Impact of Homoarginine on Myocardial Function and Remodeling in a Rat Model of Chronic Renal Failure

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Abstract

Purpose: Low plasma concentrations of the amino acid homoarginine (HA) have been shown to correlate with adverse cardiovascular outcome, particularly in patients with chronic kidney disease. The present study sought to investigate the effect of HA treatment on cardiac remodeling in rats undergoing artificially induced renal insufficiency by 5/6 nephrectomy (5/6 Nx).

Methods: A total of 33 male Wistar rats were randomly divided into sham and 5/6 Nx groups, receiving either placebo treatment or 400 mg kg⁻¹ day⁻¹ HA over a 4-week period. Results: 5/6 Nx per se resulted in adverse myocardial remodeling with aggravated cardiac function and associated cardiac overload as the most obvious alteration (-23% ejection fraction, P < 0.0001), as well as increased myocardial fibrosis (+80%, P = 0.0005) compared to placebo treated sham animals. HA treatment of 5/6 Nx rats has led to an improvement of ejection fraction (+24%, P = 0.0003) and fractional shortening (+21%, P = 0.0126), as well as a decrease of collagen deposition (-32%, P = 0.0041), left ventricular weight (-14%, P = 0.0468), and myocyte cross-sectional area (-12%, P < 0.0001). These changes were accompanied by a downregulation of atrial natriuretic factor (-65%, P < 0.0001) and collagen type V alpha 1 chain (-44%, P = 0.0006). Sham animals revealed no significant changes in cardiac function, myocardial fibrosis, or any of the aforementioned molecular changes after drug treatment. Conclusion: Dietary HA supplementation appears to have the potential of preventing cardiac remodeling and improving heart function in the setting of chronic kidney disease. Our findings shed new light on HA as a possible new therapeutic agent for patients at high cardiovascular risk.

Keywords
cardiac remodeling, cardiorenal syndrome, chronic kidney disease, amino acids, fibrosis, rats

Introduction

Patients with chronic kidney disease (CKD) are at risk to die of cardiovascular events, which is rising along with the decline in kidney function.1,2 Therefore, prevention of disease progression and treatment of associated risk factors is highly important.1,2

L-homoarginine (HA) is a nonproteinogenic amino acid derivative, which attracted scientific attention as a risk marker for adverse cardiovascular outcome and renal diseases.3,4 Given its structural similarity with L-arginine, HA is supposed to be involved in nitric oxide (NO) synthesis increasing NO either by serving as an alternative substrate for NO synthase or...
by inhibiting arginase.\textsuperscript{3} The main production site of HA is the kidney by transaminidation from its precursor amino acid lysine.\textsuperscript{4,5} Two genetic studies identified arginine-glycine amidinotransferase (AGAT) as the key enzyme responsible for endogenous HA formation.\textsuperscript{6-8} Considering the fact that the kidney is one of the major sites of AGAT expression, a strong association between HA metabolism and kidney function seems plausible.\textsuperscript{9}

In animal studies, nephrectomized (Nx) mice showed lower HA concentrations as compared to their sham-operated controls.\textsuperscript{10} Several clinical studies have also reported that impaired kidney function is accompanied by a reduction of HA plasma concentrations.\textsuperscript{1,11,12} The European mild to moderate kidney disease study, a prospective cohort study of 227 patients with CKD, demonstrated that HA plasma concentrations are directly associated with kidney function and the progression of CKD.\textsuperscript{1} Drechsler et al identified HA as a risk factor for sudden cardiac death in patients undergoing hemodialysis.\textsuperscript{13} Beyond its key role in HA metabolism, AGAT is also known to participate in creatine synthesis.\textsuperscript{14} HA deficiency is therefore supposed to be associated with myocardial dysfunction and adverse cardiovascular events.\textsuperscript{3,4} März et al reported a strong correlation between low plasma concentrations of HA and increased cardiovascular mortality in patients referred for coronary angiography and patients with type 2 diabetes mellitus receiving hemodialysis.\textsuperscript{3} Instead of being merely a risk marker, direct protective effects on cardiac function have been attributed to HA in several animal studies.\textsuperscript{6,15-17} However, the underlying mechanisms remain largely unknown, and it is unclear whether exogenously applied HA can impede or ameliorate myocardial remodeling in the clinical or experimental setting of CKD. The 5/6 Nx rat is considered as an outstanding animal model for simulation of CKD. It has been shown that rats with removal of about 85\% of kidney tissue inevitably develop progressive CKD including concomitant abnormalities such as hypertension and left ventricular hypertrophy.\textsuperscript{18}

Therefore, the aim of the study was to evaluate whether dietary HA supplementation ameliorates the severity of cardiac impairment and remodeling in a rat model of CKD.

**Methods**

The current study was conducted on 33 male Wistar rats at the age of 6-7 weeks (Charles River Laboratories, Sulzfeld, Germany). Male animals were consistently used to prevent distortion of data by hormonal fluctuations, and to ensure comparability to previous investigations. The animals were housed under standardized conditions with water and food ad libitum. L-homoarginine hydrochloride was purchased by Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Hamburg, Germany). All experiments adhered to the “Guide for the Care and Use of Laboratory Animals” published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996). The animal protocol was approved by the local authorities of the Regierungspräsidium Karlsruhe (Karlsruhe, Germany), and conformed with the ARRIVE guidelines (Animal Research: Reporting of In Vivo Experiments).

**Study Design**

At arrival, rats were randomly assigned to the experimental or control group, treated with either placebo or 400 mg·kg\(^{-1}\)·day\(^{-1}\) HA by oral gavage: (1) Sham-operated rats with placebo treatment (n = 8), (2) Sham-operated rats with treatment of 400 mg·kg\(^{-1}\)·day\(^{-1}\) HA (n = 8), (3) 5/6 Nx rats with placebo treatment (n = 8), and (4) 5/6 Nx rats with treatment of 400 mg·kg\(^{-1}\)·day\(^{-1}\) HA (n = 9). Numbers were written on cards and then shuffled, allocating a distinct number to each animal. Hereafter, all animals were identified by their number rather than their treatment group and stayed blinded until euthanasia. All animals received equal doses of either 400 mg·kg\(^{-1}\)·day\(^{-1}\) HA or placebo by oral gavage. Ready-to-use 0.9\% sodium chloride served as carrier solution for the administration of HA (B. Braun Melsungen AG, Melsungen, Germany). Pharmacological treatment was initiated on the third day after surgery to ensure sufficient recovery from surgery and continued for 4 weeks, respectively. HA was administered by oral gavage, which guaranteed a constant daily medication for all animals. After 4 weeks of treatment, the animals were anesthetized by an intraperitoneal injection of ketamine (70 mg/kg) and xylazine (2-5 mg/kg). Echocardiographic and invasive pressure measurements were performed prior to excision and weighing of hearts. Cardiac arrest was induced by injection of saturated potassium chloride (B. Braun Melsungen AG, Melsungen, Germany). Myocardial tissue samples from the left ventricle (LV) were either snap frozen for subsequent biochemical investigations or fixed in formalin for histological assessment. Analysis parameter included body weight (BW, g), heart weight (HW, mg), tibia length (TL, mm), left ventricular weight (LV, mg), right ventricular weight (RV, mg), lung weight (LuW, mg), and liver weight (LiW, g). Plasma samples of HA, taken from the tail vein, were stored at −80°C and measured by using tandem-mass spectrometry.\textsuperscript{6} Plasma HA levels were measured in blood samples from 3 placebo- and HA-treated sham animals, respectively.

**5/6 Nephrectomy Procedure**

Chronic renal insufficiency in rats was carried out in a 2-step procedure according to the method described by Baraka et al.\textsuperscript{18,20} Briefly, animals were anesthetized by an intraperitoneal injection of ketamine (70 mg/kg) and xylazine (2 to 5 mg/kg). Following orotracheal intubation and ventilation with 2.5\% isoflurane in 100\% oxygen to maintain the anesthesia (Harvard Apparatus Inc., Holliston, Massachusetts, USA), a left flank incision was performed with exposure of the left kidney. After a temporarily occlusion of the renal artery, the upper and lower thirds of the kidney were ligated and excised so that the middle third of the left kidney remained. Bleeding was controlled by application of careful pressure, until it stopped. The muscle and

**Data Analysis**

Data are presented as mean ± SD. One-way ANOVA was used to evaluate differences between groups. Differences were considered significant at p < 0.05.
skin incisions were sutured with polypropylene suture. The rats were returned to their cages for recovering. One week later, a right flank incision was made, and the right kidney excised after ligation of renal vessels and ureter. Nx rats developed a renal insufficiency over the following 4-week period of drug treatment. Sham-operated rats (same surgical procedure without excision of the kidneys) served as control groups. All experiments were performed by the same investigator (J.H.R., cardiologic resident with 7 years of experience in animal surgery). For the alleviation of pain, buprenorphine was administered subcutaneously at a dose of 0.05 mg/kg (Temgesic, Indivior, Brandenburg, Germany) once a day. Carprofen at doses of 5 mg/kg (Rimadyl, Zoetis, Berlin, Brandenburg, Germany) once a day.

Echocardiography
Transthoracic echocardiography was conducted after 4 weeks of HA treatment using a 10 MHz probe in combination with an ATL 5000 echocardiography device (ATL Ultrasound, Philips, Bothell, Washington, USA). The procedure for echocardiography has been previously reported.21 Rats were imaged in the left lateral decubitus position including the parasternal short-axis view of the heart to obtain LV dimension measurements in diastole and systole. M-mode measurements of LV dimensions were calculated from average values of more than 3 cycles. Analysis was conducted with the Scion Image software package for Windows (Scion Image, Scion Corporation, Frederick, Maryland, USA). All investigations were performed by a single investigator who was unaware of the treatment status.

Cardiac Catheterization
Pressure measurements were performed before tissue collection, as previously reported. Briefly, rats were anesthetized and mechanically ventilated as described above. A 2.0 F impedance micromanometer catheter (SPR-838, Millar Instruments Inc., Houston, Texas, USA) was inserted into the right carotid artery to obtain aortic blood pressure. To simultaneously measure LV hemodynamics, a 1.4 F micromanometer catheter (Millar Instruments Inc., Houston, Texas, USA) has been placed in the LV. The micromanometer catheter was calibrated with known volumes of heparin-treated blood for absolute volume measurements. Data was digitized with a sampling rate of 1,000 Hz and recorded on a personal computer using dedicated software (ADInstruments, Colorado Springs, Colorado, USA). Subsequent analysis of pressure measurements was performed with PVAN Software (Millar Instruments Inc., Houston, Texas, USA).

Quantitative Real-Time Polymerase Chain Reaction (PCR)
Extraction of total RNA from the apical LV was performed using Invitrogen TRIzol reagent (Thermo Fisher Scientific, Waltham, USA) by subsequent generation of cDNA from 500 ng of RNA with a Revert Aid first-strand cDNA synthesis kit (Thermo Fisher Scientific, Waltham, USA). Hereafter, real-time PCR was performed on a MyIQ real time PCR detection system (Bio-Rad Laboratories, Hercules, USA) with the SYBR Green PCR Master Mix (Applied Biosystems, Foster City, USA). All PCR investigations were conducted in duplicates with a 1:100 dilution of cDNA using 96-well plates (ThermoFast 96 PCR Plate, Thermo Fisher Scientific, Waltham, Massachusetts, USA). Copy numbers of the atrial natriuretic factor (ANF), beta-myosin heavy chain (β-MHC), brain natriuretic peptide (BNP), and collagen type V alpha 1 chain (col5a1) were measured. We have chosen type V collagen as it plays an important role in the formation of collagen fibers and stabilization of injured tissue, although with lower cardiac expression than other types of collagens.24 The housekeeping gene hypoxanthine-guanine phosphoribosyltransferase (HPRT) served as the reference gene to normalize gene expression between the samples. All measurements of the dilution series were combined with a standard curve. Automated analysis of raw datasets was performed with dedicated software (Bio-Rad qPCR analysis software, Bio-Rad Laboratories, Hercules, USA). Each PCR sample was assessed using the 2−ΔΔCt method to calculate relative quantification.25 A detailed description of primers and sequences is provided in Table S1 (Supplemental Material).

Pathology
After harvesting of hearts, LVs were isolated and further fixed in formalin, paraffin embedded, and processed for sectioning. Myocyte size was measured on LV cross-sections obtained between base and apex and stained with hematoxylin/eosin, while sirius red stainings were used to quantify areas of collagen deposition. Cardiomyocyte cross-sectional area was assessed with the ImageJ software (National Institute of Mental Health, Bethesda, Maryland, USA) and expressed in μm². All histological examinations included the analysis of at least 150 cardiomyocytes and 20 paraffin slices per heart. Two independent investigators evaluated evidence of fibrosis under bright-field and polarized light with a 10× objective lens (Zeiss, Peabody, Massachusetts, USA). Histological images were recorded under consideration of the entire cross-section of LV tissue. The amount of fibrosis was calculated for each tissue section using threshold area fraction determination and expressed as percentage of the total area of the cross-section (CAF, collagen area fraction).26,27 To avoid bias, the investigator was blinded to the treatment status.

Statistical Analysis
Data are expressed as mean ± standard error of the mean (SEM). Statistical analysis was conducted using software packages from MedCalc (MedCalc Software Ltd., Ostend, Belgium) and GraphPad Prism (La Jolla, California, USA). Normality of datasets was assessed by Kolmogorov–Smirnov test.
test. Unpaired student’s t test (two-tailed) was used when appropriate. Group comparisons were analyzed by one-way ANOVA, followed by post hoc comparisons between groups with Dunnett’s test. A P value <0.05 was considered statistically significant.

### Results

#### Increase of HA Plasma Levels Following Oral Gavage

Male Wistar rats weighed 188 ± 27 g. Before initiating experiments, we first assured that oral supplementation of HA by oral gavage leads to an adequate rise of plasma HA concentration in accordance with other studies.\(^6,15,16\) The baseline HA level in animals with placebo treatment was 0.9 ± 0.6 mg/L (0.04 mM). Following 2 weeks of HA supplementation, animals treated with 400 mg·kg\(^{-1}\)·day\(^{-1}\) showed an increase up to 274 ± 9 mg/L (1.22 mM). Effectiveness of 5/6 Nx was confirmed in preliminary experiments showing a decrease in glomerular filtration rate of approximately 77% compared to sham animals 4 weeks following 5/6 Nx surgery (9.3 ± 0.9 vs. 2.1 ± 0.5 mL/min/kg). Renal function was determined in 5 sham- and 5/6 Nx animals (n = 10), respectively.
Renal Injury by 5/6 Nephrectomy Is Associated With Characteristics of Adverse Myocardial Remodeling

As shown in Tables 1-3 and Figures 1–5, 5/6 Nx per se resulted in adverse myocardial remodeling with aggravated cardiac function as the most obvious alteration and increasements of collagen deposition and heart weight when compared to sham animals with placebo treatment. In 5/6 Nx rats with placebo treatment, ejection fraction (EF) and fractional shortening (FS) decreased by 23% (P < 0.0001) and 24% (P = 0.0004), respectively, whereas blood pressure increased by 16% (P = 0.0041), CAF by 80% (P = 0.0005), myocyte size by 17% (P < 0.0001), col5a1 by 71% (P = 0.0021), and ANF by 73% (P = 0.0020) in comparison to placebo treated sham animals. We observed no significant increasements of HW/BW ratio by 6% (P = 0.0880), RV/BW ratio by 15% (P = 0.0684), β-MHC by 52% (P = 0.0919), and BNP by 50% (P = 0.0704).

Effects of HA Treatment on Gross Pathology, Histopathology, Cardiac Function, Blood Pressure and Gene Expression in Sham-Operated Animals

The HA treated sham group presented a lower BW as compared to sham controls with placebo treatment, both at operation (P = 0.0093) and euthanasia (P = 0.0001). Therefore, HW, TL, HW/TL ratio, LV weight, LV/TL ratio, RV weight, RV/TL ratio, LuW, and LiW of fresh tissue were significantly (P ≤ 0.0037) decreased in the HA treated group as compared to animals with placebo treatment. Correlating HW, LV weight, RV weight, and LuW to BW instead of TL revealed no statistical differences (Table 1, Figure 1).

In accordance with gross pathology, histological assessment showed a significant decrease in cardiomyocyte cross-sectional area after HA treatment (Table S2, Figure 3). Herein, myocytes from sham animals treated with 400 mg·kg⁻¹·day⁻¹ HA (207 ±
4 \mu m^2) were approximately 8% smaller than myocytes from placebo treated rats (225 \pm 4 \mu m^2, P = 0.0007).

Quantitative assessment of collagen deposition (Table S3, Figure 4) revealed no significant reduction of regions with interstitial fibrosis after drug treatment as shown by sirius red staining (\% after HA treatment [2.6 \pm 0.2\%] in comparison to untreated sham controls [3.0 \pm 0.2\%, P = 0.2517]). Sham animals presented unaffected glomeruli and tubuli (Figure 6).

Cardiac function was not negatively affected by HA treatment and remained on a high level (Table 2, Figure 2). Invasive pressure measurements revealed a trend toward lowered blood pressure after HA intake (111 \pm 4 \text{mmHg}) as compared to untreated controls (119 \pm 4 \text{mmHg}, P = 0.2371) (Table 3).

Drug treatment had no significant effect on gene expression of the molecular markers ANF (P = 0.4419), BNP (P = 0.8920), or \beta-MHC (P = 0.8203) (Figures 3, 4, 5). However, transcription levels of col5a1 were decreased by 34\% after 4-week HA treatment (P = 0.0491).

**Figure 2.** Impact of HA treatment on cardiac function. Top: Exemplary echocardiographic M-mode images obtained from the parasternal short axis of the left ventricle from sham- and 5/6 Nx animals treated with 0 mg kg\(^{-1}\) day\(^{-1}\) or 400 mg kg\(^{-1}\) day\(^{-1}\) HA. Bottom: EF (A), FS (B), EDD (C), and ESD (D) of the LV estimated from transthoracic echocardiographic M-mode images. * P < 0.05. 5/6 Nx indicates 5/6 nephrectomy; HA, homoarginine; EF, ejection fraction; FS, fractional shortening; EDD, end-diastolic diameter; ESD, end-systolic diameter.

**Figure 3.** Effect of HA treatment on cardiac myocyte size and \beta-MHC expression. A, Exemplary H&E stainings of representative left ventricular sections after 4-week HA treatment. Cardiomyocyte size (\mu m\(^2\)) is given in brackets as mean \pm standard error of the mean. B, Cross-sectional area of cardiomyocytes. * P < 0.05. 5/6 Nx indicates 5/6 nephrectomy; HA, homoarginine; \beta-MHC, beta-myosin heavy chain; HPRT, hypoxanthine-guanine phosphoribosyltransferase; H&E, hematoxylin-eosin.
interstitial fibrosis (Table S3, Figure 4) after drug treatment (3.6 ± 0.2%) in comparison to untreated 5/6 Nx controls (5.3 ± 0.5%, \( P = 0.0041 \)). Hematoxylin and eosin staining showed histological changes in the renal cortex of animals from the 5/6 Nx group in comparison to the unaltered structure in the kidneys of the control group, regardless of HA treatment (Figure 5). Histologic examination of remaining kidneys after 5/6 resection showed minimal segmental glomerular sclerosis and hypertrophy of remnant glomeruli, mesangial matrix accumulation, capillary occlusion, hyalinization, and protein casts in the tubules, similar to previously reported studies.\(^{10,28}\)

Nx hearts from drug treated animals showed a significant improvement of EF upon HA treatment (64 ± 2%) when compared with placebo treated Nx hearts (52 ± 2%, \( P = 0.0003 \)). Likewise, drug treatment of 5/6 Nx rats was accompanied by an increase in FS (29 ± 2 [5/6 Nx 400 mg·kg\(^{-1}\)·day\(^{-1}\)] vs. 24 ± 1% [5/6 Nx 0 mg·kg\(^{-1}\)·day\(^{-1}\)], \( P = 0.0126 \)) (Table 2, Figure 2). Invasive pressure measurements detected significantly reduced arterial blood pressure after HA intake (115 ± 2 mmHg) as compared to untreated 5/6 Nx controls (137 ± 3 mmHg, \( P < 0.0001 \)) (Table 3).

HA treated 5/6 Nx rats demonstrated a reduction of ANF by 65% (\( P < 0.0001 \)) and col5a1 by 44% (\( P = 0.0006 \)) as compared to placebo treated 5/6 Nx animals (Figures 3, 4, 5). Results from BNP (−34%, \( P = 0.0731 \)) and β-MHC (−33%, \( P = 0.1291 \)) remained not significant.

**Adverse Side Effects**

Treatment with HA was well tolerated without any side effects. Potential interactions of HA on growth, infection rate, and cardiac function were examined. We could not observe an increased proneness for wound infections. Animals in the treatment group showed no elevation of their body weight at euthanasia. Supplementation of HA did not lead to deterioration of heart function or any other serious side effect. None of the animals died due to HA treatment.

**Discussion**

The present study has demonstrated attenuated adverse effects of renal insufficiency on cardiac remodeling and blood pressure following oral administration of HA to 5/6 Nx rats. HA treatment was found to improve cardiac function and prevent an increase of cardiac hypertrophy and collagen deposition.

**Attenuated Cardiac Remodeling Response of 5/6 Nx Animals Upon HA Treatment**

In an experimental setting, the 5/6 Nx is a classic animal model of CKD with progressive renal scarring characterized by glomerulosclerosis and interstitial fibrosis.\(^{29}\) Rats subjected to 5/6 resection of their renal tissue are known to inevitably develop progressive CKD with associated LV hypertrophy, arterial hypertension, and myocardial dysfunction.\(^{18}\) Hearts of drug treated 5/6 Nx animals showed a lesser degree of interstitial fibrosis as shown by sirius
red staining compared to placebo treated 5/6 Nx controls (−32%, P = 0.0041). The histopathological results were underlined by a reduction in LV gene expression of the fibrotic marker col5a1 (−44%, P = 0.0006). Myocardial fibrosis represents one crucial process in cardiac remodeling leading to detrimental functional alterations.30 Post-mortem analysis of heart tissue from uremic patients showed diffuse non-coronary intermyocardiocytic fibrosis in 91% of chronically uremic patients.31 A high extent of collagen deposition leads to myocardial wall stiffness with impaired ventricular diastolic distensibility.23 Accordingly, HA treated Nx animals showed a downward trend in end-diastolic and end-systolic diameter. A potential, currently underestimated mechanistic pathway might lie in the biological function of HA as a specific noncompetitive inhibitor of TNAP (tissue-nonspecific alkaline phosphatase).32 TNAP is expressed in different tissues, including endothelial cells, bone, or kidney, and takes part in a multitude of biological processes such as inflammation and bone mineralization.33 Epidemiological studies have identified a positive correlation between elevated blood concentrations of TNAP and overall mortality in patients with coronary heart disease.34,35 As such, it is tempting to argue that inhibition of TNAP by HA represents a therapeutic approach to suppress cardiac collagen deposition.

Given the detrimental effects of adverse cardiac remodeling on LV hypertrophy in the context of renal impairment, placebo treated 5/6 Nx rats experienced a significant increase in cardiomyocyte cross-sectional area compared to untreated sham-operated animals (+17%, P < 0.0001). Following HA treatment, myocyte cross-sectional area was significantly reduced in both, HA treated rats bearing remnant kidney (−12%, P < 0.0001) and sham animals (−8%, P = 0.0007), compared to untreated animals of the respective groups. Consistent with our histopathological results, left ventricular weight was found to be decreased on gross pathology after adjustment for tibia length (−13% for 5/6 Nx, P = 0.0487, and −25% for sham, P < 0.0001). Although transcription levels of the molecular hypertrophic marker β-MHC did not differ significantly between animals with HA or placebo treatment, HA treatment was accompanied by decreased levels of ANF (−65%, P < 0.0001), indicating attenuated adverse myocardial remodeling after 5/6 Nx. The exact mechanistic pathway responsible for prevention of the hypertrophic response upon 5/6 Nx remains elusive. However, previous research suggests an important role of TNAP in the regulation of cardiac hypertrophy and calcification processes by modification of transcriptional changes due to α1 adrenergic receptor activation and regulation of pyrophosphate levels.36,37 Upregulated TNAP expression in vascular endothelium has been shown to result in generalized arterial calcification in animal models.38,39 TNAP inhibition by tetramisole or DBSA (2,5-dimethoxy-N-(quinolin-3-
Improved Cardiac Function in the Context of Previous Studies

Experimental studies on the cardiovascular effects of orally applied HA are rare.\textsuperscript{6,16,41,42} An animal study from 2017 demonstrated for the first time that dietary supplementation with 14 mg/L HA in the drinking water preserves cardiac function in a murine model of post-myocardial infarction heart failure.\textsuperscript{16} In the clinical setting, HA was found to be inversely associated with biomarkers of heart failure, especially N-terminal pro-B-type natriuretic peptide.\textsuperscript{4,13,43,44} Consistent with this finding, assessment of HA levels in a cohort of 3,037 subjects referred for coronary angiography showed a positive correlation between HA and EF, pronounced in patients with decreased kidney function.\textsuperscript{12} Increasing evidence points to a pivotal role of the mitochondrial enzyme AGAT, which is regarded as the key enzyme for the synthesis of HA and creatine.\textsuperscript{45} AGAT mRNA is expressed at different levels in various tissues with high levels in the kidney and intermediate levels in heart, skeletal muscle, and brain.\textsuperscript{45} Our experiments revealed a notable increment of EF (\(+24\%\), \(P = 0.0003\)) after treatment of Nx rats with 400 mg·kg\(^{-1}\)·day\(^{-1}\) HA when compared to untreated Nx controls. Attenuation of adverse myocardial remodeling after 5/6 Nx might be attributed to the fact that AGAT is elevated in the failing heart and returns to normal levels after recovery.\textsuperscript{45,46} Given that AGAT is also involved in the synthesis of creatine, its upregulation could represent a response to creatine depletion of the myocardium by enabling local creatine synthesis counteracting the decreased intracellular levels.\textsuperscript{45} In this context, overexpression of the creatine transporter with enhanced myocardial creatine levels has been shown to protect mice from acute myocardial infarction.\textsuperscript{47} However, the role of creatine in cardiovascular health remains to be elucidated if considering other experiments that demonstrated detrimental effects of chronically increased intracellular creatine levels.\textsuperscript{4,48} Interestingly, AGAT deficient mice with a whole-body creatine-deficiency and low plasma HA levels showed only corrected systolic pressure after creatine supplementation, whereas HA application rescued impaired cardiac contractile function.\textsuperscript{17} The findings implicate that cardiac dysfunction is mainly driven by HA deficiency rather than creatine deficiency.\textsuperscript{17}

The ability of HA to improve cardiac function might also be explained by its participation in the metabolism of the vasodilator NO, whose deficiency is associated with endothelial and myocardial dysfunction.\textsuperscript{3} Experimental data from rats demonstrated lowered blood pressure after intravenous HA infusions along with simultaneously increased urinary excretion of nitrate as the degradation product of NO.\textsuperscript{49} Accordingly, application of 400 mg·kg\(^{-1}\)·day\(^{-1}\) HA in our study has led to blood pressure lowering in Nx rats as confirmed by invasive measurements (\(-16\%\), \(P < 0.0001\)). Nonetheless, a direct relationship between HA and NO metabolism remains controversial. Current research in this field indicates that HA act via modulation of mitochondrial processes rather than changing of nitric oxide synthase expression.\textsuperscript{50}

Decreased Body Weight of Animals After HA Supplementation

HA supplementation as artificial compensation for the loss of HA in Nx rats has resulted in different changes of body weight between sham- and 5/6 Nx animals following HA treatment. Given the deviation of body weight in percent considering the a priori elevated body weight at surgery of HA treated sham- and 5/6 Nx animals, HA treated 5/6 Nx animals showed an approximately 20\% higher increase in body weight compared to placebo treated 5/6 Nx animals at euthanasia, whereas body weight of sham animals decreased by about 20\% after 4-week HA treatment. Growth-promoting effects of HA are described in several previous studies showing a positive correlation between HA plasma levels and indicators of malnutrition and inflammation, including body mass index, albumin, and C-reactive protein.\textsuperscript{9,44,51} Indeed, the decreased body weight in treated sham animals represents an interesting study finding opposite to so far published literature. A possible explanation might be different outcomes after surgery with varying intensities of postsurgical cachexia.

Dose Considerations

Little is known about the optimal dosage of HA for experimental purposes. In a murine study using a model of post-myocardial infarction heart failure, a preserved contractile reserve and diastolic function was observed following the administration of 14 mg/L HA in the drinking water (approximately 2 mg·kg\(^{-1}\) body weight).\textsuperscript{16} The authors observed a strong correlation between HA levels in plasma and myocardial tissue. A beneficial effect was obtained from a threefold increase in plasma HA concentration corresponding to a human dose equivalent of about 250 mg daily.\textsuperscript{16} In humans, supplementation of 125 mg HA once a day (approximately 2 mg·kg\(^{-1}\) body weight) resulted in a sevenfold increase in HA concentration, indicating a different metabolism of HA in animals and humans.\textsuperscript{52} The HA concentration of 400 mg·kg\(^{-1}\)·day\(^{-1}\) in the present study refers to several experiments on its derivative L-arginine.\textsuperscript{53,54} Dietary supplementation with L-arginine up to 3.6 g·kg\(^{-1}\)·day\(^{-1}\) has been shown to be safe in rats for at least 91 days.\textsuperscript{55,56} In our study, HA plasma concentration went from baseline levels of 0.9 ± 0.6 mg/L (0.04 mM) to 274 ± 9 mg/L (1.22 mM) after treatment. Compared to other studies, our measured plasma HA concentrations were approximately 1,000 to 6,000-fold higher.\textsuperscript{6}
**Limitations**

Several study limitations have to be noted when interpreting our results. First, size of animal groups should be extended in forthcoming studies to confirm our preliminary findings. Second, the 4-week period of HA administration might have been too short for the full manifestation of the cardiorenal syndrome. In this context, only moderate hypertensive values were found in placebo treated 5/6 Nx animals. Third, the high dose of 400 mg·kg$^{-1}$·day$^{-1}$ HA in our study has to be noted which limits the comparability with other studies. Fourth, despite of a carefully performed and strictly standardized 5/6 Nx procedure renal function might differ within certain limits across the subgroups. Laboratory measurements of renal function were performed in preliminary experiments on ten rats, not comprising the whole stock of laboratory animals (n = 33). However, standard deviation of the glomerular filtration rate was small suggesting almost equal surgical outcomes. Fifth, experiments were conducted on immature rats with an average body mass of about 200 g at surgery. Considering the different metabolic of immature and mature myocardium, additional studies are warranted to elucidate the impact of HA in mature animals. Finally, our study findings are restricted to young male Wistar rats without knowledge of HA interactions in other species.

**Conclusions**

The present study is the first study that has systematically examined the underlying cardiac changes of a dietary HA supplementation in the setting of renal insufficiency. Reflecting this data, our study adds important new knowledge by identifying beneficial effects of exogenously applied HA on myocardial remodeling and cardiac function. Our findings have clinical implications with regard to the pathophysiology of the cardiorenal syndrome, suggesting novel targets for pharmaceutical treatment. Alleviating detrimental effects of the so-called cardiorenal syndrome is crucial for increasing patients’ quality of life. Whether the effects of HA seen in animal studies are transferable to humans and truly favorable in the long term, has to be tested in clinical studies.

**Authors’ Note**

Compliance with Ethical Standards: Research involving Human Participants and/or Animals: Experimental study on animals (rats). Ethics Approval: Approval of the local Institutional Review Board was obtained.

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**Author Contributions**

All authors have contributed significantly to this work, participating in conception, design, analysis, interpretation of data, and writing/editing.

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