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Ferric Citrate Attenuates Cardiac Hypertrophy and Fibrosis in a Rat Model of Chronic Kidney Disease

Asaki Goto,[1,2,3,*] Yasunori Suematsu,[3,*] Ane CF Nunes,[1] Wanghui Jing,[1] Mahyar Khazaeli,[1] Wei Ling Lau,[1] Nosratola D Vaziri[1]

[1]Division of Nephrology and Hypertension, Department of Medicine, University of California, Irvine, USA
[2]Nephrology Section, Tibor Rubin VA Hospital, Long Beach, CA, USA
[3]Department of Cardiology, Fukuoka University School of Medicine, Fukuoka, Japan
*Both authors contributed equally to this manuscript.

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Introduction. Chronic kidney disease (CKD) promotes hypertrophy and fibrosis in heart, and increases the risk of cardiovascular mortality. Ferric citrate is a dietary phosphate binder used to control hyperphosphatemia in CKD patients. It has been shown to raise iron stores, improve anemia and secondary hyperparathyroidism, and decrease vascular calcification in CKD patients. The present study was done to explore the effects and mechanism of actions of ferric citrate on cardiac hypertrophy and fibrosis.
Materials and Methods. Male SD rats were randomized to CKD (5/6 nephrectomized) and sham-operated control groups. CKD rats were fed regular diet or a diet containing 4% ferric citrate. After 8 weeks, hemoglobin, renal function and cardiovascular endpoints including blood pressure, heart/body weight ratio, serum N-terminal prohormone of brain natriuretic peptide (NT-proBNP), cardiac histology and markers of hypertrophy, fibrosis and inflammation were assessed.
Results. Compared to the controls, untreated CKD group exhibited hypertension, elevated serum urea, creatinine, phosphate, and NT-proBNP concentrations, anemia, cardiomegaly,cardiac hypertrophy and fibrosis. Treatment with ferric citrate significantly increased hemoglobin and serum iron concentrations, reduced serum phosphate and NT-proBNP levels and ameliorated hypertension, heart/body weight ratio, cardiac hypertrophy, fibrosis and inflammation. In addition, ferric citrate administration reduced the size of cardiomyocytes and expressions of myocardin, transforming growth factor-β, interleukin-6 and monocyte chemotactic protein 1.
Conclusions. Treatment with ferric citrate attenuated renal failure and cardiovascular abnormalities including myocardial hypertrophy and fibrosis in CKD rats.

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INTRODUCTION

Chronic kidney disease (CKD) has emerged as a major public health issue worldwide. The prevalence of CKD in the United States has increased from 10% during the 1988-1994 periods to 13.1% in 1994-2004.[1] CKD results in hypertension, accelerated atherosclerosis, arteriosclerosis, and heart failure.[2] Cardiovascular disease (CVD) is the leading cause of premature mortality and accounts for 50% of death from congestive heart failure, acute myocardial infarction, and sudden cardiac death in the CKD population.[3] The kidneys and

heart have a complex relationship whereby renal failure can cause heart failure and vice versa; an interplay, which is termed cardiorenal syndrome (CRS). CRS type 4 is defined as chronic abnormalities in renal function that lead to cardiac disease and is characterized by left ventricular hypertrophy, fibrosis and diastolic dysfunction.[4,5] Ferric citrate is used as a dietary phosphate binder to control hyperphosphatemia in CKD and end stage renal disease (ESRD) patients.[6-9] It concurrently improves iron deficiency anemia by replenishing iron stores and has been shown to ameliorate secondary hyperparathyroidism and vascular calcification in CKD.[10-12] Based on the aforementioned data present study was undertaken to explore the nature and the underlying mechanisms of the effects of ferric citrate on CKD-associated cardiac abnormalities using the well-established rat model of experimental CKD induced by 5/6 nephrectomy.

MATERIALS AND METHODS

Study Groups

The study was approved by the University of California, Irvine Institutional Committee for the Use and Care of Experimental Animals. We purchased 8-week old male Sprague-Dawley rats from Charles River Labs (Raleigh, NC). They were housed in a climate-controlled vivarium with 12h day/night cycles and provided access to food and water ad libitum. The rats were randomized to the sham-operated control and CKD groups. The CKD rats were subjected to 5/6 nephrectomy by removing the upper and lower thirds of the de-capsulated left kidney, followed by right nephrectomy a week later. The control (CTL) group underwent sham operation. We induced anesthesia with 5% inhaled isoflurane (Piramal Clinical Care, Bethlehem, PA) which was maintained with 2-4% isoflurane. For pain relief, rats were given 0.05 mg/kg Buprenex (Reckitt Benckiser Pharmaceutical Inc., Richmond, VA, USA). We randomly assigned the CKD rats to regular diet or diet containing 4% ferric citrate for 6 weeks. The animals were then placed in metabolic cages for a 24h urine collection. We measured systolic blood pressure (SBP) by tail plethysmography as described previously.[13] The animals were euthanized by cardiac exsanguination under general anesthesia and plasma and heart tissue were collected. The heart was weighed and the left ventricle (LV) was dissected and cross-sectioned into two parts. The apex was fixed in 10% buffered formalin for histological analysis and the other portion was snap frozen in liquid nitrogen and stored at -80°C for protein analysis.

Blood and Urine Biochemical Assays

Serum urea nitrogen (BUN), calcium, phosphorus, iron, and serum and urine creatinine were determined using Quanti Chrom Assay Kits purchased from Bio Assay Systems (Hayward, CA). The blood hemoglobin was determined using the Aim Strip Hb meter (Ermarine Laboratories Inc., San Antonio, TX). Plasma N-terminal prohormone of brain natriuretic peptide (NT-proBNP) was measured using NT-proBNP ELISA Kit (Catalog, OKEH00475, Aviva Systems Biology Corporation, San Diego, CA).

Histologic Analysis

The heart apex was fixed in 10% buffered formalin and embedded into paraffin. Five-micron sections were stained with hematoxylin and eosin (H&E) and images were captured on a Nikon Eclipse 80i (Nikon Instruments Inc., Melville, NY) and analyzed using Image J software (version 1.47, National Institutes of Health, Bethesda, MD). Images of 10 fields per LV section at 400× magnification were obtained, and the size of cardiomyocytes was determined after calibration with a stage micrometer. The mean values were calculated per LV section and reported as square-micron.

Western Blot Analyses

Cytoplasmic protein extracts from the LV tissue were prepared and proteins of interest were quantified as previously described[14,15] using the following primary antibodies purchased from Abcam (Cambridge, MA): Rabbit antibodies against rat myosin heavy chain 7B (MyH7B, ab172967), myocardin (ab203614) and tropomyosin (ab133292), collagen 1 (ab34710), transforming growth factor-β (TGF-β, ab92486), interleukin-6 (IL-6, ab9324). Antibody against monocyte chemotactic protein 1 (MCP-1, NBP1-07035) was obtained from Novus Biologicals (Littleton, CO). Mouse antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (ab8245, Abcam) was utilized as the housekeeping control. Band intensities were measured using Image J software (version 1.47, National Institutes of Health, Bethesda, MD).

Statistical Analysis

Data are expressed as the mean ± standard error. Group differences were analyzed by one-way analysis of variance (ANOVA) with Tukey's post-test. Statistical significance was defined as a *P* value of less than .05. Statistical analysis was performed using GraphPad Prism, version 6.01 (GraphPad Software, Inc. San Diego, CA).

RESULTS

General Data

Data are summarized in Table 1. Compared to the CTL group the CKD animals had significantly higher arterial blood pressure, serum creatinine, BUN and phosphate levels and significantly lower creatinine clearance, blood hemoglobin and serum iron levels. Consumption of the 4% ferric citrate-containing diet resulted in significant reduction of blood pressure and serum phosphate values and significant increase in blood hemoglobin and serum iron concentration in the CKD animals. Serum NT-proBNP concentration was significantly increased in CKD animals when compared to the CTL group. Ferric citrate therapy reduced serum NT-proBNP levels in the CKD animals (Figure 1).

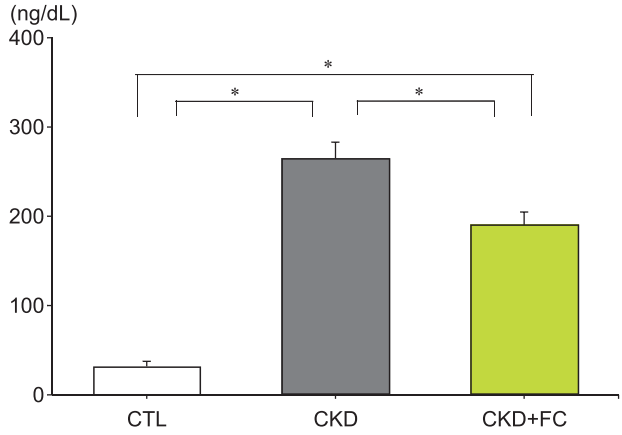

Figure 1. Serum N-terminal Pro Brain Natriuretic (NT-proBNP) Concentration
Data are mean ± SEM, n = 6 per group, * *P* < .05 (ANOVA)

Impact of CKD and Ferric Citrate Treatment on Cardiac Hypertrophy

Data are shown in Figure 2. The heart to body weight ratio (a marker of cardiac hypertrophy) was significantly increased in CKD animals when compared to the CTL group. Ferric citrate therapy resulted in significant improvement in the heart to body weight ratio in the CKD animals. Representative photomicrographs of H & E stained LV tissue cardiomyocytes are shown in Figure 2. The LV tissue from CKD animals exhibited significant hypertrophy with increased size of cardiomyocytes. Ferric citrate therapy significantly reduced the size of the LV cardiomyocyte in CKD rats. Furthermore, cardiac biomarkers of hypertrophy including MyH7B, myocardin and tropomyosin protein levels were significantly increased in the untreated CKD animals when compared to the CTL group. Ferric citrate therapy reversed upregulations of myocardin in the CKD animals. Although the levels of MyH7B and tropomyosin in ferric citrate treated group were lower than the untreated CKD group, the difference did not reach statistical significance.

Impact of CKD and Ferric Citrate Treatment on Biomarkers of Cardiac Fibrosis and Inflammation

Data are shown in Figure 3. The molecular

Table 1. Body Weight, Tail Blood Pressure, and Serum and Urine Biochemistries in the 3 Study Groups

	CTL (N = 6)	CKD (N = 6)	CKD+FC (N = 6)
Final Body Weight (g)	588.4 ± 31.4	497.3 ± 15.2	485.6 ± 8.2
Systolic Blood Pressure (mmHg)	111.2 ± 3.6	145.4 ± 2.1[a]	111.4 ± 4.1[b]
Serum Creatinine (mg/dL)	0.36 ± 0.1	0.46 ± 0.14[a]	0.35 ± 0.12[a]
Serum BUN (mg/dL)	10.1 ± 0.3	23.6 ± 1.5[a]	24.3 ± 1.3[a]
Creatinine Clearance (mL/min*kg)	298.6 ± 160.6	164.5 ± 25.3[a]	198.8 ± 23.9[a]
24h Urine Volume (mL)	14.3 ± 1.0	26.3 ± 1.9[a]	20.0 ± 0.5[a,b]
Serum Phosphate (mg/dL)	8.5 ± 0.2	12.2 ± 0.2[a]	8.7 ± 0.6[b]
Serum Calcium (mg/dL)	9.8 ± 0.7	9.0 ± 0.5	9.8 ± 0.3
Hemoglobin (g/dL)	13.4 ± 0.09	11.4 ± 0.5[a]	13.7 ± 0.1[b]
Hematocrit (%)	40.6 ± 0.6	39.1 ± 0.4	40.3 ± 0.3
Serum Iron (µg/dL)	218.4 ± 20.4	167.1 ± 7.5[a]	196.2 ± 21.8[b]

Abbreviations. BUN, blood urea nitrogen; CKD, chronic kidney disease; FC, 4% ferric citrate in diet.
a) *P* < .05 (ANOVA) vs. CTL; b) *P* < .05 (ANOVA) vs. CKD. Results are shown as mean ± SEM.

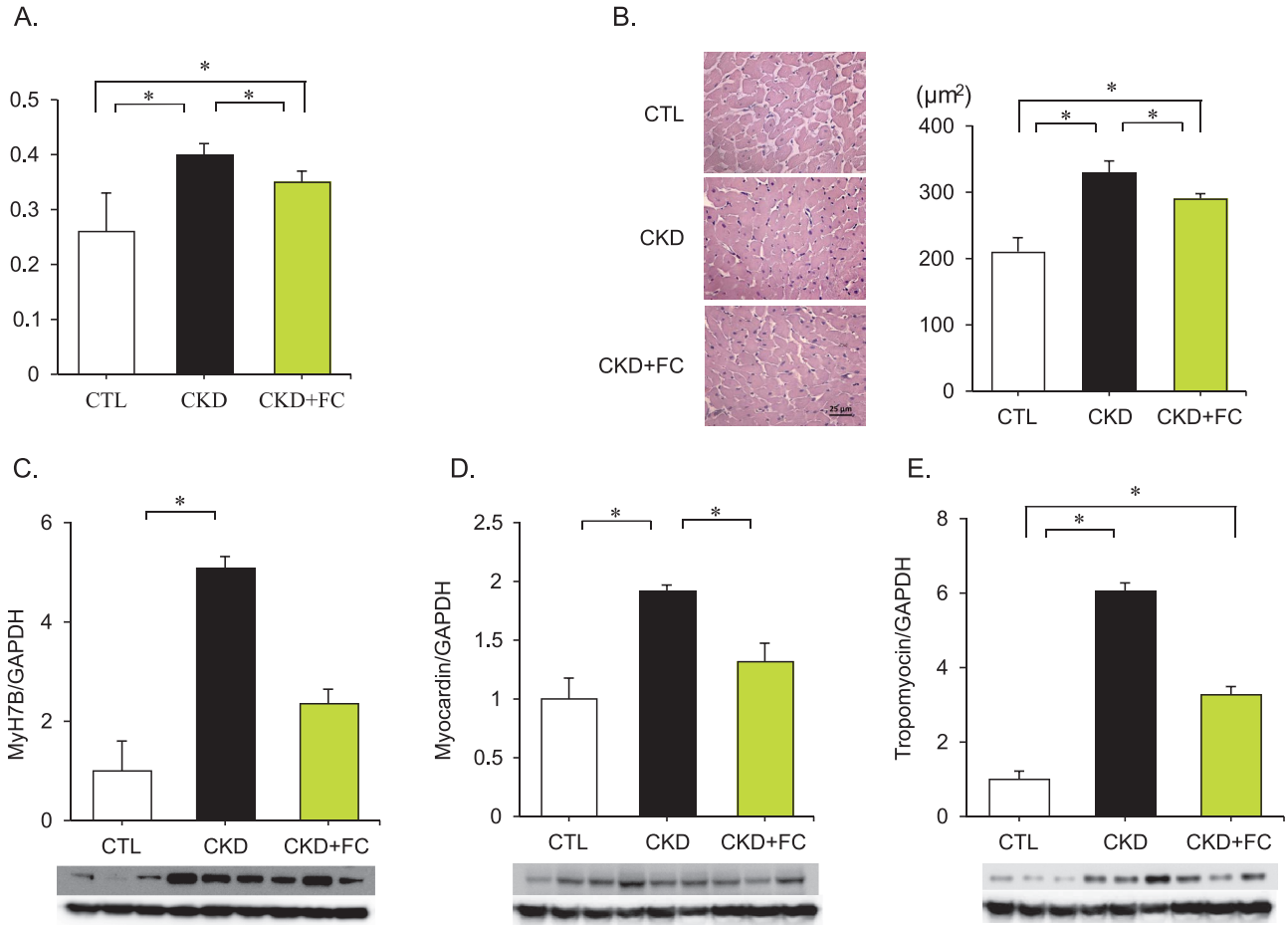

Figure 2. Impact of CKD and Ferric Citrate Treatment on Cardiac Hypertrophy
A) Heart (mg) / Body Weight Ratio (mg). B) Representative microphotographs of LV tissue stained with hematoxylin & eosin and quantification analysis for mean cross sectional areas of cardiomyocytes in each group. C-F) Representative western blots and group data depicting the LV protein abundance of C. MyH7B, D) Myocardin, and E) Tropomyocin. Data are mean ± SEM, n = 6 per group, * $P < .05$ (ANOVA)

markers of cardiac fibrosis including the LV tissue collagen 1 and TGF-β were significantly higher in the untreated CKD group when compared to the CTL animals. Ferric citrate therapy significantly reduced the TGF-β protein abundance. Although the collagen-1 abundance in ferric citrate treated group was lower than that found in the untreated CKD group, the difference did not reach statistical significance. The biomarkers of cardiac inflammation including IL-6 and MCP-1 were significantly higher in the left ventricles of the untreated CKD group compared to the CTL animals. Ferric citrate therapy reversed upregulations of IL-6 and MCP-1 in the CKD animals.

DISCUSSION

Using rats with 5/6 nephrectomy-induced CKD, which is known to cause cardiac remodeling and fibrosis,[16-18] we found that treatment with ferric citrate ameliorated anemia hypertension, cardiac hypertrophy and slowed progression of renal failure. Biomarkers of cardiomyocyte hypertrophy and cardiac fibrosis and inflammation including myocardin, IL-6, MCP-1 and TGF-β, were significantly elevated in untreated CKD rats and were significantly lowered with ferric citrate administration. Likewise, circulating NT-proBNP levels, which were elevated in untreated CKD rats, were significantly reduced with ferric citrate administration.

Impaired kidney function results in cardiorenal syndrome (CRS) type 4 which is characterized by left ventricular hypertrophy and diastolic dysfunction and contributes to the high rate of cardiovascular morbidity and mortality in the CKD population. Despite common use of the cardiovascular protective

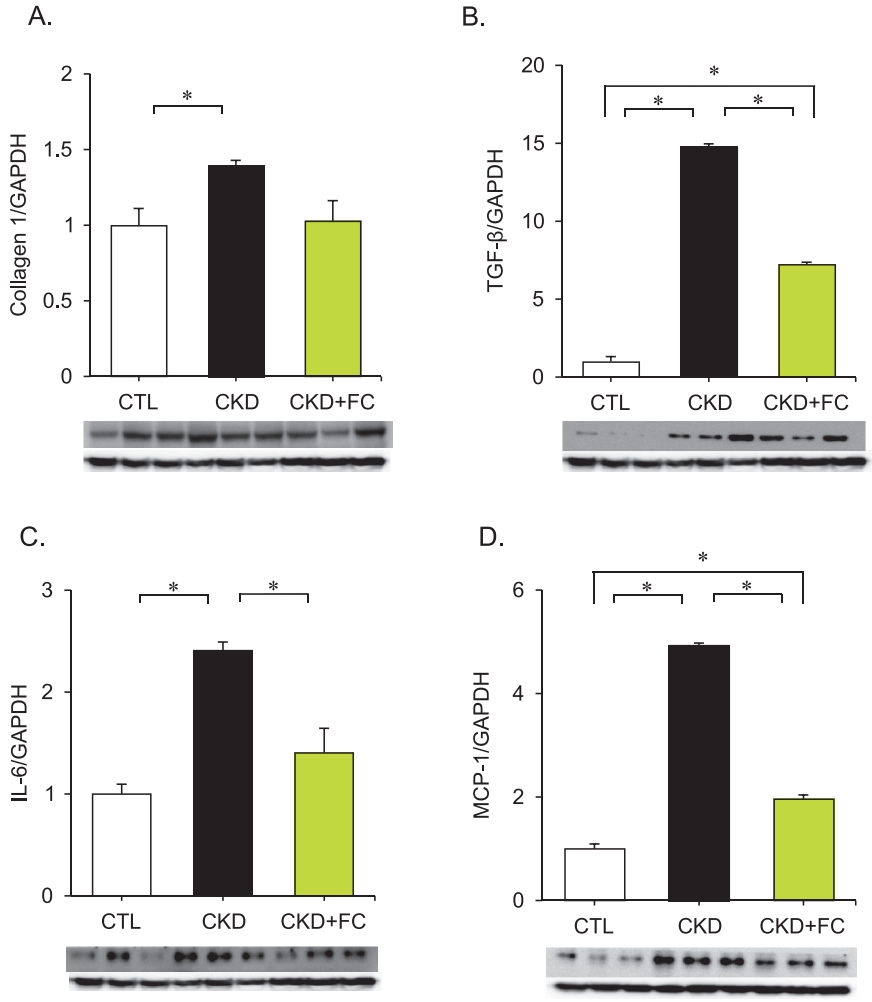

Figure 3. Impact of CKD and Ferric Citrate Treatment on Biomarkers of Cardiac Fibrosis and Inflammation.
A-D) Representative western blots and group data depicting the LV protein abundance of A. Collagen 1, B) TGF-β, C) IL-6, and D) MCP-1. Data are mean ± SEM, n = 6 per group, * *P* < .05 (ANOVA)

medications such as angiotensin converting enzyme inhibitors,[9] angiotensin receptor blockers[20] and beta blockers,[21] the rate of adverse cardiovascular events remains extremely high in CKD patients.[22] Cardiovascular risk factors in CKD patients include anemia, volume overload, electrolyte disorders, hypertension, hyperphosphatemia, oxidative stress, systemic inflammation, dyslipidemia, and accumulation of uremic toxins which work in concert to promote, arrythmia, LVH, CHF, atherosclerosis, and arteriosclerosis in patients with CKD.[23-30] In particular, hyperphosphatemia has been linked to heart failure and increased risk of cardiac mortality by promoting vascular calcification via induction of osteogenic transformation and apoptosis of vascular smooth muscle cells.[31-34] In the current study, in addition to ameliorating hyperphosphatemia, treatment with the phosphate binder, ferric citrate, ameliorated anemia, attenuated hypertension and improved renal function in CKD animals. These composite effects had protective benefits on the heart by suppressing cardiac hypertrophy and fibrosis.

Calcium loading can promote vascular calcification in patients and animals with CKD.[35] Administration of ferric citrate, which is a calcium-free phosphate binder, did not change serum calcium levels in CKD rats. This represents an advantage of ferric citrate over calcium containing phosphate binders. Indeed, meta-analyses have shown a lower rate of cardiovascular calcification and mortality in CKD patients treated with non-calcium-based phosphate binders, as compared to those treated with calcium-based phosphate binders.[36-37]

Ferric citrate has dual benefits in that besides

controlling hyperphosphatemia it improves anemia by enhancing availability of iron for erythropoiesis. In a phase 3 randomized clinical trials, treatment of CKD patients with ferric citrate for 16 weeks, increased mean transferrin saturation and ferritin levels by 18.4% and 170 ng/ml respectively.[12] Iron deficiency has been shown to correlate with pathophysiologic changes in the myocardium including enhanced inotropic responsiveness and decreased expression of the type-1 transferrin receptor.[38,39] Several clinical trials have shown beneficial effects of iron repletion in heart failure including improved LV ejection fraction and patient's quality of life.[0-43] Anemia in itself has been implicated in deterioration of kidney function by mediating hypoxia-induced tubular epithelial cell injury and interstitial fibrosis.[44] Furthermore, by enhancing oxygen delivery to myocardium and reversing tachycardia, improvement of anemia attenuates cardiac hypertrophy and fibrosis.

CONCLUSIONS

In addition to controlling hyperphosphatemia administration of ferric citrate attenuated anemia and left ventricular hypertrophy and fibrosis in CKD rats. Long-term clinical trials are needed to investigate its impact on cardiovascular outcomes in CKD and ESRD patients.

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DISCLOSURES

The authors declare no conflict of interest.

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Correspondence to:
Nosratolah D Vaziri
Division of Nephrology and Hypertension, Department of Medicine University of California, C362 Medical Sciences I, Irvine, CA 92697, USA
Tel: +1 949 824 4964
Fax: +1 714 456 6034
E-mail: ndvaziri@uci.edu

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