take place during cardiocyte loss. Studies in neonatal cardiomyocytes have shown activation of a mitochondrial pathway characterized by the increase in cytosolic cytochrome C and the activation of caspase-3 and 9 [8]. Another important pathway of caspase activation is through a mitochondrion-independent mechanism that uses cell death receptors (e.g., Fas and tumor necrosis factor receptor). Expression of the death receptor Fas has been shown to be upregulated on cardiomyocytes during myocardial ischemia [10,12].

In the present study, we evaluated the levels of cell death of cardiac cells derived from a rat model for CKD by flow cytometry. In addition, we assessed the levels of caspase-dependent apoptosis in these cells. The general purpose was to evaluate whether cardiac cells undergo enhanced apoptosis in the setting of CKD. We anticipate that the data obtained in the current study will help us better understand the patho-mechanisms of CRS type IV.

MATERIALS AND METHODS

IN VIVO MODEL

The experimental protocol was approved by the Institutional Review Board Committee for Animal Experimentation at the Tel Aviv Sourasky Medical Center and conforms to the Guide for the Care and Use of Laboratory Animals published by the U.S, National Institutes of Health. We employed a rat model for cardio-renal syndrome, as previously described by us [13]. Briefly, Lewis rats (300–350 g body weight) underwent subtotal nephrectomy (STN) in two subsequent surgeries under anesthesia with ketamine (50 mg/kg) and xylazine (10 mg/ml): two-thirds of the left kidney was removed initially, followed by removal of the right kidney one week later. This phase of the experiment was associated with 70% survival of the animals that underwent STN. Renal dysfunction was validated by creatinine clearance test (CCT) and by measuring the blood urea nitrogen (BUN) levels, as previously reported [4].

Acute myocardial infarction (acute MI) was induced by ligation of the left anterior descending (LAD) coronary artery after chest opening. Prior to all surgical procedures, animals were anesthetized with ketamine and xylazine. The study included four experimental arms (6–10 animals/group): (i) CKD only: the animals were terminated 4 weeks after completion of the 5/6 STN; (ii) acute MI: the animals were terminated 5 days following LAD ligation; (iii) left ventricular dysfunction (LVD) due to acute MI: the animals were sacrificed 30 days later; and (iv) sham-operated control animals, which included abdominal and chest opening only. Prior to termination the animals were weighted. Promptly after termination, kidneys and hearts were removed and weighed.

ECHOCARDIOGRAPHY

Cardiac function was evaluated 1 week prior to termination by transthoracic echocardiography (Acuson, Sequoia, Siemens, Washington DC, USA) as described previously [14]. Left ventricle end-diastolic diameter (LVEDD), left ventricle end-systolic diameter (LVESD), intraventricular septal wall thickness and posterior wall thickness were measured in systole and diastole to determine the presence of hypertrophy.

EX VIVO ANALYSIS

Following termination, the hearts were removed. The left ventricles (LV) were cut into transverse sections which were further divided for the two assays described below:

- *Flow cytometry analysis of the percentage of apoptotic cells:* LV cells were isolated after mechanically mincing the LV sections followed by 1 hour incubation with collagenase type IV, as we previously reported [15]. The cells were then stained with annexin V and propidium iodide (PI) (Abcam, USA), and the percentages of the non-viable cells (i.e., cells that stained positive for annexin, PI or both annexin and PI) were determined by flow cytometry (BD Biosciences, FACSCanto II, USA).
- *Caspase-3 determined by Western blot analysis:* Briefly, LV sections were extracted using a commercial lysis buffer (Sigma, Israel). Equal protein amounts (80 μg) were loaded on a 4–20% acrylamide gel followed by electric transfer to nitrocellulose membranes. Following an overnight blocking with 5% low fat milk diluted in TBS-tween, the membranes were incubated with an anti-cleaved caspase-3 (Cell Signaling, USA), anti-TRPV2 (Alamone, acc-039) or with an anti-GAPDH antibody used for validating equal loading (Abcam, clone 6C5, USA). The primary antibodies were followed by blotting with HRP-conjugated secondary antibodies. After rapid incubation with an ECL substrate (Biological Industries, Israel), the membranes were exposed to an imaging film.

STATISTICAL ANALYSIS

Groups were compared using one-way ANOVA. The Tukey post hoc correction was taken to account for multiple testing (IBM SPSS statistics 20 software). Significance was set at $P < 0.05$ ($P < 0.05$, $P < 0.01$). Results are expressed as means ± SE.

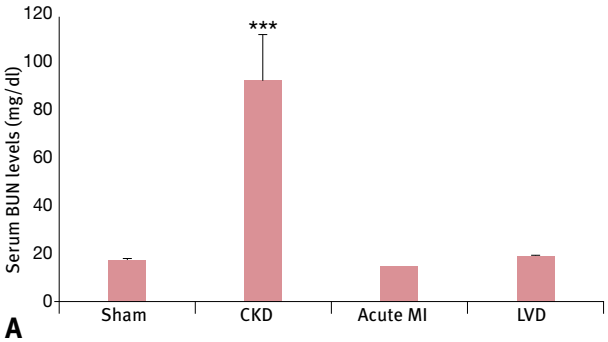
RESULTS

CKD INDUCES HYPERTROPHY

In the current study we induced short-term CKD by 5/6 nephrectomy (4 weeks) as well as acute MI by LAD ligation (the animals were sacrificed 5 days post-infarction) and LVD

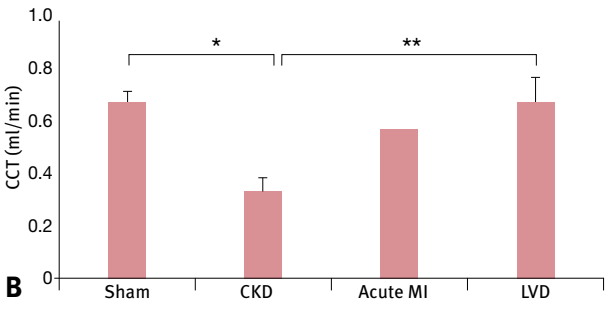
due to acute MI (animals were terminated 30 days post-LAD occlusion). In order to validate establishment of the CKD model we calculated the following parameters following termination: mean BUN and CCT values as well as mean heart weight/body weight (BW) and kidney weight/BW ratios. BUN levels were significantly higher in the sera of the CKD animals (93.7 ± 19 mg/dl) compared to all other experimental groups (17.6 ± 0.4, 18.9 ± 0.5 and 14.3 ± 0.3 mg/dl) for sham, LVD and acute MI, respectively; $P < 0.001$ [Figure 1A], indicating that 5/6 nephrectomy indeed induces a chronic state of CKD. In addition, as documented in Figure 1B, the mean CCT value of the CKD animals (0.29 ± 0.06 ml/min) was significantly lower relative to the sham animals (0.67 ± 0.046 ml/min) and LVD (0.67 ± 0.1 ml/min, $P < 0.02$), and also insignificantly lower compared to acute MI (0.57 ± 0.003 ml/min, $P = 0.5$). Thus, the mean CCT value of the acute MI group was slightly lower compared to the mean values of the sham or LVD, though this reduction was not statistically significant ($P < 0.74$), suggesting that acute MI may cause short-term renal injury.

Figure 1. 5/6 nephrectomy induces CKD in the experimental animals
[A] Mean values of sera BUN levels (mg/dl) in the experimental groups determined after termination, i.e., 4 weeks upon completion of the 5/6 nephrectomy (CKD group), 5 days following LAD ligation (acute MI group) and 30 days after LAD occlusion (LVD)



***$P < 0.001$; n=7 (CKD), n=10 (LVD), n=6 (acute MI), and n=6 (sham)

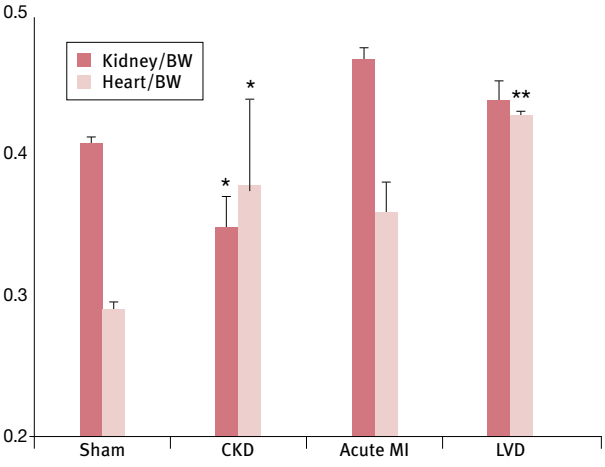
[B] CCT levels calculated using the following formula: [Cr (urine)/ Cr (serum)* urine volume]/ 1440 [4]



*$P < 0.05$, ***$P < 0.001$; n=6 (CKD), n=9 (LVD), n=6 (acute MI) and n=4 (sham)

In order to assess potential renal and cardiac hypertrophy, the animals were weighed prior to termination and the kidneys/remaining kidneys as well as the hearts were weighed immediately after necropsy. As expected, due to the 5/6 nephrectomy, the kidney/BW ratio was lower in the CKD group (mean ratio 0.34 ± 0.02) compared to the three other experimental groups (mean ratio > 0.4). Interestingly, the mean ratio for kidney/BW was slightly higher in the LVD (0.44 ± 0.014) and acute MI (0.46 ± 0.08) compared to the sham-operated controls (0.40 ± 0.005), though non-significantly ($P < 0.1$), indicating that the cardiac disease may lead to renal hypertrophy. Interestingly, heart weight/BW ratio was significantly increased in the CKD group relative to the sham group (0.38 ± 0.06 and 0.29 ± 0.006 for CKD and sham groups respectively, $P = 0.028$). Concomitantly, as expected, heart weight/BW was markedly elevated in the LVD group (0.42 ± 0.02) compared to sham (0.29 ± 0.006), $P < 0.001$. Acute MI also resulted in a non-significant elevation in the heart weight/BW ratio relative to sham (0.36 ± 0.008, $P = 0.13$). The data indicate that short-term CKD (4 weeks) is sufficient to induce pathological changes in the heart [Figure 2]. It is important to note, however, that the calculated decline in heart/body weight ratio does not only reflect cardiac hypertrophy but also body weight loss of 10%, which can be attributed to uremia in the CKD setting (406.5 ± 8.6 g in sham vs. 362.8 ± 15.1 g in CKD) [Table 1]. Cardiac hypertrophy was further supported by the echocardiography data indicating wall thickness and the significant decrease in LVESD. Surprisingly, echocardiography indicated a moderate

Figure 2. CKD promotes cardiac hypertrophy. Prior to termination, the animals were weighed and the mean values for each experimental group obtained. After the animals were sacrificed, one kidney (or remaining kidney) and the heart were weighed. The mean heart weight/BW and kidney weight/BW were calculated



*$P < 0.05$, **$P < 0.01$. n=7 (CKD), n=10 (LVD), n=6 (acute MI) and n=6 (sham)

increase in fractional shortening, demonstrating that LVH did not impair systolic cardiac function [Table 1].

CARDIAC HYPERTROPHY AND APOPTOSIS OF CARDIAC CELLS

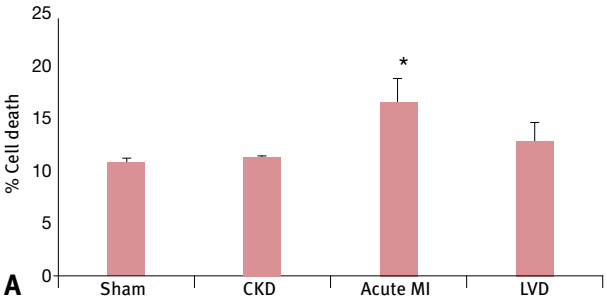
Cardiac cell suspensions from LV sections, which may consist of various cell populations, including cardiomyocytes as well as cardiac fibroblasts, endothelial cells and immune cells, were analyzed for cell viability by flow cytometry analysis. We assessed the percentage of non-viable cells (annexin-FITC-positive, PI-positive or annexin-positive and PI-positive) in total cells isolated from the LV tissues of CKD, acute MI, LVD or sham animals (6 animals/arm). As expected, the data showed a 50% increase in the non-viable cardiac cells of the acute MI arm compared to sham (17.0 ± 2.3% vs. 11.0 ± 0.53 in acute MI versus

Table 1. Cardiac parameters derived from echocardiography and post-necropsy analysis of the heart

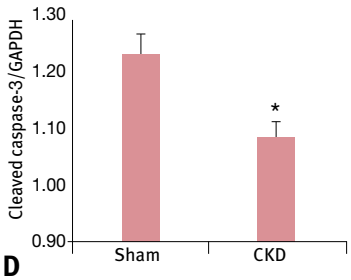
Cardiac parameters	Sham (n=6)	CKD (n=8)	*P* value
Body weight (g)	406.5 ± 8.6	↓ 362.8 ± 15.1	0.03
Heart weight (mg)	1.17 ± 0.008	↑ 1.31 ± 0.05	0.04
Heart/Body weight (mg/g, %)	0.36 ± 0.025	↓ 0.29 ± 0.006	0.004
Intraventricular septum diastole (mm)	1. 45 ± 0.08	↑ 1.62 ± 0.08	0.005
LV posterior wall thickness in diastole (mm)	1.62 ± 0.09	↑ 1.77 ± 0.08	0.04
LV end-diastolic diameter (mm)	6.18 ± 0.48	5.87 ± 0.44	NS (0.55)
LV end -systolic diameter (mm)	3.47 ± 0.29	↓ 2.78 ± 0.11	< 0.05
Fractional shortening (%)	44.7 ± 1.3	↑ 51.9 ± 1.4	0.005

Figure 3. Cardiac cell death is promoted following acute MI but not upon CKD. LV sections were mechanically and enzymatically digested to single cell suspensions. First, the cells were double-stained with annexin-FITC and PI, and then subjected to cell death analysis by FACS

[A] Bar graph showing the average percentage of cell death (cells stained positive with either annexin only, PI only or both annexin-PI)

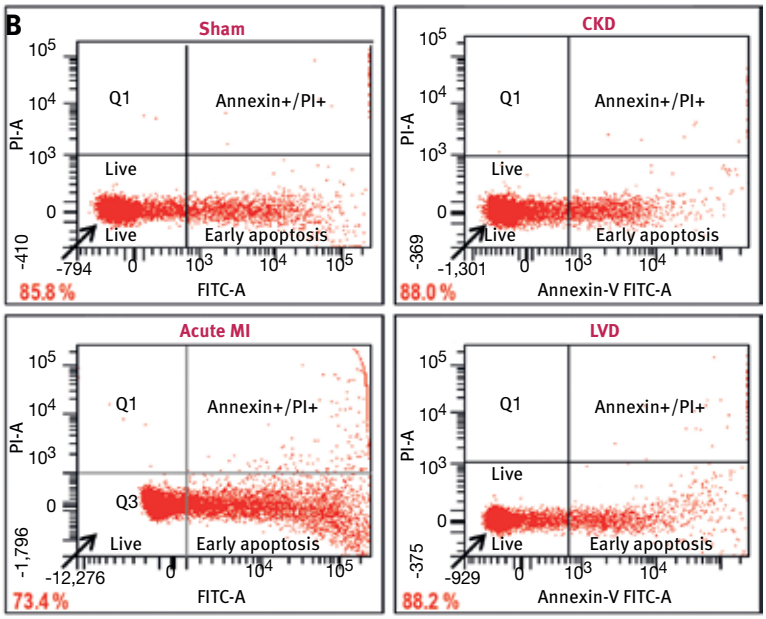


[C] In a second set of experiments, LV lysates of CKD or sham-operated animals were subjected to Western analysis with an antibody that recognizes the cleaved form of caspase-3. GAPDH antibody served as control for equal loading of the samples



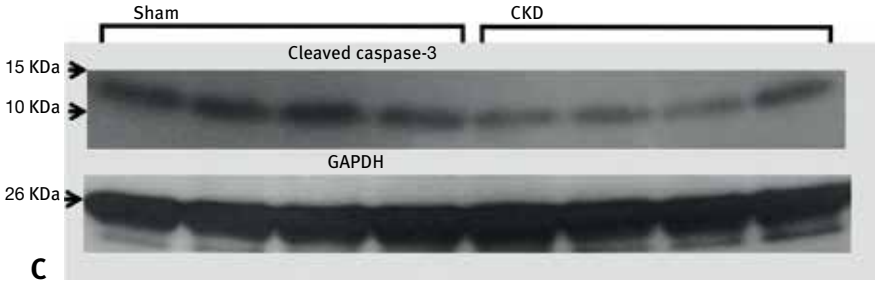
*P < 0.05, n=4 (sham and CKD)

[B] Representative captures of the annexin-PI staining in each one of the four experimental groups



*P < 0.05. n=6 (CKD, LVD, acute MI, sham). An = Annexin-FITC only

[D] A representative Western blot capture



sham respectively, $P = 0.02$). However, as documented in Figure 3 A and B, the CKD as well as the LVD groups did not show any significant enhancement of cardiac cell death compared to sham (12.0 ± 2.3%, 13 ± 2.1% and 11.0 ± 0.53% in CKD, LVD and sham groups, respectively, $P > 0.05$). The data show that the cardiac hypertrophy induced in CKD does not seem to involve loss of cardiac cells. Further, since enhanced caspase-3 activity is a known hallmark of apoptosis-induced cell death, we thought to assess the expression levels of cleaved caspase-3 in LV sections of CKD animals in order to confirm that CKD does not induce apoptosis of cardiac cells. To this end, we performed a Western blot analysis for LV sections of CKD and sham animals (n=4/group) and quantitated the levels of the cleaved form of caspase-3. In line with the flow cytometry data, the Western analysis did not show any augmentation in the cleaved form of caspase-3. On the contrary, the data pointed to a 15% decrease in the levels of cleaved caspase-3 in the CKD samples compared to sham ($P = 0.02$, Figure 3 C and D). The data indicate that the clinical presentation of CKD does not lead to cardiac cell death.

DISCUSSION

Cardiac remodeling as well as myocardial fibrosis is frequently observed in early-stage CKD patients [16,17]. Cardiac hypertrophy, the primary mechanism for cardiac remodeling, is associated with a significant increase in the risk of heart failure, dilated cardiomyopathy, ischemic heart disease, and sudden death, leading to increased cardiovascular mortality [18,19]. In line with the data gathered from CKD patients, using a rat model for short-term CKD we showed that CKD leads to increased cardiac interstitial fibrosis with no apparent reduction in systolic LV function [13]. On the other hand, we have shown that LVD leads to prominent expansion of the tubuli in the medullari sections of the kidney, accompanied by interstitial fibrosis and interstitial inflammation, a pathological presentation that deteriorates markedly when CKD is followed by LVD due to acute MI [4]. Using an Affimetrix GeneChip array analysis of cardiac sections in CKD versus sham-operated controls, we observed two major gene clusters being modified in CKD compared to sham [13]. The first cluster included upregulation of genes affiliated with ribosome biogenesis and translational elongation. This process represents cardiac hypertrophy which occurs in CKD. The upregulation of protein synthesis combined is highly associated with tissue hypertrophy, as it is well established that protein synthesis is a classical feature of cardiomyocyte hypertrophy [20,21]. Nevertheless, it should be noted that our gene chip array analysis did not show any increase in the expression of specific cardiac hypertrophy genes such as *BNP*, *ANP* or *ACTA1* coding for skeletal actin [13]. We thus believe that enhanced protein synthesis may represent the first steps of cardiac hypertrophy, which may finally culminate in overexpression of the classical hypertrophic genes during the course of disease progression. This process may eventually lead to heart failure, even if ejection fraction is still preserved, as documented in the current study and as previously suggested [22]. Indeed it is currently established that about 74% of stage 5 CKD patients suffer from left ventricular hypertrophy, with or without LV systolic impairment, at the initiation of renal replacement therapy [23].

The other major gene cluster that was dramatically downregulated in CKD included genes affiliated with ubiquitin-dependent proteolysis and proteosome biosynthesis, suggesting reduced cell death in the CKD setting. The GeneChip data also demonstrated that while the expression level of the *Casp3* gene, coding for caspase-3, is mildly overexpressed in the setting of acute MI (+1.22-fold, $P = 0.02$), this gene is slightly and non-significantly downregulated in CKD (-1.09-fold change, $P = 0.27$). The data presented here confirm the GeneChip data at the protein level. We indeed failed to observe induction of cell death in LV sections of CKD animals. Moreover, the expression level of cleaved caspase-3 was slightly reduced in cardiac sections of CKD compared to sham. Altogether, the results presented here suggest that cardiac hypertrophy in the setting of CKD is not associated with increased apoptosis or necrosis of the cardiac cells. The lack of enhanced cell death may "help" in the process of cardiac hypertrophy as part of a general attempt to maintain normal cardiac function, at least in the early stages of CKD.

STUDY LIMITATIONS

Previous studies using a similar rat model for 5/6 nephrectomy demonstrated a rise of systolic blood pressure from 120 mmHg in the control animals to 180 mmHg in the experimental animals [24,25]. We thus cannot exclude that the evolving cardiac hypertrophy described here is not a consequence of the uremic state but may indicate the combined effects of uremia and its resultant hypertension.

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