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Tissue ACE inhibition and sodium status in left ventricular dysfunction

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Chapter 5

Adverse Renal Effects of Hydrochlorothiazide in Rats with Myocardial Infarction Treated with ACE Inhibition.

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Abstract

Background: Diuretics reduce fluid retention and can potentiate the response to ACE inhibition (ACE-I). However, volume depletion during ACE-I can also have adverse renal structural and functional effects. We studied renal effects of adding diuretic to ACE-I after myocardial infarction (MI), i.e. a condition with intrinsically normal kidneys.

Methods: MI was induced in rats by coronary ligation. After 2 weeks, rats were randomized to ACE-I quinapril alone (QUI, n=34) or with add-on hydrochlorothiazide (QUI+HCTZ, n=46). Survival was monitored for 14 months. Plasma creatinine (pCr) was measured at 4 months. Subgroups were sacrificed to study renal morphology after 8 and 14 months. In addition, untreated MI rats were studied at 8 months. Kidneys were studied for interstitial damage, myofibroblast transformation and fibrosis, and macrophage influx.

Results: At 4 months, pCr was increased by 40% in QUI+HCTZ compared to QUI (46 vs. 33 µmol/L, p=0.001). Though 14-month mortality was similar in QUI+HCTZ and QUI, stratification based on pCr showed increased mortality in the tertile with highest pCr (p=0.03, Log rank). Remarkably, add-on HCTZ caused severe renal interstitial lesions, i.e. tubular dilatation and fibrosis. Interstitial SMA was increased at 8 and 14 months, and coincided with collagen deposition and macrophage influx. HCTZ did not affect blood pressure or plasma K^+. In rats with QUI monotherapy or untreated MI renal structure was normal.

Conclusions: Adding HCTZ to ACE-I detrimentally affected not only renal function, but also renal structure in rats with MI. As decreased renal function was associated with increased mortality, adverse renal effects of volume depletion by adding HCTZ to ACE-I may exert unfavorable effects on long-term prognosis after MI.

Introduction

The progression of left ventricular (LV) dysfunction towards overt chronic heart failure (CHF) after myocardial infarction (MI) is associated with progressive cardiac remodeling. Activation of the renin angiotensin aldosterone system (RAAS) plays a central role in this process, and blocking the RAAS with angiotensin-converting enzyme inhibitors (ACE-I) effectively reduces remodeling and prolongs survival after MI.

Diuretics are often chronically added to ACE-I treatment to prevent fluid retention, although clinical trials to show their effect on mortality are lacking. On one hand, a diuretic-induced negative sodium balance generally elicits an optimal therapeutic effect of ACE inhibition[1-3]. On the other hand, diuretics may have adverse effects, i.e. electrolyte disturbances and renal function loss. We previously reported improved survival and cardiac function by add-on diuretic therapy with hydrochlorothiazide in rats with MI in the early chronic phase[4]. However, this survival benefit was not maintained during prolonged long-term follow-up (14 months). Addition of diuretic treatment to ACE inhibition may have adverse renal structural and functional effects, which may in turn affect long-term survival after MI, as poor renal function is strongly and independently associated with worsened long-term prognosis after MI[5]. Addition of a diuretic to ACE inhibition however may decrease renal function[6].

In the present study we investigated the long-term effects of adding a diuretic to ACE inhibition on renal morphology and function in relation to prognosis. We hypothesized that hydrochlorothiazide added to ACE inhibition in rats with myocardial infarction causes a sustained decrease in renal function and alterations in renal morphology, which is associated with worsened long-term survival.

Methods

The investigation conforms to the Guide for the Care and Use of Laboratory Animals (Published by the US National Institutes of Health, NIH publication No. 85-23, revised 1996). The animal research committee of the University of Groningen approved this study protocol.

Male, Sprague Dawley rats (Harlan, Zeist, The Netherlands), weighing 280 ± 25g were subjected to coronary ligation as previously described[7]. Since the study was aimed at the chronic phase after myocardial infarction, and not at interfering with the early healing process and scar formation, treatment was started 14 days post-MI. Rats were randomly assigned to: quinapril (QUI, 15 mg kg^{-1} day^{-1}), quinapril + hydrochlorothiazide (HCTZ), quinapril + low sodium diet (LS).

Quinapril was mixed through food (Hope Farms, Woerden, The Netherlands). This dose results in optimal ACE-I therapy[8,9]. HCTZ (50 mg kg^{-1} day^{-1}) was dissolved in drinking water. This dose causes RAAS activation and an increase in diuresis, but no blood pressure reduction in rats with myocardial infarction[10]. The LS-group was fed with food pellets (Hope Farms, Woerden, The Netherlands containing 0.05% NaCl instead of 0.3% (normal diet) (LS-diet;). To ensure constant drug intakes during the entire study period,

concentrations of quinapril and HCTZ were adjusted weekly. This was done by measuring food/water intake and average body weight per cage weekly, and calculating the required drug concentration in food and water (per cage) for the week after. Rats were fed *ad libitum*, and housed group-wise in clear polyethylene cages in temperature (22^0 C)- and humidity (50%)-controlled rooms with a 12h light/dark cycle.

Study 1- Renal function and mortality
Rats were allocated to one of the three active treatment groups, and survival was monitored - in a blinded fashion – for a period of 14 months after onset of therapy. Use of color-tags ensured appropriate housing and treatment by caretakers. Cages were checked for dead animals at least once daily; tissues of dead rats were collected, weighed and stored for analysis.
After 4 months of treatment 1 mL blood was obtained from the retro-orbital plexus under isoflurane anesthesia. The blood was anti-coagulated with EDTA, centrifuged at 1600G for 10 minutes at 4°C, and stored at -80°C until measurement of plasma creatinine concentrations according to routine clinical methods.

Study 2 - Renal morphology during and after chronic treatment
Rats were operated and treated according to the same procedures as in the above study, and sacrificed 8 months after onset of treatment to study renal morphology. In addition, subgroups of randomly chosen surviving rats from the study described above were sacrificed for assessment of renal morphology after the 14-month follow-up period.

Tissue processing
Rats were anesthetized with isoflurane (2%) in a mixture of O_2 and N_2O (1:2), the carotid artery was cannulated and a pressure tip catheter (Micro-Tip 3French, Millar instruments, Houston TX, USA) advanced into the aortic arch, and arterial blood pressure was recorded. Subsequently, arterial blood and tissues were collected for further analysis.
Blood was drawn from the abdominal aorta, anti-coagulated with EDTA, centrifuged at 1600G for 10 minutes at 4°C, and stored at -80°C until assay. Plasma K^+ and Na^+ concentrations were determined according to routine clinical methods. Hearts and kidneys were rinsed with ice cold NaCl (0.9%), and fixed in 2% paraformaldehyde. The kidneys were cut longitudinally in two parts. The fixated tissues were then processed for paraffin embedding according to standard procedures.
LV infarct size was determined, on sections stained with Sirius Red/Fast Green, as percentage of LV circumference, as described before[7]. Only rats with MI sizes larger than 20% of LV were included for analysis, since smaller infarcts are fully compensated, and do not result in LV dysfunction.

Kidney histology
Routine morphology was evaluated using periodic acid shift-stained sections by a qualified pathologist. Renal interstitial α-smooth muscle actin (α-SMA) was detected in 3-μm paraffin sections with a mouse monoclonal antibody. First, the antibody was incubated for 60 minutes, and subsequently its binding was detected with peroxidase (PO)-labeled

rabbit anti-mouse antibody for 30 minutes. The expression of interstitial α-SMA was quantified by computerized morphometry (50 fields/kidney, magnification 200x); Glomeruli and vessels were excluded from measurement by tracing them with a cursor along Bowman's capsule or the vessel wall. The medulla was not evaluated.

Renal interstitial macrophage infiltration was detected using a mouse anti-rat monoclonal antibody against ED1 (Serotec, Oxford, England). Subsequently sections were incubated with PO-labeled secondary antibody. Peroxidase activity was visualized by 3-amino-9-ethylcarbazol (AEC). Interstitial macrophages were counted using morphometry; per kidney, 50 fields were evaluated.

Statistics

Data are presented as mean ± standard error of the mean. Survival analysis was done using log rank analysis with pairwise comparison over strata. Other parameters were compared using oneway analysis of variances (ANOVA) with least square difference post hoc analysis for multiple comparisons. Differences were considered significant at the level of 0.05 (two tailed).

Results

Study 1 - Renal function and mortality

To investigate whether renal function affects mortality in rats with MI, we analyzed long-term survival based on plasma creatinine concentrations assessed after 4 months of treatment (figure 1A). Stratification of groups into tertiles based on plasma creatinine concentrations, regardless of treatment, showed significantly increased mortality in the highest tertile after 14 months treatment, as compared to the group with lowest plasma creatinine concentrations. The middle tertile showed intermediate survival. The three groups stratified on plasma creatinine had similar infarct sizes; 32±1, 34±1, and 33±1 % of LV, for low, intermediate and high plasma creatinine, showing that the extent of myocardial damage was no confounder in the relation between renal function and long-term survival.

The effects of treatment on renal function are shown in figure 1B. The three treatment groups had comparable infarct sizes: 34±1 %, 32±1 %, and 33±1 %, for quinapril, quinapril + HCTZ, and quinapril + LS, respectively. Addition of HCTZ to quinapril caused a ˜40% increase in plasma creatinine concentrations after 4 months of treatment (p=0.001, figure 1B). Notably, average plasma creatinine concentrations in the HCTZ-treated rats corresponded with the highest tertile in the survival analysis described above. Dietary sodium restriction did not influence plasma creatinine concentrations.

Study 2 – Renal morphology

Table 1 shows general characteristics of the rats sacrificed for pathological analyses after 8 and 14 months of treatment. Comparison of untreated and quinapril-treated rats at 8 months showed a marked reduction in systolic blood pressure (p<0.005) and an increase in plasma K^+ concentrations (p<0.05). Addition of hydrochlorothiazide or low sodium diet to quinapril did not further alter blood pressure or plasma K^+ concentrations, neither at 8 nor at 14 months. Plasma Na^+ concentrations were slightly lower in rats

treated with HCTZ or dietary sodium in addition to quinapril when compared to quinapril alone. Treatment groups were well balanced for infarct size (table 1).
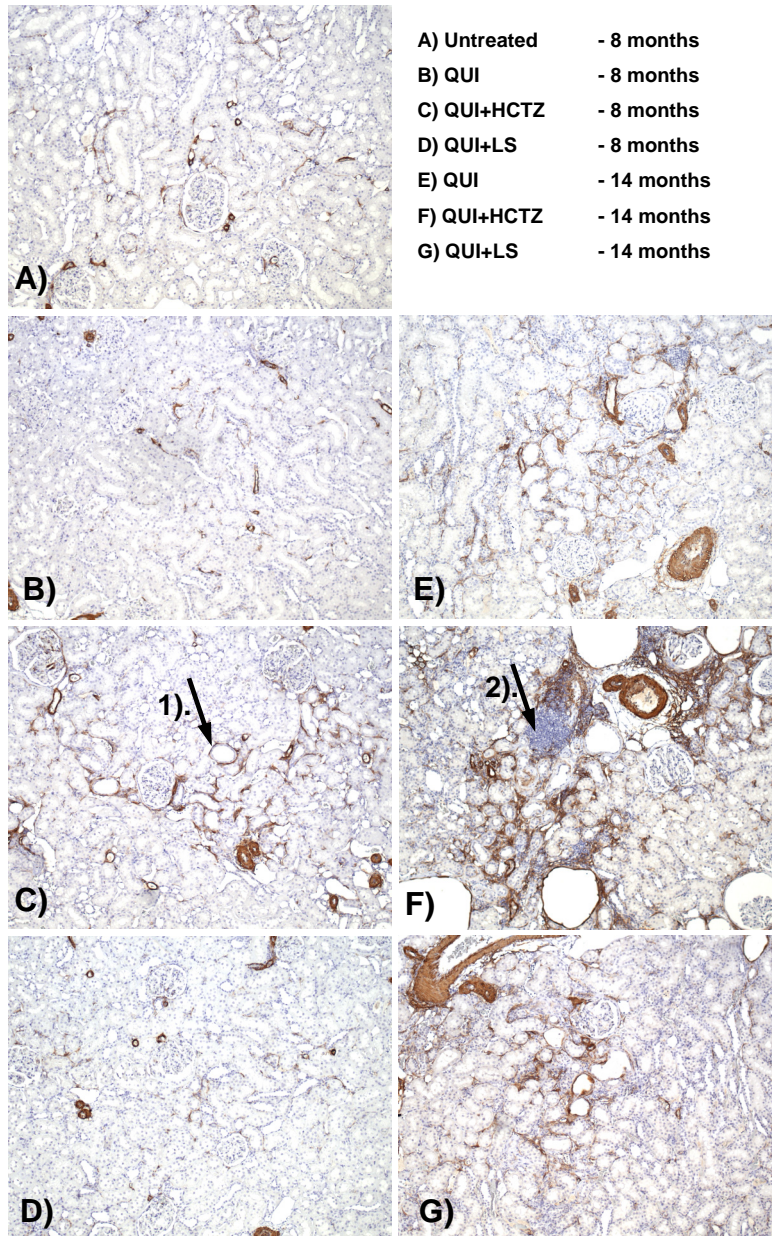
Table 1. General characteristics of rats included in histological measurements, after 8 and 14 months treatment.

| | Month | MI-Quinapril | | | MI |
		Control	+ HCTZ	+ LS	Control
N=	8	6	5	5	4
	14	11	12	10	-
MI size (% of LV)	8	25±1	28±1	29±3	27±3
	14	34±2	33±1	37±2	-
Plasma K+ (mmol/L)	8	4.4±0.1 *	4.4±0.2 *	4.4±0.2 *	3.9±0.2
	14	4.7±0.2	4.5±0.2	5.0±0.1	-
Plasma Na+ (mmol/L)	8	137.4±0.3	136.6±0.5 *	134.5±2.1 *	142.8±4.4
	14	131.1±0.9	130.3±0.4	130.6±0.6	-
SBP (mm Hg)	8	92±2 *	91± 3 *	89±4 *	125±9
	14	94±5	94±3	91±3	-

p<0.05 versus untreated control. HCTZ; Hydrochlorothiazide, LS; Low sodium diet, SBP; systolic blood pressure at termination.
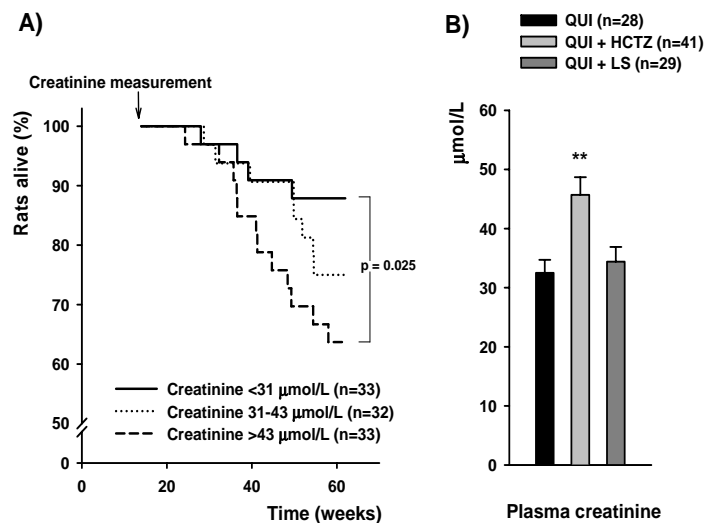

Figure 1. Renal function during ACE inhibition after myocardial infarction, and relation with mortality. A) Survival curves represent groups stratified into tertiles based on plasma creatinine. B) Effect of add-on HCTZ and dietary sodium restriction on renal function as reflected by plasma creatinin concentrations. ** p<0.005 versus QUI.

A) Untreated - 8 months
B) QUI - 8 months
C) QUI+HCTZ - 8 months
D) QUI+LS - 8 months
E) QUI - 14 months
F) QUI+HCTZ - 14 months
G) QUI+LS - 14 months

Figure 2. Renal tubular degeneration and increased expression of the fibrosis marker α-smooth muscle actin (brown) by addition of hydrochlorothiazide to ACE inhibitor therapy in rats with myocardial infarction. A) 8 Months untreated MI; B-D) 8 months treatment with QUI, QUI+HCTZ, and QUI+LS, respectively; E-G 14 months treatment with QUI, QUI+HCTZ, and QUI+LS, respectively. 1) tubular dilatation, 2) infiltration of inflammatory cells.

A) α-smooth muscle actin
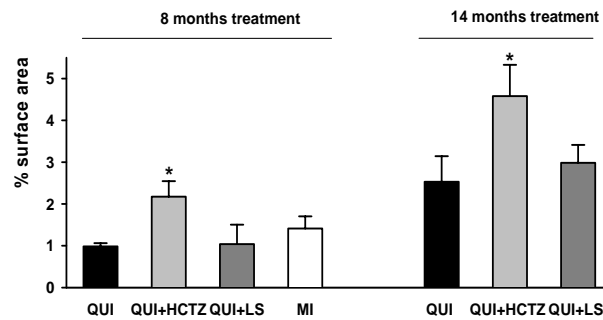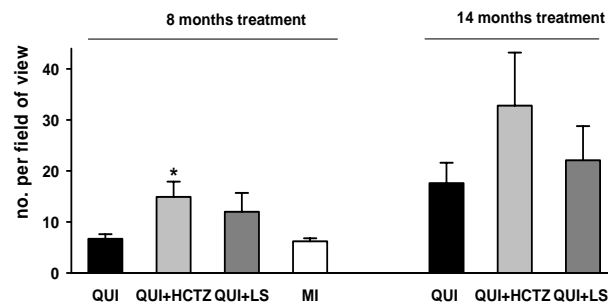

B) macrophage infiltration

Figure 3. Effects of sodium depletion on parameters representing renal interstitial fibrosis in rats with myocardial infarction, after 8 and 14 months treatment with ACE inhibitor quinapril, and. α-SMA; interstitial alpha-smooth muscle actin. * $p<0.05$ versus quinapril treated control.

Quinapril treatment did not result in abnormal renal morphology, except for proximal arteriolar wall thickening (not shown). However, addition of HCTZ to quinapril treatment had profound effects on tubulo-interstitial morphology. Most notably marked tubular dilatation and degeneration of tubular cells occurred (figure 2). This was accompanied by myofibroblast transformation, as indicated by significantly increased α-SMA expression (figures 2 and 3). Furthermore, marked peritubular interstitial fibrosis was confirmed by intense collagen-positive staining (with Sirius Red) around the dilated tubuli (figure 4, right panel). Fibrosis coincided with inflammation, as shown by significantly increased interstitial macrophage influx (figures 2 and 3). Comparison between quinapril-treated and untreated MI rats showed that the quinapril *per se* or combined with dietary sodium restriction did not cause any sign of tubulo-

interstitial damage. These interstitial lesions were also not observed in rats treated with a low sodium diet in addition to quinapril.

Discussion

We studied the effects of long-term add-on diuretic treatment or dietary sodium restriction to ACE inhibition on kidney structure and function in rats with myocardial infarction. Main observation of this study was that hydrochlorothiazide caused a marked increase in plasma creatinine concentrations, and renal tubular degeneration and interstitial fibrosis in rats treated with the ACE inhibitor quinapril. In contrast, dietary sodium restriction did not have these effects.

As reduced kidney function was associated with increased mortality in the current study, adverse renal effects of adding HCTZ treatment to ACE inhibition may hence overwhelm the favorable effects of combination therapy on cardiac function and survival on the long term. This would explain why diuretic treatment failed to improve long–term outcome of ACE inhibition in rats with myocardial infarction, despite early beneficial effects on cardiac function and survival [4].

Renal effects of add-on HCTZ
Plasma creatinine concentrations were determined as a measure for renal function. The increase in plasma creatinine concentration caused by addition of HCTZ to quinapril may have been caused by volume depletion, resulting in hypoperfusion of the kidneys[6]. This especially holds true for LV dysfunction, which is intrinsically characterized by decreased cardiac output and low renal perfusion[11].

The mechanism underlying the tubulo-interstitial abnormalities cannot be determined from the current study. The tubular lesions described in the current study could reflect hypoxia-induced damage as a result of renal hypoperfusion after volume depletion by combining HCTZ and ACE inhibitor treatment. However, this explanation is unlikely, as we saw no tubular degeneration in rats treated with quinapril only or quinapril + low sodium diet, whereas the effects of these regimens on blood pressure in these rats were as pronounced as in rats treated with add-on HCTZ. Furthermore, low renal perfusion by ACE-I is generally reflected by prominent afferent arteriolar wall thickening[8], but morphometry analysis did not show further afferent wall thickening in HCTZ-treated rats (data not shown).

Alternatively, direct effects of HCTZ on the tubular cells may have caused the lesions reported in the present study. Low sodium diet did not influence renal morphology; indicating that the effects of HCTZ are independent of sodium status. This is in accordance with a previous study, which showed that a comparable dose of HCTZ caused degeneration and apoptosis of distal tubular cells and interstitial infiltration of inflammatory cells in normotensive, sodium-repleted rats[12]. It was postulated that this effect was caused by complete inhibition of Na^+ entry or by increased Ca^{2+} entry into these cells. In theory, the interstitial lesions could also have been caused by diuretic-induced hypokalemia[13]. However, this is unlikely for the current study, as we found no effect of HCTZ on plasma K^+ concentrations.

Renal function and post-MI survival

This is to our knowledge the first experimental animal study showing a relation between renal function and prognosis after myocardial infarction. This finding is of particular interest when considering the mechanisms underlying this cardio-renal interaction. It is firmly established that kidney function is strongly associated with long-term prognosis in patients with left ventricular dysfunction after myocardial infarction[14-17]. It has been suggested that the relation between mild renal impairment and progression of cardiac disease in human patients is non-causal, and that both are the consequence of traditional cardiovascular risk factors, such as atherosclerosis and hypertension. However, these confounding factors are not present in our experimental model. Hence, decreased GFR (reflected by increased plasma creatinine concentrations) itself may be a risk factor triggering development of heart failure. Accordingly cardiomyocyte apoptosis, and reduced capillary/cardiomyocyte ratios have been observed in rats with mild renal impairment[18,19]. Furthermore rats with mild renal impairment by nephrectomy showed reduced ischemia tolerance, through a yet unresolved mechanism, but independent of confounding effects of hypertension, sympathetic overactivity, and salt retention[20]. Hence, a decrease in renal function caused by addition of HCTZ to ACE inhibition may on the long-term affect survival after myocardial infarction.
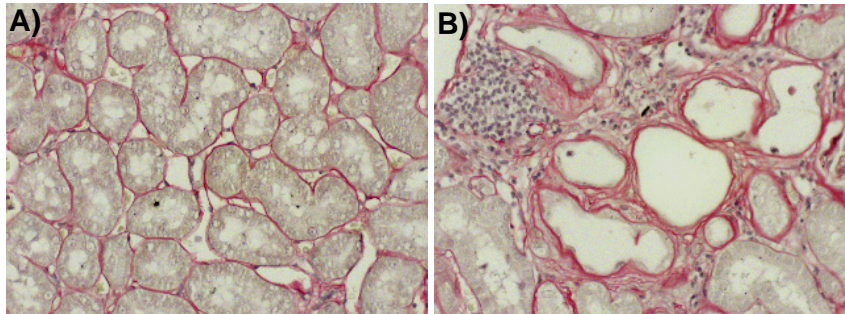
Figure 4. Collagen staining of renal cortical tubuli. A) Typical example of normal tubuli, B) Tubular degeneration by HCTZ+quinapril treatment coincided with collagen deposition.

Study limitations

The current study does not show a causal relation between the HCTZ-induced decrease in renal function and long-term mortality. However, it shows that this combination causes on average an increase in plasma creatinine concentrations to an extent that is associated with increased long-term mortality.

The dose of hydrochlorothiazide used in the present study (50/mg/kg/day) is much higher than the dose generally given to patients with LV dysfunction (±0.5-1 mg/kg/day). However, as drug absorption and kinetics are different in rat and man, this does not mean that we used a toxic, supra-pharmacological dose of HCTZ. Furthermore, previous studies showed that this dose causes no blood pressure reduction and

hypokalemia in rats with myocardial infarction[10], and much higher dosages were tolerated in long-term toxicological studies[21-23].

As we did not include a group treated with HCTZ monotherapy, it cannot be determined whether the renal functional and morphological abnormalities are caused by HCTZ per se, or by the combination of HCTZ with quinapril. Nevertheless, as all patients with LV dysfunction post-MI should be treated with RAAS inhibition, primary aim of our studies was to investigate whether diuretic therapy can be safely applied in a setting mimicking the clinical situation.

Conclusion

Adding the diuretic hydrochlorothiazide to ACE inhibitor treatment detrimentally affected not only renal function, but also renal structure in rats with myocardial infarction. As decreased renal function was associated with increased mortality, adverse renal effects of volume depletion by adding HCTZ to ACE-I may exert unfavorable effects on long-term prognosis after MI.

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