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N-acetylcysteine attenuates the progression of chronic renal failure

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N-acetylcysteine attenuates the progression of chronic renal failure.

Background. Lipid peroxidation impairs renal function. Aldosterone contributes to renal injury in the remnant kidney model. This study aimed to determine the effects of the antioxidant N-acetylcysteine (NAC) on renal function and aldosterone levels in chronic renal failure.

Methods. Adult male Wistar rats were submitted to 5/6 nephrectomy or laparotomy (sham-operated) and received NAC (600 mg/L in drinking water, initiated on postoperative day 7 or 60), spironolactone (1.5 g/kg of diet initiated on postoperative day 7), the NAC-spironolactone combination or no treatment. Clearance studies were performed on postoperative days 21, 60, and 120.

Results. Mean daily NAC and spironolactone ingestion was comparable among the treated groups. Mean weight gain was higher in NAC-treated rats than in untreated rats. A significant decrease in urinary thiobarbituric acid reactive substances (TBARS) concentrations, a lipid peroxidation marker, was observed in NAC-treated rats. By day 120, glomerular filtration rate (GFR), which dropped dramatically in untreated rats, was stable (albeit below normal) in NAC-treated rats, which also presented lower proteinuria, glomerulosclerosis index, and blood pressure, together with attenuated cardiac and adrenal hypertrophy. These beneficial effects, observed even when NAC was initiated on postnephrectomy day 60, were accompanied by a significant reduction in plasma aldosterone and urinary sodium/potassium ratio. The NAC-spironolactone combination lowered blood pressure and improved GFR protection.

Conclusion. The NAC-spironolactone combination improves renal function more than does NAC alone. In the remnant kidney model, early or late NAC administration has a protective effect attributable to decreased plasma aldosterone and lower levels of lipid peroxidation.

Key words: acetylcysteine, kidney failure, chronic, lipid peroxidation, TBARS, kidney function tests, inulin, aldosterone, spironolactone.

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Oxidative stress, resulting from the imbalance between reactive oxygen species (ROS) and the antioxidant system, contributes to the pathogenesis of different diseases. Several studies have demonstrated that chronic renal failure is associated with oxidative stress [1, 2]. An antioxidant deficient diet increased the progression of renal disease in animals with nephrectomy [3]. However, the pathogenesis of oxidative stress in chronic renal failure patients remains poorly defined [4]. Levels of malondialdehyde (MDA), a product of lipid peroxidation, have been shown to increase after 5/6 nephrectomy in rats. The antioxidant N-acetylcysteine (NAC) is a source of sulfhydryl groups in cells and, due to its interaction with ROS, is a scavenger of free radicals [5]. It has been shown that NAC administration improves endothelial function while reducing inflammation, fibrosis, cartilage explants, and acetaminophen liver metal toxicity [5]. In addition, NAC decreases MDA levels in chronic hemodialysis patients [6] and ameliorates ischemic renal failure [7]. It has also been used in the prevention of radiocontrast-induced renal failure [8]. The effect of NAC on progressive renal disease has not been previously evaluated.

Aldosterone contributes to renal injury in the remnant kidney model [9]. In the heart, aldosterone-induced inflammation is also mediated by oxidative stress and attenuated by NAC [10]. Aldosterone, partly via increased oxidative stress, may mediate some of the angiotensin II–induced vascular effects seen in hypertension [11]. Treatment with NAC reduces composite endpoints of cardiovascular events in patients with end-stage renal failure [12].

This study was carried out in order to determine the effects of NAC on kidney function and aldosterone production in the remnant kidney model.

METHODS

A total of 70 adult male Wistar rats (150 to 200 g) were provided by the University of São Paulo School of Medicine for use in this study. All rats were anesthetized

with 50 mg/kg of body weight of sodium pentobarbital, administered intraperitoneally, and were subjected to either 5/6 nephrectomy, induced by right nephrectomy and ligation of two branches of the renal artery, or laparotomy (sham operation). After recovering from the anesthesia, the animals were returned to their original cages and given free access to water and standard rat chow (Nuvilab®) (Curitiba, Brazil). Clearance studies were performed, variously, on postoperative days 21, 60, and 120. One day prior to each clearance study, rats were housed in metabolic cages without food or water. The Ethics in Research Committee of the University of São Paulo School of Medicine approved the study design.

Early NAC administration after 5/6 nephrectomy or sham operation

In order to assess the effect of NAC administration in the early stages of nephrectomy-induced renal failure, animals were assigned to one of various groups, some receiving NAC (600 mg/L in drinking water), initiated on postoperative day 7, and some receiving no treatment. The groups were defined as follows: sham/EV21 (sham operated and evaluated on postoperative day 21)($N = 6$); nephrectomized/EV21 (nephrectomized and evaluated on day 21) ($N = 6$); nephrectomized/EV60 (nephrectomized and evaluated on day 60) ($N = 6$); nephrectomized/EV120 (nephrectomized and evaluated on day 120) ($N = 6$); sham + NAC-IN7/EV21 (sham operated, treated with NAC from day 7 and evaluated on day 21) ($N = 6$); nephrectomized + NAC-IN7/EV21 (nephrectomized, treated with NAC from day 7 and evaluated on day 21) ($N = 6$); nephrectomized + NAC-IN7/EV60 (nephrectomized, treated with NAC from day 7 and evaluated on day 60) ($N = 6$); and nephrectomized + NAC-IN7/EV120 (nephrectomized, treated with NAC from day 7 and evaluated on day 120) ($N = 6$).

NAC administration in the later stages of renal failure

To evaluate the effect of NAC on end-stage renal failure, another group was created, composed of nephrectomized rats receiving NAC (600 mg/L in drinking water) initiated on postoperative day 60. These rats were evaluated on postoperative day 120, and the group was designated nephrectomized + NAC-IN60/EV120 ($N = 6$).

Early administration of spironolactone and the NAC-spironolactone combination

Two additional groups were created, both composed of nephrectomized rats. The animals in one group received spironolactone (1.5 g/kg of diet, initiated on postoperative day 7) and were evaluated on postoperative day 60. This group was designated nephrectomized + spironolactone-IN7/EV60 ($N = 8$). Animals in the other group, designated nephrectomized +spironolactone-NAC-IN7/EV60 ($N = 8$) also received spironolactone (in the same quantity), together with NAC (600 mg/L in drinking water), both initiated on postoperative day 7, and were also evaluated on postoperative day 60.

Urinary protein excretion and urinary levels of thiobarbituric acid reactive substances (TBARS)

As previously mentioned, rats were housed in metabolic cages without food or water before each clearance study, and a 24-hour urine sample was collected in order to determine urinary protein excretion and levels of TBARS. Urinary protein excretion was determined using the Sensiprot Kit (Labtest, São Paulo, Brazil). Urinary TBARS levels were assessed using the thiobarbituric acid method, diluting a 0.2 mL urine sample in 0.8 mL of distilled water, to which 1 mL of 17.5% trichloroacetic acid was immediately added. Subsequently, 1 mL of 0.6% thiobarbituric acid, pH 2, was added. The sample was then placed in a boiling water bath for 15 minutes. After the sample had cooled, 1 mL of 70% trichloroacetic acid was added, and the mixture was allowed to incubate for 20 minutes. The sample was then centrifuged for 15 minutes at 2000 rpm. The optical density of the supernatant was read at 534 nm against a reagent blank using a spectrophotometer. The concentration of lipid peroxidation products was calculated as MDA equivalent using a molar extinction coefficient for the MDA-thiobarbituric acid complex of 1.56×10^5 mol^{-1}/cm^{-1}. Urinary levels of TBARS were expressed as nmol/24 hours [3].

Clearance studies

Prior to the performance of the clearance studies, each designated animal was anesthetized intraperitoneally with sodium thiopental (50 mg/kg body weight). The trachea was cannulated with a polyethylene (PE)-240 catheter, and spontaneous breathing was maintained. To control mean arterial pressure and allow blood sampling, the right carotid artery was catheterized with a PE-60 catheter. For infusion of inulin and fluids, the left jugular vein was cannulated with a PE-60 catheter. In order to collect urine samples, a suprapubic incision was made, and the urinary bladder was cannulated with a PE-240 catheter. Following the surgical procedure, a loading dose of inulin (100 mg/kg body weight diluted in 0.9% saline) was administered through the jugular vein. A constant infusion of inulin (10 mg/kg body weight in 0.9% saline) was then started and continued at 0.04 mL/min throughout the experiment. A total of three urine samples were collected at 30-minute intervals. Blood samples were obtained at the beginning and end of the experiment. At the clearance study end point, the kidneys were flushed with saline and perfused with Dubosq-Brazil (modified Bouin) fixative in situ at the measured arterial pressure.

The renal tissue was then weighed, and two sections were postfixed in buffered 10% formaldehyde solution. The material was embedded in paraffin for later assessment of glomerular and renal cortical interstitial injury. Hearts and adrenal glands were also removed and weighed.

Blood and urine inulin were determined by the anthrone method, and sodium and potassium concentrations were measured using a flame photometer (model 143) (Instrumentation Laboratory, Lexington, MA, USA). Glomerular filtration rate (GFR) and urinary sodium/potassium (UNa/UK) ratio were calculated. Serum aldosterone in blood samples obtained at the end of the clearance study was determined by radioimmunoassay (Coat-A-Count) DPC, Los Angeles, CA, USA).

Histomorphometric analysis

Paraffin-embedded renal tissue was deparaffinized using standard techniques, and 2- to 3 m thick sections were stained with periodic acid-Schiff (PAS) and Masson trichrome technique. A single blinded observer performed all histomorphometric measurements. The extent of glomerular sclerosis was evaluated in four animals on postnephrectomy day 60 and in six animals on postnephrectomy day 120. A score was attributed to each glomerulus according to the extent of sclerotic injury: 0, intact; 1, up to 10% damaged; 2, 11% to 20% damaged; 3, 21% to 30% damaged; 4, 31% to 40% damaged; 5, 41% to 50% damaged; 6, 51% to 60% damaged; 7, 61% to 70% damaged; 8, 71% to 80% damaged; 9, 81% to 90% damaged; and 10, 91% to 100% damaged. For each rat, the glomerulosclerosis index (GSI) was calculated. The GSI is a weighted average, multiplied by 100, of all individual glomeruli thus obtained. At least 50 glomeruli per rat were examined.

Fractional interstitial area

In kidneys obtained from nephrectomized/EV120 and nephrectomized + NAC-IN7/EV120 group animals, the fractional interstitial area of the renal cortex was determined by morphometry with a light camera connected to an image analyzer (KS300) (Kontron, Munich, Germany) [13]. Twenty 0.174 mm^2 grid fields were evaluated in the renal cortex of each kidney. Interstitial areas were first manually encircled on a video screen and then determined by computerized morphometry.

Immunohistochemical analysis

Immunohistochemical analysis was performed for kidneys obtained from animals in the nephrectomized/EV120 and nephrectomized + NAC-IN7/EV120 groups. The primary antibodies used were a monoclonal anti-rat endothelin-1 (ED-1) antibody to a cytoplasmic antigen present in macrophages and monocytes (Serotec Product Datasheet, Oxford, UK) and a monoclonal IgG antibody to rat lymphocytes (CD3) (Harlan Sera-Lab, Loughborough, England). The sections were incubated for 1 hour with anti-ED-1 antibodies (macrophages) (1:1000) or CD3 antibodies (lymphocytes) (1:1000). The reaction product was detected with an avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA, USA). The color reaction was developed with 3,3′-diaminobenzidine (DAB) (Sigma Chemical Company, St. Louis, MO, USA), and the material was counterstained (with methyl green for ED-1 or hematoxylin for ED-3), then dehydrated and mounted. Nonspecific protein binding was blocked by incubation with 20% goat serum in phosphate-buffered saline (PBS) for 20 minutes. Replacing the primary antibody with mouse IgG, at an equivalent concentration, created a negative control.

Infiltrating macrophages/monocytes and lymphocytes in the renal cortex were counted in the tubulointerstitium and in the glomeruli using a 0.245 mm^2 grid field or 30 glomeruli, respectively.

Statistical analysis

Data were analyzes using one-way analysis of variance (ANOVA) and Bonferroni's post test using GraphPad Prism (version 3.0) and Stata (version 8.0) statistical software.

Data are mean ± SEM, a P value < 0.05 was considered to be significant at a two-tailed level. The ANOVA and Bonferroni's post test were used to analyze the GSI. Analysis of the immunohistochemistry was performed using *t test* with Welch's correction. Statistical significance was established at $P < 0.05$.

RESULTS

Early NAC administration after 5/6 nephrectomy or sham operation

There was a significant and progressive increase in the body weight of all rats submitted to 5/6 nephrectomy. However, nephrectomized + NAC-IN7/EV60 and nephrectomized + NAC-IN7/EV120 rats presented significantly higher mean body weight gain than did untreated nephrectomized rats (Table 1).

As illustrated in Table 1, NAC ingestion was similar in all NAC-IN7–treated groups. Urinary protein was significantly higher in nephrectomized rats than in sham-operated rats. However, nephrectomized + NAC-IN7/EV60 and nephrectomized + NAC-IN7/EV120 rats presented lower levels of protein excretion than did untreated nephrectomized rats.

Urinary TBARS excretion was also lower in nephrectomized + NAC-IN7 group rats than in untreated nephrectomized rats. In nephrectomized + NAC-IN7/

Table 1. Mean body weight gain, daily N-acetylcysteine (NAC) ingestion and 24-hour urinary excretion of protein and thiobarbituric acid reactive substances (TBARS) on postoperative days 21, 60, and 120 in untreated rats and in rats receiving NAC initiated on postoperative day 7

	ΔBody weight	NAC ingestion *mg/100 g body weight/day*	Urinary protein *mg/24 hours*	Urinary TBARS *nmol/24 hours*
Sham/EV21	106 ± 10	—	4.74 ± 1.02	18.22 ± 2.41
Nephrectomized/EV21	133 ± 14	—	21.46 ± 2.71[b]	28.83 ± 3.22
Nephrectomized/EV60	141 ± 11	—	26.57 ± 2.99[b]	109.0 ± 17.95[a]
Nephrectomized/EV120	248 ± 20	—	207.5 ± 9.50[a]	254.0 ± 51.51[a]
Sham + NAC-IN7/EV21	98 ± 9	14.0 ± 0.5	3.41 ± 0.45	14.85 ± 1.64
Nephrectomized + NAC-IN7/EV21	146 ± 16	13.7 ± 0.6	14.78 ± 1.93	15.50 ± 0.89[d]
Nephrectomized + NAC-IN7/EV60	188 ± 13[c]	14.5 ± 0.3	15.62 ± 2.64	63.21 ± 6.32[a,e]
Nephrectomized + NAC-IN7/EV120	308 ± 16[d]	15.7 ± 0.8	158.6 ± 9.82[a,d]	91.69 ± 6.03[a,e]

Data expressed as mean ± SEM. Abbreviations are: ΔBW, variation (increase) in body weight; sham, sham-operated; IN7, initiated (treatment) on postoperative day 7; EV21, evaluated on postoperative day 21; EV60, evaluated on postoperative day 60; EV120, evaluated on postoperative day 120.
[a]$P < 0.001$; [b]$P < 0.05$, vs. sham-operated animals; [c]$P < 0.01$; [d]$P < 0.005$, [e]$P < 0.02$; comparison between treated and untreated groups by one-way analysis of variance (ANOVA) and Bonferroni's post test.

Table 2. Renal and systemic functional parameters on postoperative days 21, 60, and 120 in untreated rats and in rats receiving N-acetylcysteine (NAC) initiated on postoperative day 7

	Serum aldosterone *ng/dL*	Glomerular filtration rate *mL/min/100 g body weight*	Blood pressure *mm Hg*	Fractional excretion of potassium %	Uninary sodium/ potassium ratio	Heart weight *g*	Adrenal weight *mg*
Sham/EV21	11 ± 2	0.83 ± 0.05	109 ± 3	28.4 ± 1.6	0.87 ± 0.03	0.85 ± 0.03	29 ± 0.25
Nephrectomized/EV21	288 ± 34[a]	0.25 ± 0.06[a]	138 ± 4[a]	34.0 ± 4.7	4.74 ± 1.61[b]	1.04 ± 0.03[b]	41 ± 2.70[a]
Nephrectomized/EV60	319 ± 50[a]	0.22 ± 0.06[a]	158 ± 3[a]	219.8 ± 46.9[a]	5.90 ± 2.03[b]	1.37 ± 0.06[a]	59 ± 0.84[a]
Nephrectomized/EV120	134 ± 12[b]	0.16 ± 0.03[a]	174 ± 5[a]	100.1 ± 22.1[a]	5.13 ± 0.69[b]	1.51 ± 0.07[a]	59 ± 2.02[a]
Sham + NAC-IN7/EV21	15 ± 2	0.93 ± 0.02	103 ± 1	24.0 ± 1.6	1.76 ± 0.32	0.81 ± 0.05	27 ± 0.88
Nephrectomized + NAC-IN7/EV21	54 ± 16[a,c]	0.48 ± 0.05[a,f]	134 ± 2[a]	28.3 ± 4.5	1.89 ± 0.62	1.04 ± 0.06[b]	45 ± 4.20[a]
Nephrectomized + NAC-IN7/EV60	125 ± 24[a,c]	0.47 ± 0.05[a,e]	154 ± 2[a]	46.7 ± 4.6[a]	1.63 ± 0.27	1.16 ± 0.04[a,f]	35 ± 2.10[a,e]
Nephrectomized + NAC-IN7/EV120	59 ± 2[b,e]	0.45 ± 0.04[a,d]	150 ± 7[a,d]	18.24 ± 2.2[d]	2.23 ± 0.72[f]	1.35 ± 0.05[a]	42 ± 1.7[a,e]

Data expressed as mean ± SEM. Abbreviations are: Sham, sham-operated; IN7, initiated (treatment) on postoperative day 7; EV21, evaluated on postoperative day 21; EV60, evaluated on postoperative day 60; EV120, evaluated on postoperative day 120.
[a]$P < 0.001$; [b]$P < 0.05$, vs. sham-operated animals; [c]$P < 0.0001$; [d]$P < 0.005$; [e]$P < 0.01$; [f]$P < 0.05$, comparison between treated and untreated groups by one-way analysis of variance (ANOVA) and Bonferroni's post test.

Table 3. Renal and systemic functional parameters on postoperative day 60 in nephrectomized rats receiving spironolactone alone or the combination of spironolactone and N-acetylcysteine (NAC) initiated on postoperative day 7

Group	Blood pressure *mm Hg*	Glomerular filtration rate *mL/min/100 g body weight*	Proteinuria *mg/24 hours*	Urinary sodium/ potassium ratio	Aldosterone *ng/dL*	TBARS *nmol/24 hours*	Heart weight *g*	Adrenal weight *mg*
Nephrectomized + spironolactone-IN7/EV60	155 ± 3	0.28 ± 0.04	18 ± 1	0.90 ± 0.1	325 ± 77	118 ± 14	1.38 ± 0.03	57 ± 1.5
Nephrectomized + spironolactone + NAC-IN7/EV60	136 ± 2[a]	0.59 ± 0.04[a]	14 ± 1[b]	0.66 ± 0.1	183 ± 20	81 ± 5[b]	1.31 ± 0.03	41 ± 1.4[a]

Data expressed as mean ± SEM. Abbreviations are: TBARS, thiobarbituric acid reactive substances; IN7, initiated (treatment) on postoperative day 7; EV60, evaluated on postoperative day 60
[a]$P < 0.0001$ vs. nephrectomized + spironolactone only; [b]$P < 0.03$ vs. nephrectomized + spironolactone only, by unpaired *t* test.

EV21 rats, levels of TBARS were comparable to those observed in sham-operated rats, whereas there was a significant increase in this parameter in untreated nephrectomized rats. In nephrectomized + NAC-IN7/EV60 and nephrectomized + NAC-IN7/EV120 rats, respectively, TBARS levels were approximately 40% and 60% lower than in untreated nephrectomized rats (Table 1). These data suggest that NAC reduces lipid peroxidation and attenuates the renal injury responsible for the high protein excretion in the 5/6 nephrectomy model.

Table 2 shows that inulin clearance (and therefore GFR) fell progressively in nephrectomy rats in comparison to sham-operated rats ($P < 0.001$). Nevertheless, rats in all nephrectomy + NAC-IN7 groups presented less dramatic drops in GFR than did sham-operated rats, with values almost 50% higher than those seen in untreated nephrectomized rats.

Serum aldosterone increased significantly and progressively in nephrectomized rats compared with sham-operated rats. However, rats in all nephrectomized +

NAC-IN7 groups presented significantly lower serum levels of this hormone, approximately five times lower in nephrectomized + NAC-IN7/EV21 rats and 2.5 times lower in nephrectomized + NAC-IN7/EV60 rats than those seen in untreated nephrectomized rats. On postoperative day 120, all nephrectomized rats presented lower serum aldosterone levels, although rats in the nephrectomized + NAC-IN7 groups continued to present considerably lower values than did untreated nephrectomized rats (Table 2).

On postoperative days 60 and 120, fractional excretion of potassium was significantly lower in nephrectomized + NAC-IN7 group rats compared with untreated nephrectomized rats. On postoperative day 120, the UNa/UK ratio was significantly lower in all nephrectomized + NAC-IN7 rats than in untreated nephrectomized rats (Table 2).

Heart and adrenal weights also increased significantly and progressively in nephrectomized rats (Table 2). By postoperative day 21, the weights of these organs remained unchanged. However, nephrectomized + NAC-IN7/EV60 and nephrectomized + NAC-IN7/EV120 rats presented significantly lower heart and adrenal weights, indicating that NAC might have attenuated the hypertrophy of these organs during the progression of chronic renal failure.

There were no differences in blood pressure between nephrectomized + NAC-IN7–treated and untreated nephrectomized rats evaluated at 21 and 60 days. Nevertheless, a significant lower blood pressure was observed in nephrectomized + NAC-IN7/EV120 rats (Table 2).

As illustrated in Figure 1, the relative interstitial area in nephrectomized/EV120 rats was 22.47 ± 3.66%, compared with 10.17 ± 0.66% for nephrectomized + NAC-IN7/EV120 rats ($P < 0.03$).

As can be seen in Figures 2 and 3, the number of cells demonstrating ED-1 staining for macrophages/monocytes was significantly lower in nephrectomized + NAC-IN7/EV120 rats than in nephrectomized/EV120 rats, both in the glomeruli (1.46 ± 0.25 vs. 4.16 ± 0.70) ($P < 0.02$) and in the tubulointerstitium (13.26 ± 1.80 vs. 34.59 ± 6.16) ($P < 0.02$).

The immunohistochemical analysis also showed significantly lower numbers of infiltrating lymphocytes in nephrectomized + NAC-IN7/EV120 rats than in nephrectomized/EV120 rats, both in glomeruli (1.19 ± 0.26 vs. 2.10 ± 0.30) ($P < 0.05$) and in the tubulointerstitium (10.45 ± 0.61 vs. 27.12 ± 2.93) ($P < 0.05$) (Figs. 4 and 5).

NAC administration in the later stages of renal failure

In nephrectomized + NAC-IN60/EV120 rats, a protective effect similar to that seen in nephrectomized + NAC-IN7/EV120 rats was observed. Inulin clearance was 0.51 ± 0.03 mL/min/100 g body weight; mean arterial pressure was 159 ± 8 mm Hg; proteinuria was 134 ± 22 mg/day; urinary TBARS excretion was 127 ± 15 nmol/day; aldosterone 31 ± 4 ng/dL, UNa/UK was 1.3 ± 0.1, heart weight was 1.42 ± 0.07 g, and adrenal weight was 43 ± 3 mg. Mean body weight gain in the nephrectomized + NAC-IN60/EV120 group (336.7 ± 10.1 g) was comparable to that of the nephrectomized + NAC-IN7/EV120 group. These results indicate that NAC is protective even in end-stage renal failure.

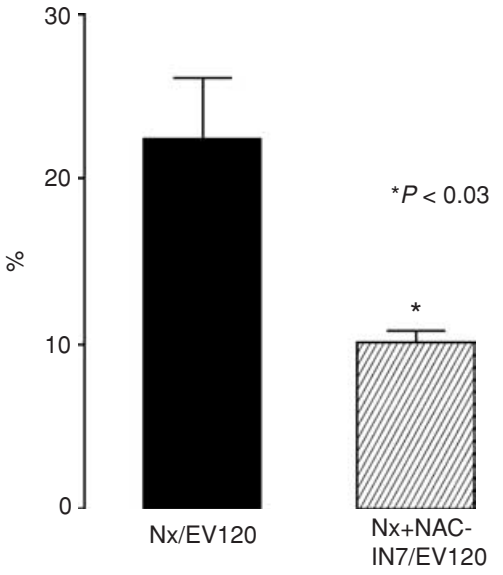

Fig. 1. Fractional interstitial area in cortex of nephrectomized and evaluated on postoperative day 120 (Nx/EV120) and nephrectomized plus N-acetylcysteine initiated treatment on day 7/evaluated on postoperative day 120 (Nx + NAC-IN7/EV120) rats. Data are expressed as mean ± SEM (unpaired *t* test with Welch's correction at $P < 0.03$).

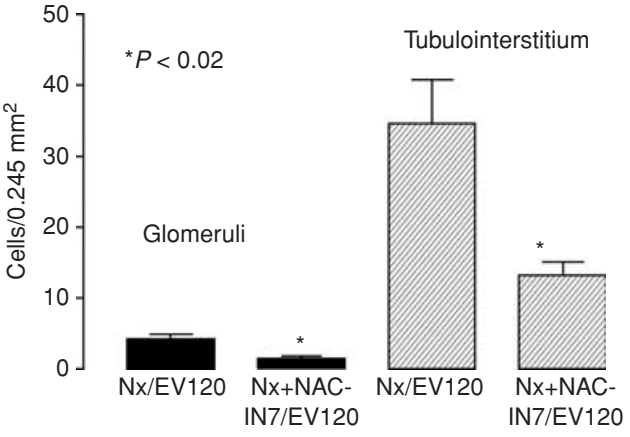

Fig. 2. Number of endothelin-1 (ED-1)–positive cells/mm^2 in the glomeruli and tubulointerstitium of nephrectomized/evaluated on postoperative day 120 (Nx/EV120) and nephrectomized plus N-acetylcysteine-initiated treatment on day 7/evaluated on postoperative day 120 (Nx+NAC-IN7/EV120) rats. Data are expressed as mean ± SEM (unpaired *t* test with Welch's correction at $P < 0.02$).

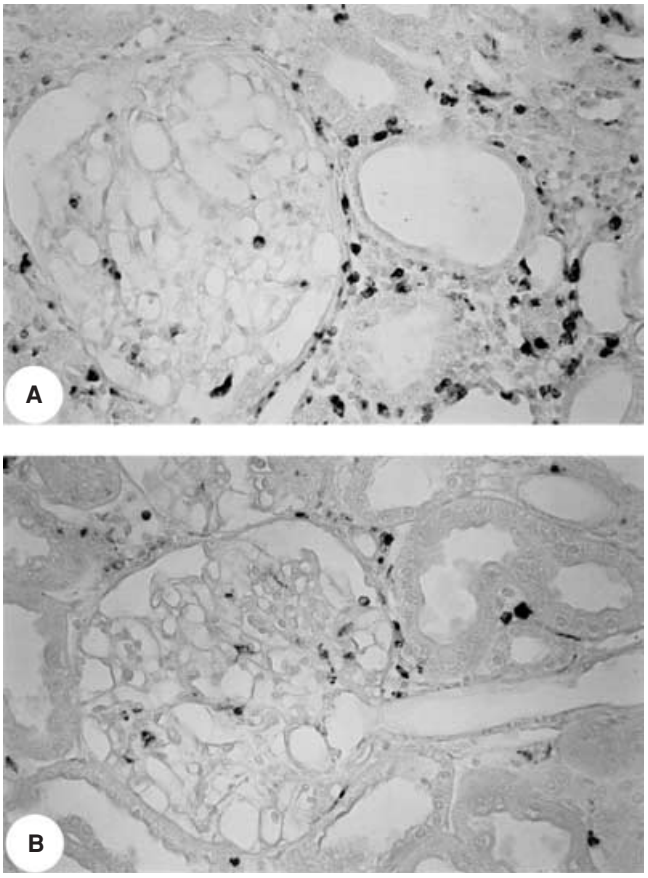

Fig. 3. Immunostaining for endothelin-1 (ED-1) (monocytes/macrophages) in the renal cortices from nephrectomized/evaluated on postoperative day 120 (Nx/EV120) rats (*A*) and nephrectomized plus N-acetylcysteine initiated on day 7/evaluated on postoperative day 120 (Nx+NAC-IN7/EV120) rats (*B*). Note that the number of ED-1–positive cells is higher in (A).

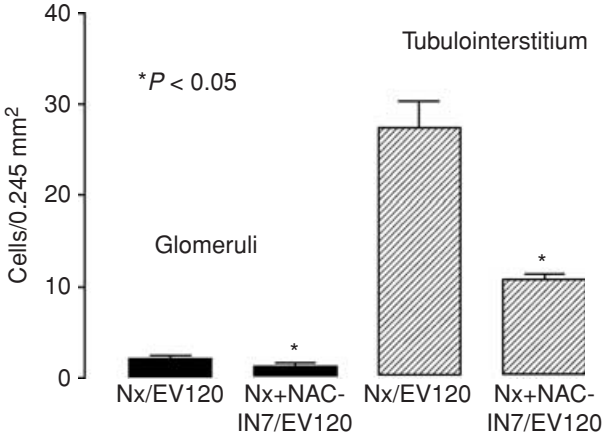

Fig. 4. Number of CD3-positive cells/mm^2 in the glomeruli and tubulointerstitium of nephrectomized/evaluated on postoperative day 120 (Nx/EV120) and nephrectomized plus N-acetylcysteine initiated on day 7/evaluated on postoperative day 120 (Nx + NAC-IN7/EV120) rats. Data are expressed as mean ± SEM (unpaired *t* test with Welch's correction at $P < 0.05$).

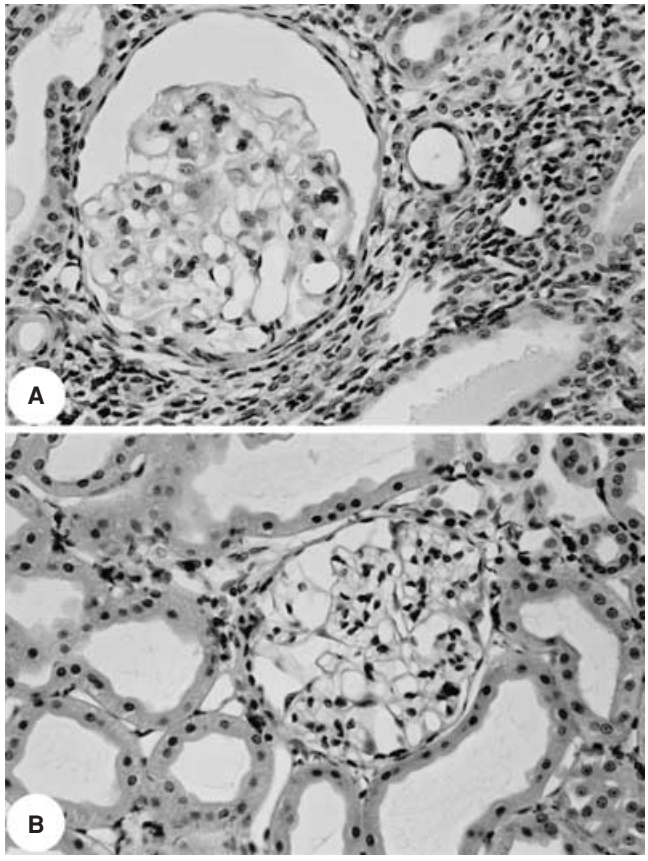

Fig. 5. Immunostaining for CD3 (T lymphocytes) in renal cortices from nephrectomized/evaluated on postoperative day 120 (Nx/EV120) rats (*A*) and nephrectomized plus N-acetylcysteine initiated on day 7/evaluated on postoperative day 120 (Nx + NAC-IN7/EV120) rats (*B*). Note that the number of CD3-positive cells is higher in (A).

As illustrated in Figure 6A, the GFR was higher in nephrectomized + NAC-IN7/EV120 rats and in nephrectomized + NAC-IN60/EV120 rats than in nephrectomized/EV120 rats, whereas the GSI after nephectomy was considerably lower in nephrectomized + NAC-IN7/EV120 rats and in nephrectomized + NAC-IN60/EV120 rats than in nephrectomized/EV120 rats (Fig. 6B)

The clearance data are from the surviving animals. In nephrectomized/EV120 we started with nine rats, one of which died on day 78, another one on day 98, and one on day 103, as a result, we performed clearance studies in six animals (33%). In the nephrectomized + NAC-IN7/EV120 group, we started with eight rats. One died on day 96 and another one on day 110, so six animals were studied (25%). In the nephrectomized + NAC-IN60/EV120 group, we started with seven animals and only one died on day 97 (14.3%).

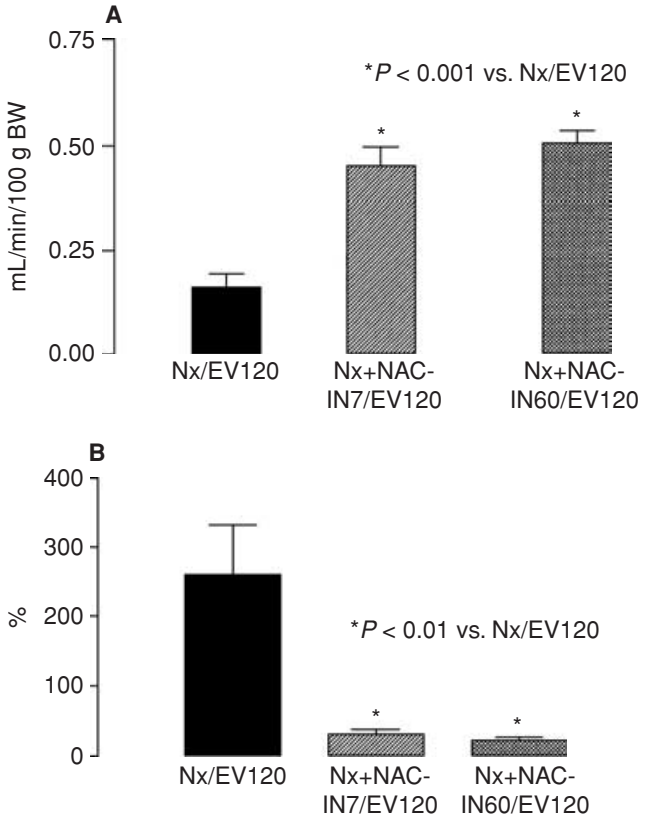

Fig. 6. (*A*) Inulin clearance and (*B*) glomerulosclerosis index in nephrectomized/evaluated on postoperative day 120 (Nx/EV120), nephrectomized plus N-acetylcysteine initiated on day 7/evaluated on postoperative day 120 (Nx + NAC-IN7/EV120) and nephrectomized plus N-acetylcysteine initiated on day 60/evaluated on postoperative day 120 (Nx+NAC-IN60/EV120) groups. Data are expressed as mean ± SEM [analysis of variance (ANOVA) and Bonferroni's post test, $P < 0.05$].

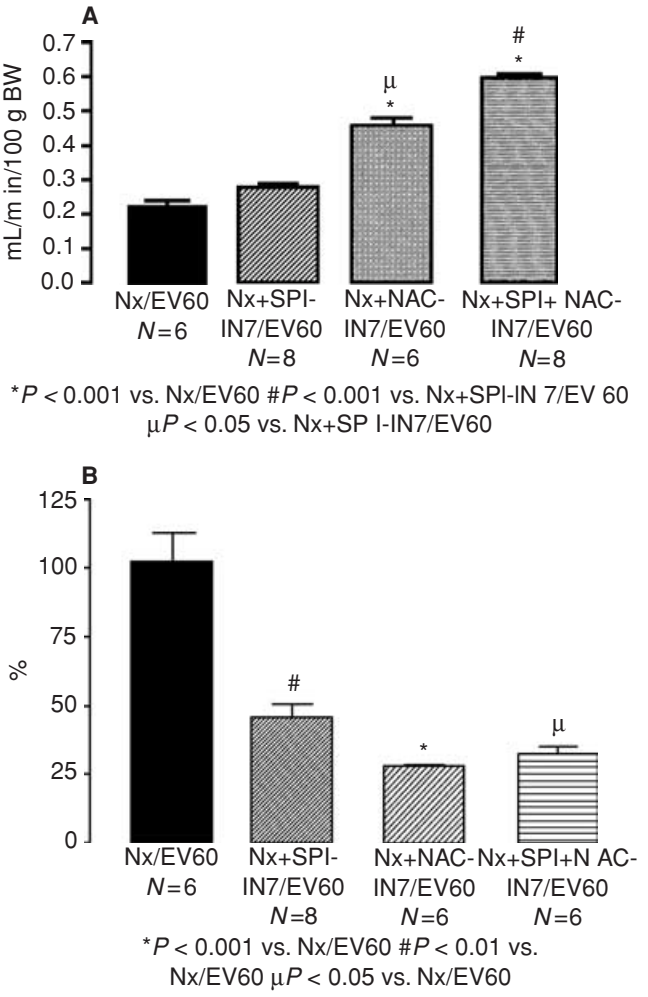

Fig. 7. (*A*) Inulin clearance and (*B*) glomerulosclerosis index in the nephrectomized/evaluated on postoperative day 60 (Nx/EV60), nephrectomized plus spironolactone initiated on day 7/evaluated on postoperative day 60 (Nx + SPI-IN7/EV60), nephrectomized plus N-acetylcysteine initiated on day 7/evaluated on postoperative day 60 (Nx + NAC-IN7/EV60) and nephrectomized plus spironolactone plus N-acetylcysteine initiated on day 7/evaluated on postoperative day 60 (Nx + SPI + NAC-IN7/EV60) groups. Data are expressed as mean ± SEM [analysis of variance (ANOVA) and Bonferroni's post test, $P < 0.05$].

Spironolactone and the NAC-spironolactone combination

Mean daily spironolactone ingestion was comparable between the two groups (nephrectomized + spironolactone-IN7/EV60 134 ± 7 mg/kg/day and nephrectomized + spironolactone + NAC-IN7/EV60 126 ± 5 mg/kg/day (not significant).

In the nephrectomized + spironolactone-IN7/EV60 group (Table 3), urinary protein excretion was lower than in the nephrectomized/EV60 group (Table 1) (18 ± 1 mg/24 hours vs. 27 ± 2 mg/24 hours) ($P < 0.001$); as was the UNa/UK ratio (0.90 ± 0.10 vs. 5.90 ± 2.0) ($P < 0.001$). The inulin clearance was greater in nephrectomized + spironolactone-IN7/EV60 (Table 3) than in the nephrectomized/EV60 group (0.28 ± 0.04 vs. 0.21 ± 0.06 mL/min/100g body weight) ($P < 0.05$). The same was observed in the case of arterial pressure (155 ± 3 vs. 158 ± 3 mm Hg) ($P < 0.05$), whereas no differences were observed in aldosterone, TBARS levels, heart, and adrenal weight (Tables 1–3).

The NAC-spironolactone combination resulted in lower mean blood pressure than did spironolactone only (nephrectomized + spironolactone + NAC-IN7/EV60 136 ± 2 mm Hg; nephrectomized + spironolactone-IN7/EV60 155 ± 3 mm Hg) ($P < 0.001$). In addition, inulin clearance in the nephrectomy + spironolactone + NAC-IN7/EV60 group was 0.59 ± 0.04 mL/min/100 g body weight, demonstrating an additive effect in comparison to treatment with NAC alone (nephrectomized + NAC-IN7/EV60 0.47 ± 0.05) ($P < 0.001$) (Fig. 7A, Table 3).

Mean GSI was significantly higher in the nephrectomized/EV60 group than in any of the other groups evaluated on postoperative day 60, the lowest GSI being

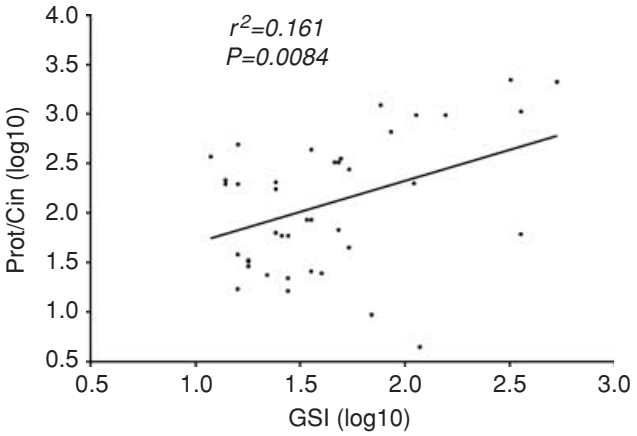

Fig. 8. Correlation between proteinuria, corrected by inulin clearance (UV prot/Cin), and the respective glomerulosclerosis index (GSI). Data are from nephrectomized/evaluated on postoperative day 60, nephrectomized plus spironolactone initiated on day 7/evaluated on postoperative day 60, nephrectomized plus N-acetylcysteine initiated on day 7/evaluated on postoperative day 60, nephrectomized plus spironolactone plus N-acetylcysteine initiated on day 7/evaluated on postoperative day 60, nephrectomized/evaluated on postoperative day 120, nephrectomized plus N-acetylcysteine initiated on day 7/evaluated on postoperative day 120, and nephrectomized plus N-acetylcysteine initiated on day 60/evaluated on postoperative day 120 groups.

observed in the nephrectomized + spironolactone + NAC-IN7/EV60 group (Fig. 7B).

If we plot the rate of urinary excretion of protein/inulin clearance with the respective glomerulosclerosis index of animals, we obtain a positive correlation (Fig. 8) ($r = 0.161$, $P < 0.008$).

DISCUSSION

Our data demonstrate that TBARS urinary excretion increases progressively in the remnant kidney model, indicating that lipid peroxidation plays an important role in the progression of chronic renal failure. Nath, Carott, and Hostetter [14] showed that MDA per nephron increases in a model of subtotal nephrectomy and is accompanied by an increase in fractional and absolute urinary excretion of MDA. A recent study showed that, in evaluating patients with chronic kidney disease, the measurement of oxidative damage markers (such as MDA) in urine is a more sensitive method than the measurement of such markers in plasma [15].

In the present study, we showed that NAC lowers lipid peroxidation significantly and consistently for a period of at least 120 days after subtotal nephrectomy. This was accompanied by a significant protective effect on GFR and renal inflammation, as well as by significant decreases in aldosterone levels.

Subtotal (5/6) nephrectomy is widely used to create experimental models of progressive nephropathy. Renal inflammation is prominent in such models. Various methods of reducing renal injury in nephrectomized rats, such as the administration of angiotensin-converting enzyme (ACE) inhibitors, angiotensin II antagonists or immunosuppressive agents (such as mycophenolate mofetil), have been employed [16–19]. All of these agents decreased proteinuria and reduced renal inflammation, as evaluated by the GSI and tubulointerstitial injury scores. However, significant protection of GFR, as determined by inulin clearance, has not been reported.

The major new finding of the present study is that NAC administration to 5/6 nephrectomized rats produced significant protection against a decrease in GFR, maintaining a mean inulin clearance of 0.45 mL/min (50% of normal) that remained stable at 120 days after nephrectomy. In contrast, GFR decreased progressively in the untreated rats. Reductions in inflammation and in fractional interstitial volume were observed in NAC-treated rats at 120 days after nephrectomy. Although reductions in proteinuria and blood pressure were also observed in these rats, the protective effect that NAC had on GFR was more impressive than the reduced proteinuria.

The data regarding GFR and glomerulosclerosis are quite interesting. However, the antiproteinuric effect of NAC at 120 days appears quite modest. Nevertheless, if we correct the mean daily proteinuria by the respective mean inulin clearance, nephrectomized/EV120 rats presented a four to five times greater protein excretion/inulin clearance when compared to nephrectomized + NAC-IN7/EV120 rats and nephrectomized + NAC-IN60/EV120 rats. The same is true if we compare urinary excretion of protein/inulin clearance in nephrectomized/EV60 rats to that observed in nephrectomized + NAC-IN7/EV60 rats and nephrectomized + spironolactone + NAC-IN7/EV60 rats.

In addition, a significant reduction in blood pressure was not observed in NAC-treated rats until day 120. Similar results have been obtained with administration of the antioxidant vitamin E in a rat model of chronic nitric oxide synthase inhibition [20] in which renal injury was reduced, but there was no concomitant improvement in hypertension. In spontaneously diabetic rats fed a high-sodium diet, treatment with angiotensin II receptor blockers has been shown to improve parameters without affecting blood pressure [21].

Proteinuria and elevated blood pressure are predictors of progressive kidney injury. Some studies suggest that albumin can stimulate the production of proinflammatory cytokines in proximal tubule cells by nuclear factor-kappaB (NF-κB) activation [22, 23]. It is not known whether the proinflammatory state and oxidative stress in the kidney can be reduced without a significant reduction in proteinuria and blood pressure.

A recent study was conducted involving patients with chronic kidney disease who were being treated with

an ACE inhibitor in combination with other antihypertensive agents [15]. The authors submitted the patients to additional angiotensin II blockade with losartan and observed improvement in GFR with no effect on blood pressure or proteinuria. The additional angiotensin II blockade induced a 35% reduction in oxidized albumin and a reduction in urinary excretion of monocyte chemotactic protein-1, corresponding to the degree of renal inflammation. In another study of chronic kidney disease patients, angiotensin II blockade also induced a 38% reduction in urinary excretion of the fibrogenic cytokine transforming growth factor-β (TGF-β([24]. Therefore, as previously demonstrated, treatment with angiotensin II receptor blockers improves oxidative stress parameters in proteinuric patients with chronic kidney disease without affecting blood pressure [25].

In our study, the combination of NAC and spironolactone (nephrectomized spironolactone + NAC-IN7/EV60 rats) was found to lower blood pressure and reduce oxidative stress, thereby conferring additive protection of GFR. This finding suggests a need for further studies evaluating the effectiveness of treatment with antioxidant-antihypertensives combinations in models of chronic renal failure.

Administration of NAC has been shown to be protective in several types of organ injury. This is due to the fact that NAC inhibits the activation of some protein kinases and of NF-κB, which is an important transcription factor for a number of cytokine genes that may be activated as a result of endothelial shear stress [5, 26, 27]. The expression of NF-κB–dependent genes may be important in inducing endothelial cell death, as well as in generating a local inflammatory reaction characterized by the release of endothelial-derived cytokines [28].

Based on our data, we can conclude that NAC directly regulates the proinflammatory effects of oxidative stress in chronic kidney disease. These data may be significant since current strategies for treating chronic kidney disease address the reduction of blood pressure and proteinuria. Therefore, the addition of NAC to these treatment regimens could be promising as a means of preventing the oxidative stress complications of chronic kidney disease.

It is remarkable that aldosterone levels remained elevated on day 120 after 5/6 nephrectomy, albeit to a lesser degree than on day 60. A previous study carried out in our laboratory showed that aldosterone levels were lower in 8-month-old rats than in younger rats [29]. In the present study, the lower aldosterone levels seen at 120 days are probably attributable to the aging of the animals and are consistent with the lower fractional excretion of potassium seen in these rats.

Rats treated with NAC presented noticeable decreases in aldosterone levels as early as 21 days after nephrectomy. Our data suggest that aldosterone makes only a modest contribution to the induction of arterial hypertension in the remnant kidney model since the marked decrease in aldosterone levels in NAC-treated rats 21 and 60 days following nephrectomy was accompanied by only a slight decrease in blood pressure.

Since high levels of aldosterone are necessary to maintain potassium balance in chronic renal failure, we can hypothesize that the lower levels of aldosterone seen in NAC-treated animals are a consequence of a lesser degree of renal injury.

Nonhemodynamic effects of aldosterone may contribute to glomerulosclerosis. Studies in vitro have shown that production of type IV collagen increased in mesangial cells incubated with aldosterone [30]. Transcripts for the mineralocorticoid receptor have been detected in the glomeruli, which may mediate the action of this hormone at this site [31]. It is possible that the beneficial effect of NAC on GFR and glomerulosclerosis is at least partly mediated by aldosterone.

Sun et al [10] and Sarnak [32] demonstrated aldosterone-induced inflammation in the rat heart. The authors found this effect to be mediated by oxidative stress and attenuated by NAC. In our study, cardiac hypertrophy, indirectly evaluated by determining heart weight, increased progressively following nephrectomy. Heart weights at 60 and 120 days after nephrectomy were significantly higher in NAC-treated rats than in untreated animals. It is possible that the lower levels of aldosterone in NAC-treated rats are responsible for this effect.

CONCLUSION

Our data demonstrate that NAC attenuates drops in GFR, as well as lowering proteinuria and blood pressure in nephrectomized rats. This is accompanied by a significant reduction in aldosterone levels. Our results indicate that ROS play an important role in the progression of chronic renal failure. It is evident that NAC has potential utility in preventing glomerulosclerosis and loss of kidney function in patients with chronic renal failure. The findings that NAC attenuated GFR drop and lowered proteinuria, even in end-stage chronic renal failure, and that the combination of NAC and spironolactone improves renal function more than does NAC alone have significant clinical implications.

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