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Endothelin type A receptor blockade increases renoprotection in congestive heart failure combined with chronic kidney disease: Studies in 5/6 nephrectomized rats with aorto-caval fistula

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ABSTRACT

Background: Association of congestive heart failure (CHF) and chronic kidney disease (CKD) worsens the patient's prognosis and results in poor survival rate. The aim of this study was to examine if addition of endothelin type A (ET_A) receptor antagonist to the angiotensin-converting enzyme inhibitor (ACEi) will bring additional beneficial effects in experimental rats.

Methods: CKD was induced by 5/6 renal mass reduction (5/6 NX) and CHF was elicited by volume overload achieved by creation of aorto-caval fistula (ACF). The follow-up was 24 weeks after the first intervention (5/6 NX). The treatment regimens were initiated 6 weeks after 5/6 NX and 2 weeks after ACF creation.

Results: The final survival in untreated group was 15%. The treatment with ET_A receptor antagonist alone or ACEi alone and the combined treatment improved the survival rate to 64%, 71% and 75%, respectively, however, the difference between the combination and either single treatment regimen was not significant. The combined treatment exerted best renoprotection, causing additional reduction in albuminuria and reducing renal glomerular and tubulointerstitial injury as compared with ACE inhibition alone.

Conclusions: Our results show that treatment with ET_A receptor antagonist attenuates the CKD- and CHF-related mortality, and addition of ET_A receptor antagonist to the standard blockade of RAS by ACEi exhibits additional renoprotective actions.

1. Introduction

Congestive heart failure (CHF) presents an extreme burden to the public healthcare worldwide. Almost 40% of CHF patients die within 1 year from the diagnosis and 70% within 5 years, even under adequate modern therapy [1,2]. The incidence and prevalence of chronic kidney disease (CKD) is also increasing [3] and CKD is one of the strongest risk factors for the development of CHF [4,5]. CHF coexists with CKD in approximately half of CHF patients [4–8]. Unfortunately, patients with estimated glomerular filtration rate $\leq$ 30 ml/min/1.73 m^2 have now largely been excluded from randomized control trials in HF, which limits the information on patients with combined CHF and CKD [4,5,7,8]. Therefore, although the patients with combined CHF and advanced CKD represent probably the highest cardiovascular risk population, their exclusion from CHF trials is a serious deontological error. Even the newest guidelines of the European Society of Cardiology for the treatment of CHF admit that there is little direct evidence to support any recommendation for the treatment of these patients [7,9]. Obviously,

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new therapeutic strategies for the treatment of CHF combined with CKD are urgently needed, and focused experimental studies should be performed to define new pharmacotherapeutic targets for prospective clinical research.

The rat with aorto-caval fistula (ACF) presents a well-defined model of CHF due to volume overload, which in many respects imitates the course of CHF in untreated humans [10–13]. The model is endorsed by the American Heart Association and the European Society of Cardiology for preclinical testing to identify new targets for CHF treatment [13]. In addition, the rat that undergoes the 5/6 renal mass reduction (5/6 NX) is acknowledged as a standard model of CKD and it thus provided a majority of the knowledge about the pathophysiology of CKD [14,15]. Importantly, we have introduced and validated a combination of 5/6 NX and ACF as a model to study the pathophysiology of CHF combined with CKD [16]; therefore the model was used in the present study.

In search for novel pharmacological strategies targeting the systems beyond the treatment currently considered and underlying fundamental drug therapy of CHF and/or CKD [3,7,9], attention has been directed to the endothelin (ET) system. Endothelin-1 (ET-1) is the most powerful mammalian vasoconstrictor [17] which plays an important role in the pathophysiology of hypertension, end-organ damage, CKD and CHF [18–21]. ET-1 actions are mediated via ET type A (ET_A) and ET type B (ET_B) receptors whose activation causes, respectively, vasoconstriction or vasodilatation with natriuresis [19,20]. ET system is markedly upregulated in animals with CHF and CKD and preclinical studies showed that activation of ET_A receptors contributes to the pathophysiology of CHF and CKD [19,21,22]. Therefore pharmacological blockade of ET might be a new approach to the treatment of CHF and CKD. Disappointingly, initial clinical studies evaluating the effects of ET blockade in patients with CHF and CKD failed to bring beneficial effects, and many of them had to be terminated prematurely, mainly due to fluid overload and worsening of CHF and even the development of acute heart failure [22–26]. Such adverse events might result from blockade of ET_B receptors in the kidney, a notion indirectly corroborated by recent results of SONAR study, which excluded patients at risk of fluid retention; pharmacological ET_A receptor blockade provided also renoprotection in type 2 diabetic patients with CKD [27]. Nevertheless, effects of selective ET_A receptor blockade on the course CHF when combined with CKD have not been thoroughly evaluated, even in preclinical studies. Therefore, utilizing our suitable experimental model and the available atrasentan, orally active and highly selective ET_A receptor antagonist [19], we first examined the effects of chronic atrasentan treatment on the morbidity and mortality in 5/6 NX + ACF normotensive Hannover Sprague-Dawley (HanSD) rats. Given that the pharmacological blockade of the renin-angiotensin system (RAS) is currently a cornerstone therapy for CHF as well as CKD, we compared the effects of pharmacological blockade of ET_A receptor antagonist with the standard RAS blockade (ACEi) as described earlier in studies in CHF and CKD [11,28–30]. Since previous experimental and clinical studies showed that renoprotective actions of ET_A receptor blockade in CKD are also present in individuals undergoing pharmacological blockade of the RAS, our third aim was to find out if the addition of the selective ET_A receptor blockade to standard treatment with ACEi would attenuate the progression of combined CHF and CKD in 5/6 NX + ACF HanSD rats. To further evaluate our hypothesis that beneficial actions of ET_A receptor blockade on the mortality in 5/6 NX + ACF HanSD rats are mediated predominantly by renoprotective actions, we assessed the degree of renal glomerular and kidney tubulointerstitial damage and organ weights in separate groups of animals after eight weeks of treatment, because at this stage the untreated 5/6 NX + ACF HanSD rats began markedly to die.

2. Methods

2.1. Ethical approval and animals

The studies were performed in accordance with the guidelines and practices established by the Animal Care and Use Committee of the Institute for Clinical and Experimental Medicine, Prague, which accord with the national law and the European Union policy and were approved by the Ministry of Health of the Czech Republic (project decision 21984/2021–5/OZV). The animals were bred at the Institute's Center of Experimental Medicine, accredited by the Czech Association for Accreditation of Laboratory Animal Care. The animals were kept on a 12-hour/12-hour light/dark cycle. Throughout experiments rats were fed a normal salt, normal protein diet (0.45% NaCl, 19–21% protein) produced by SEMED (Prague, Czech Republic) and had free access to tap water.

2.2. CHF and CKD models, exclusion criteria, therapeutic regimes and general analytic procedures

Male HanSD rats at initial age of 8 weeks, derived from several litters, were randomly assigned to experimental groups. In order to obtain reliable data regarding the effects of two treatment regimens on the survival rate, high initial *n* values were used, established using statistical power analysis [31].

The present studies were performed only in male rats, even though important sex-related differences in the progression of CHF as well as CKD are recognized and documented [2,3,32,33]. The American Physiological Society and the British Pharmacological Society recently recommended that "sex" should no longer be ignored as an experimental variable. Indeed, this is crucially important in preclinical research as a prerequisite for successful translation of the results into clinical practice, unless a strong rationale is provided for not incorporating both sexes [34–36]. In the present study, the justification for single-sex investigation is based on our original validation studies indicating no significant sex-related differences in the evaluated parameters [34–36]. Since, specifically, we found no such differences with respect to CKD- and CHF-related morbidity and mortality in 5/6 NX + ACF HanSD rats [16], we decided to perform the present study in one "sex" only, and the random choice was "male sex".

Animals were anesthetized (tiletamine + zolazepam, Virbac SA, Carros Cedex, France, 8 mg/kg; and xylasine, Spofa, Czech Republic, 4 mg/kg intramuscularly) and CHF was induced by volume overload from ACF created using needle technique as described previously [10–12,16,37–40]. Sham-operated rats underwent an identical procedure but without creating ACF.

To develop the CKD model, rats were anesthetized as usual and 5/6 NX was performed as described previously [28,29]. Briefly, the right kidney and both poles of the left kidney were excized in order to remove 5/6 of renal parenchyma. Sham-operated animals underwent the same procedure without removing renal parenchyma. Post-operative analgesia (meloxicam) wasadministered subcutaneously for two days.

To inhibit the angiotensin-converting enzyme (ACE), trandolapril (Gopten; Abbot, Prague, Czech Republic), was given at 2 mg/L in drinking water, the dose was previously shown to provide maximal blockade of the RAS [11,30]. ET_A receptor blockade was achieved with atrasentan (Abbot, Illinois, USA), 5 $mg.kg^{-1}.day^{-1}$ in drinking water; the dose was adjusted weekly to actual water intake and was previously found to effectively block ET_A receptors [28,29,41]. Importantly, there were no significant differences in water intake between experimental groups that were exposed to the treatment protocols with ET_A receptor antagonist and ACEi, alone or combined.

Albumin excretion was determined in 24-hour urine collections in individual metabolic cages; urinary albumin was measured by a quantitative sandwich enzyme immunoassay technique, using the commercially available ELISA kit (ERA3201–1, AssayPro, MO, USA). Before placing in metabolic cages, rats' body weight (BW) was monitored as well as the presence of CHF symptoms using a scoring system (used also for assessment of the onset of decompensation of heart failure in the model of spontaneously hypertensive heart failure rats) [42]. The method was found suitable also for the ACF model of CHF [16,38]. The

scoring referred to the five most apparent aspects of CHF phenotype: (1) presence of raised fur (piloerection), (2) diminished activity (lethargy), (3) peripheral cyanosis, (4) rapid or labored breathing (dyspnea), and (5) abdominal swelling (ascites). Every symptom was scored on the scale from 0 to 3 and a total CHF score was calculated for every animal as the sum of individual points; thus, the theoretical maximum that can be reached is 15. Once the score reached a threshold of ≥ 3, the advanced phase of CHF was diagnosed. It was demonstrated in the original characterization study that when an animal reached the score of 5, the onset of decompensation of CHF was seen and animals typically died within the next 7 days [43]. At the end of the study the survived animals were killed and individual organ weights were obtained, and the kidneys were used to assess glomerular damage and tubulointerstitial injury. The kidneys were fixed in 4% formaldehyde, dehydrated and embedded in paraffin. The sections stained with hematoxylin-eosin and PAS (periodic acid, for Schiff reaction) were examined and evaluated in a blind-test fashion. Fifty glomeruli in each kidney were examined on a semi-quantitative scale. The evaluation was as follows: *grade 0*, all glomeruli normal; *grade 1*, sclerotic area up to 25% (minimal sclerosis); *grade 2*, sclerotic area 25–50% (moderate sclerosis); *grade 3*, sclerotic area 50–75% (moderate-to-severe sclerosis); *grade 4*, sclerotic area 75–100% (severe sclerosis). The glomerulosclerosis index (GSI) was calculated using the following formula: $GSI = [(1 \times n_1) + (2 \times n_2) + (3 \times n_3) + (4 \times n_4)]/(n_0 + n_1 + n_2 + n_3 + n_4)$, where n_x is the number of glomeruli in each grade of glomerulosclerosis. Kidney cortical tubulointerstitial injury was evaluated as defined by Nakano et al. [44], to determine inflammatory cell infiltration, tubular dilatation, atrophy, or interstitial fibrosis. The injury was graded semi-quantitatively using the following scale of lesions: grade 0, no abnormal findings; 1, mild (<25% of the cortex); 2, moderate (25 – 50% of the cortex); 3, severe (>50% of the cortex). The lesions were assessed in at least 30 random and non-overlapping fields in the renal cortex. Thus, the maximum score for GSI is 4 and for the index of kidney tubulointerstitial injury is 3. This method is always employed in our studies evaluating the degree of kidney damage [12,28,29,45].

Systolic blood pressure (SBP) was measured with automated tail cuff system (Hatteras Instruments, Cary, N.C., USA). In accordance with recommendations for blood pressure (BP) measurements in experimental animals [46], this method is adequate for detecting intergroup differences in SBP over time, and therefore is optimal for long-term studies. Beginning from the initial age (i.e. 8 weeks), three times per week (Monday, Wednesday and Friday) the animals were immobilized for 20 min in the, heated cage designed for tail cuff SBP measurement, On the day of SBP measurement, after 5 min adaptation period, three values of SBP were taken, with 3–5 min' pause between individual measurements, and the average from these measurements was calculated. This method of training and SBP measurement was previously used and validated in our and professor Salazar's laboratory [41,45,47]; a close correlation was found between measurements by tail-plethysmography and direct BP measurements using indwelling catheter in conscious rats [36,40,42]. Admittedly, the method does not allow accurate measurements of diastolic BP and mean arterial pressure, in contrast to radiotelemetry which, however, could not be implemented in long-term studies in large groups of 5/6 NX + ACF animals. Nevertheless, as repeatedly shown, this method provides valuable information regarding the role of BP in mediating cardiovascular and renal effects of various treatment regimens in rats [48]. While being aware of this methodological limitation, we have demonstrated in preliminary experiments that the tail-cuff method and radiotelemetry provided similar results in 5/6 NX rats [41,45]. At the end of the study, mean arterial pressure (MAP) was monitored under anesthesia for 10 min using an arterial indwelling catheter. Finally, the animals were killed with an overdose of thiopental sodium and organs were collected.

2.3. Detailed experimental design

2.3.1. Series 1: Effects of long-term treatment (18 weeks) with ETA receptor antagonist and ACEi, alone or combined, on the survival rate and morbidity

The details of the design and timing of the experimental manoeuvres in this series are given in Fig. 1A. The following groups were evaluated:

(1): Sham-operated HanSD rats + placebo (initial n = 12).

(2): 5/6 NX + ACF HanSD rats + placebo (initial n = 26).

(3): 5/6 NX + ACF HanSD rats + ET_A receptor antagonist (initial n = 25).

(4): 5/6 NX ACF HanSD rats + ACEi (initial n = 24).

(5): 5/6 NX + ACF HanSD rats + ACEi + ET_A receptor antagonist (initial n = 24).

2.3.2. Series 2: Effects of 8-week treatment with ET_A receptor antagonist and ACEi, alone or combined, on kidney damage and organ weights

Animals were prepared as in series 1 and the design of this series is given in Fig. 1B. The following groups were examined:

(1): 5/6 NX + ACF HanSD rats + placebo (initial n = 16 and, final n = 10).

(2): 5/6 NX + ACF HanSD rats + ET_A receptor antagonist (initial n = 10, final n = 9).

(4): 5/6 NX ACF HanSD rats + ACEi (initial n = 10, final n = 9).

(5): 5/6 NX + ACF HanSD rats + ACEi + ET_A receptor antagonist (initial n = 11, final n = 10).

2.4. Statistical analysis

Graph-Pad Prism software (Graph Pad Software, San Diego, California, USA) was used. Comparison of survival curves was performed by log-rank (Mantel-Cox) test followed by Gehan-Breslow-Wilcoxon test. Statistical comparison of other results was made by Student's *t*-test, Wilcoxon's signed-rank test for unpaired data or one-way ANOVA when appropriate. Values are means ± S.E.M. P value < 0.05 was considered statistically significant.

3. Results

3.1. Series 1: Effects of long-term treatment (18 weeks) with ET_A receptor antagonist and ACEi, alone or combined, on the survival rate and morbidity

All sham-operated HanSD rats survived until the end of experiment (not shown in Fig. 2). Untreated 5/6 NX + ACF HanSD rats began to die at week + 2 (8 weeks after 5/6 NX operation, 4 weeks after ACF creation) and the final survival rate was 15% (4 of 26 animals) (Fig. 2). ET_A antagonist alone and ACEi alone treatments improved the survival up to the final rate of 64% (16/25) and 71% (17/24), respectively. The combined treatment improved the survival to 75% (18/24). The effectiveness of the three treatment regimens did not significantly differ.

In all groups BW progressively increased throughout study, but this weight gain was significantly arrested in untreated 5/6 NX + ACF HanSD rats between weeks + 5 to + 10; as we observed here and previously [16,38] the onset of mortality is associated with a profound BW drop (Fig. 3A).

The threshold for the advanced phase of CHF (heart failure score ≥ 3) was reached in untreated 5/6 NX + ACF HanSD rats at week + 5, and remained so until the end of the study (Fig. 3B). The score increased slightly and progressively with each of three treatment regimens but not sooner than at the end of study (week +18) did it come near the threshold value, still without reaching it (Fig. 3B). Thus, the animals that died in connection with ACF-induced CHF, in the weeks preceding the death showed the highest CHF score, hence the death caused its paradoxical improvement (as was also seen with BW changes). Therefore, reaching the HF score ≥ 3 in the whole group indicates that a significant

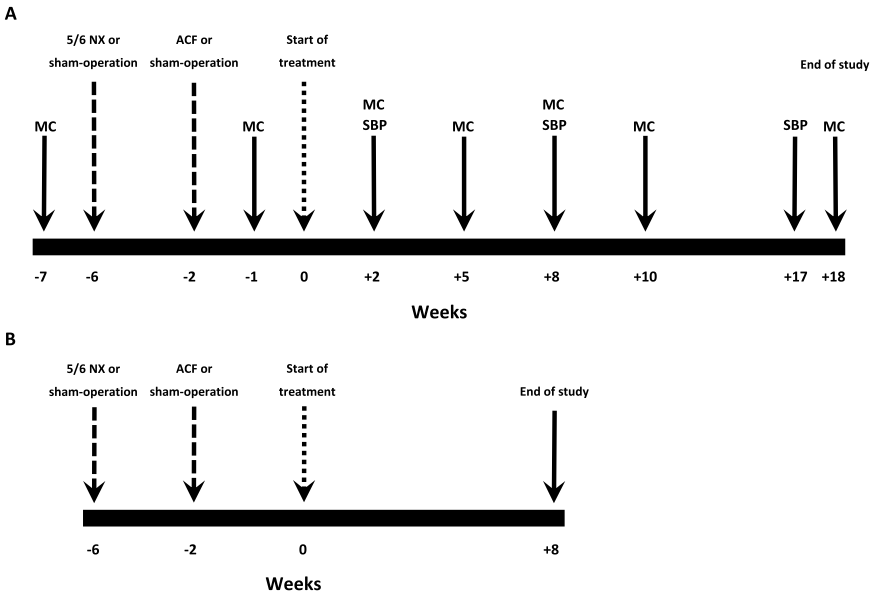

Fig. 1. The experimental design, especially the time sequence of experimental manoeuvres. 5/6 NX: 5/6 renal mass reduction, ACF: creation of aorto-caval fistula, MC: placement into an individual metabolic cage for 24-hour urine collection, SBP: systolic blood pressure measurement by tail-cuff plethysmography.

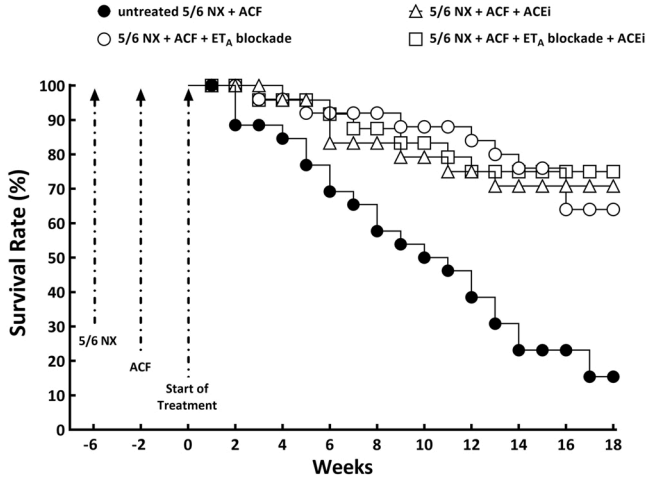

Fig. 2. Series 1: The effects of long-term treatment (18 weeks) on the survival rate in sham-operated normotensive Hannover-Sprague Dawley (HanSD) rats, in untreated HanSD rats undergoing combination of 5/6 renal mass ablation (5/6 NX) and creation of the aorto-caval fistula (ACF), in 5/6 NX + ACF HanSD rats treated with endothelin type A (ET_A) receptor antagonist, or with angiotensin-converting enzyme inhibitor (ACEi), alone or combined.

majority of animals were in the advanced phase of CHF.

At week + 2 in all 5/6 NX + ACF HanSD rats the systolic blood pressure was markedly lower than in sham-operated HanSD rats (Fig. 3C). Similar as with BW, in untreated 5/6 NX + ACF HanSD rats at weeks + 8 and + 17 SBP was significantly lower than in all other groups of 5/6 NX + ACF HanSD rats that were exposed to either treatment regimen; this SBP decrease was most pronounced in the animals that subsequently died within a few days.

Albuminuria, expressed in absolute values or normalized to urinary creatinine excretion, was negligible before 5/6 NX operation and ACF creation but progressively increased slightly with time, also in sham-operated animals. (Figs. 4A and 4B).

Untreated 5/6 NX + ACF HanSD showed a dramatic increase in proteinuria, up to the end of experiment, its values being 120-times higher than in sham-operated HanSD rats. ET_A antagonist treatment alone attenuated the progressive rise in albuminuria, however, at the end of the study no beneficial action was seen compared with untreated 5/6 NX + ACF HanSD rats. ACEi treatment attenuated the increase in albuminuria throughout the study, and in the end the animals showed ~45% reduction compared with untreated 5/6 NX + ACF HanSD rats. Remarkably, the combined treatment with ET_A antagonist and ACEi had additional beneficial effect as compared with the ACEi alone, and the reduction in albuminuria was about 70% as compared with untreated 5/6 NX + ACF HanSD rats (Figs. 4A and 4B).

Fig. 5 summarizes GSI and kidney tubulointerstitial injury at the end of study (in animals that survived until the end of experiment). Untreated 5/6 NX + ACF HanSD rats displayed marked renal glomerular and tubulointerstitial injury. ET_A antagonist treatment alone did not significantly reduce the renal glomerular damage (Fig. 5A), but significantly reduced the kidney tubulointerstitial injury (Fig. 5B). In contrast, ACE inhibition alone significantly reduced the renal glomerular damage as well as kidney tubulointerstitial injury. Remarkably, combined application of the two regimens brought additional reduction in GSI and kidney tubulointerstitial injury in 5/6 NX + ACF HanSD rats as compared with ACE inhibition alone.

Representative images of renal kidney sections of sham-operated HanSD rats and untreated as well as treated 5/6 NX + ACF HanSD rats are shown in Figs. 6 and 7.

Figs. 8 and 9 summarize MAP and organ weights at the end of study. As shown in Fig. 8A, in all groups of 5/6 NX + ACF HanSD rats MAP was significantly lower than in sham-operated HanSD rats, but in the untreated 5/6 NX + ACF HanSD rats it equalled only 71 ± 4 mmHg, markedly lower than in treated 5/6 NX + ACF HanSD rats irrespective of the treatment regimen. All groups of 5/6 NX + ACF HanSD rats exhibited marked bilateral cardiac hypertrophy as seen from whole heart weight, left ventricle (with septum) weight (Figs. 8B and 8C) and right ventricle weight (Fig. 9A), and the treatments did not attenuate it. Interestingly, the degree of right ventricle hypertrophy in 5/6 NX + ACF HanSD rats was higher than that of the left ventricle (higher right-to-left ratio) (Fig. 9B). All groups of 5/6 NX + ACF HanSD rats displayed increased lung weight as compared with sham-operated HanSD rats, and this was not altered by any of the treatment regimens (Fig. 9C).

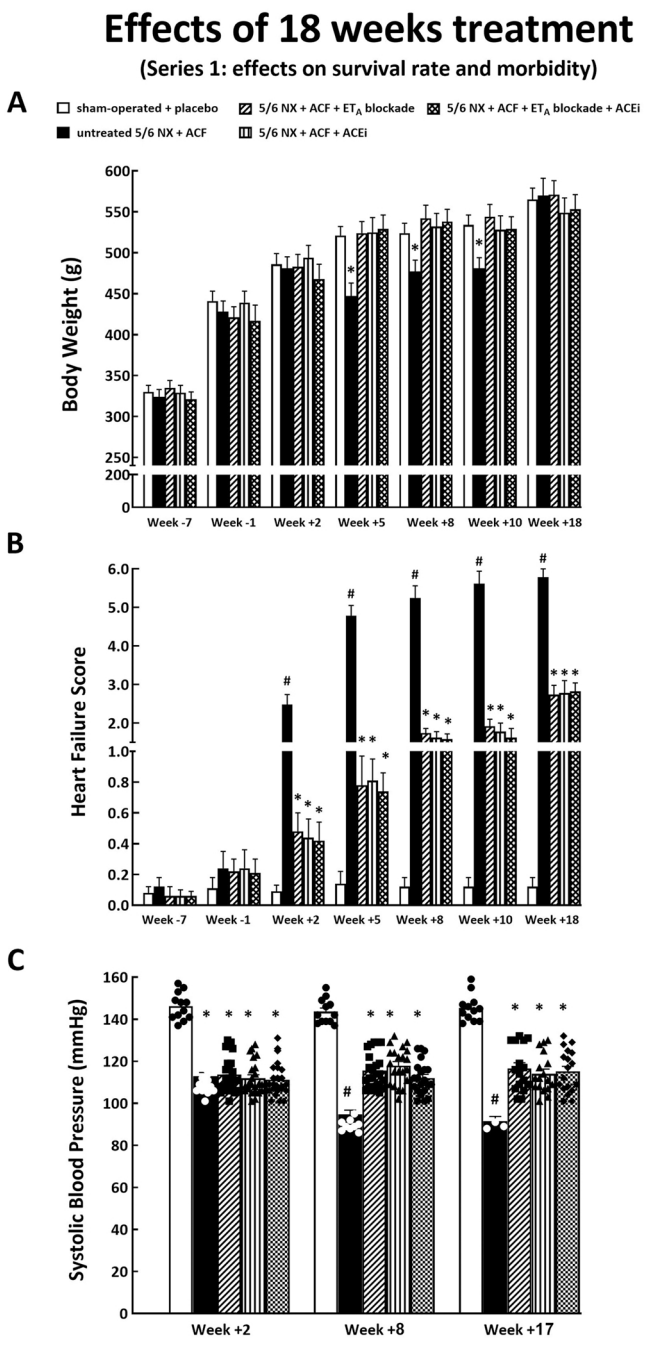

Fig. 3. Series 1: The effects of long-term treatment (18 weeks) on body weight changes (A), total heart failure score (B) and systolic blood pressure (C), in sham-operated normotensive Hannover-Sprague Dawley (HanSD) rats, in untreated HanSD rats undergoing combination of 5/6 renal mass ablation (5/6 NX) and creation of the aorto-caval fistula (ACF), in 5/6 NX + ACF HanSD rats treated with endothelin type A (ET_A) receptor antagonist, or with angiotensin-converting enzyme inhibitor (ACEi), alone or combined. Week − 7: basal values, before surgical interventions, week − 1: values 5 weeks after 5/6 NX operation and 1 week after ACF creation. * $P < 0.05$ sham-operated HanSD rats. # $P < 0.05$ versus all other groups.

3.2. Series 2: Effects of 8-week treatment with ET_A receptor antagonist and ACEi, alone or combined, on kidney damage and organ weights

Fig. 10 summarizes GSI and kidney tubulointerstitial injury 14 weeks after 5/6 NX and 10 weeks after ACF creation, the rats were treated for 8 weeks. Untreated 5/6 + ACF HanSD rats showed exceptionally high degree of renal glomerular and tubulointerstitial injury. ET_A antagonist

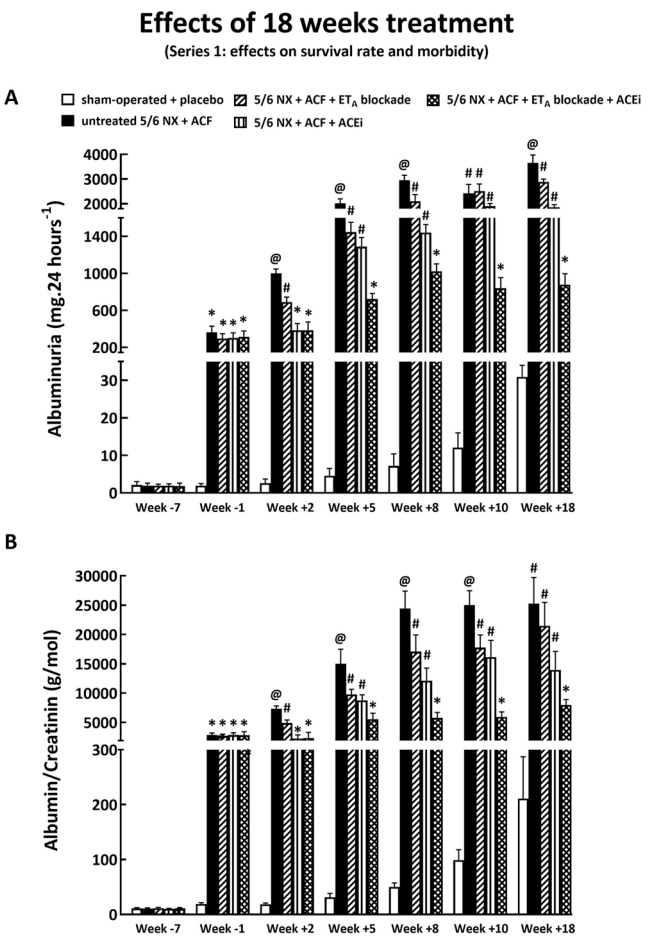

Fig. 4. Series 1: The effects of long-term treatment (18 weeks) on albuminuria (A) and albumin to creatinine ratio (B) in sham-operated normotensive Hannover-Sprague Dawley (HanSD) rats, in untreated HanSD rats undergoing combination of 5/6 renal mass ablation (5/6 NX) and creation of the aorto-caval fistula (ACF), in 5/6 NX + ACF HanSD rats treated with endothelin type A (ET_A) receptor antagonist, or with angiotensin-converting enzyme inhibitor (ACEi), alone or combined. Week − 7: basal values, before surgical interventions, week − 1: values 5 weeks after 5/6 NX operation and 1 week after ACF creation. * $P < 0.05$ sham-operated HanSD rats. # $P < 0.05$ versus 5/6 NX + ACF HanSD rat treated with ET_A receptor antagonist + ACEi. @ $P < 0.05$ versus all other groups.

treatment alone as well as ACE inhibition alone substantially reduced both GSI and kidney tubulointerstitial injury in 5/6 NX + ACF HanSD rats (Figs. 10A and 10B). Remarkably, combined application of the two drugs caused additional reduction in GSI and kidney tubulointerstitial injury in 5/6 NX + ACF HanSD rats (Figs. 10A and 10B), roughly to the levels that were observed in sham-operated HanSD rats at the end of study i.e. ten weeks later (Figs. 5A and 5B).

Fig. 11 summarizes organ weights measured at the same time point of the study as GSI and tubulointerstitial injury were analysed. As shown in Fig. 11A, the treatment with ET_A antagonist alone as well as ACE inhibition alone did not reduce the whole heart weight in 5/6 NX + ACF HanSD rats as compared with untreated counterparts, but the combined treatment reduced it significantly. As shown in Fig. 11B, in 5/6 NX + ACF HanSD rats ET_A antagonist treatment alone did not reduce left ventricle heart weight whereas ACE inhibition alone reduced it significantly. The combined treatment with ET_A receptor antagonist and ACE inhibitor tended to reduce it even more, but the difference from ACE inhibition alone did not reach statistical significance. Interestingly, only the combined treatment with ET_A receptor antagonist and ACE inhibitor significantly reduced right ventricle weight in 5/6 NX + ACF HanSD rats

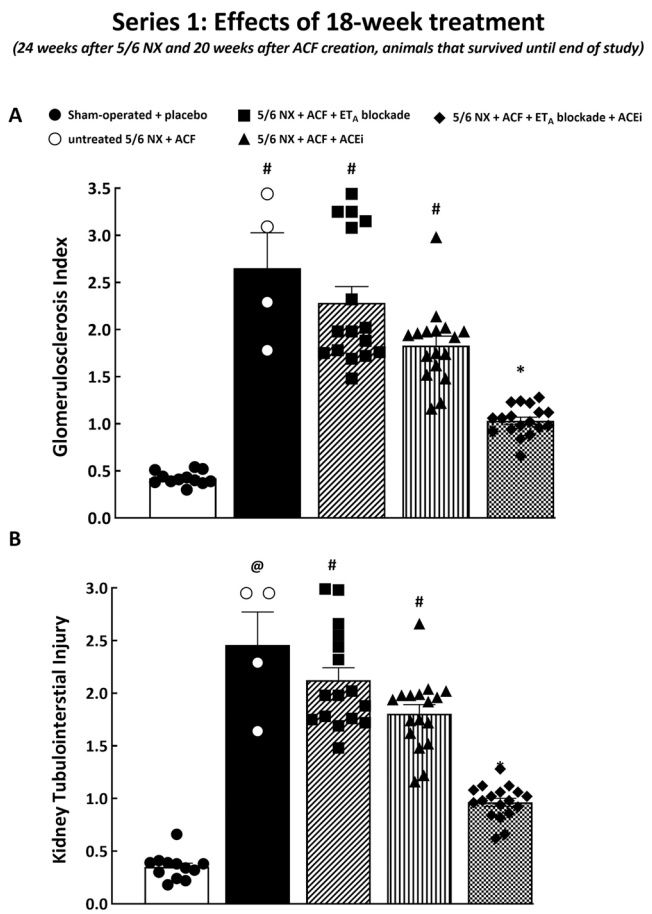

Fig. 5. Series 1: The effects of long-term treatment (18 weeks) on glomerulosclerosis index (A) and kidney tubulointerstitial injury (B) in sham-operated normotensive Hannover-Sprague Dawley (HanSD) rats, in untreated HanSD rats undergoing combination of 5/6 renal mass ablation (5/6 NX) and creation of the aorto-caval fistula (ACF), in 5/6 NX + ACF HanSD rats treated with endothelin type A (ET_A) receptor antagonist, or with angiotensin-converting enzyme inhibitor (ACEi), alone or combined. * $P < 0.05$ sham-operated HanSD rats. # $P < 0.05$ versus 5/6 NX + ACF HanSD rat treated with ET_A receptor antagonist + ACEi. @ $P < 0.05$ versus all other groups.

(Fig. 11C). There were no significant differences in the right-to-left ventricle ratio between experimental groups. In the group under ACE inhibition alone there was a tendency for an increase in this ratio as a consequence of a reduction in the left ventricle weight without alterations in the right ventricle weight. However, the difference did not reach significance level (data now shown). As shown in Fig. 11D, all treatment regimens significantly reduced the lung weight as compared with untreated 5/6 NX + ACF HanSD rats.

4. Discussion

This is the first experimental study that evaluates the role of the ET system in the progression of CHF combined with CKD. Specifically, it examines if selective ET_A receptor blockade attenuates the disease. The crucial question was if the addition of a selective ET_A receptor antagonist to the standard ACE inhibition would have additional beneficial effects in 5/6 NX + ACF HanSD rats, a model of combined CHF and CKD.

The first issue to consider was the effects of the "two-organ damage" on the mortality and morbidity and on albuminuria in this model. We found that in 5/6 NX + ACF HanSD rats the survival rate was dramatically worsened as compared with normotensive animals that were exposed only to ACF creation. The survival median in 5/6 NX + ACF animals was 12 weeks after ACF creation whereas in our original study

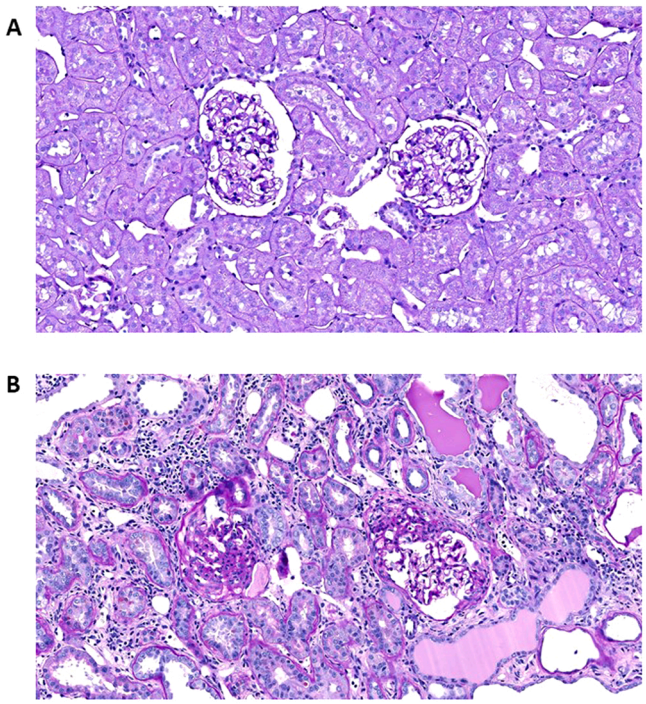

Fig. 6. Representative histological images of the renal cortex from the end of series 1, i.e. after long-term treatment (18 weeks) and 24 weeks after 5/6 renal mass ablation (5/6 NX) and 20 weeks after creation of the aorto-caval fistula (ACF) in (A) sham-operated normotensive Hannover-Sprague Dawley (HanSD) rats and in untreated HanSD rats undergoing combination of 5/6 NX and ACF creation (B).

with animals after ACF creation alone the median was 43 weeks [43]. Moreover, 5/6 NX + ACF HanSD rats exhibited albuminuria that was spectacularly higher than observed by us in Ren-2 transgenic hypertensive rats (TGR) after single organ damage (after 5/6 NX or ACF creation alone). This was so even though TGR display both features crucial for the progression of CKD and CHF: hypertension and inappropriately activated RAS [3,4,9,15]. Remarkably, in untreated 5/6 NX + ACF HanSD rats albuminuria was about 6-fold higher than in untreated 5/6 NX TGR [28,29,45,49], and even about 60-fold higher than in untreated ACF TGR [30]. Evidently, CKD is an exceptionally strong and independent risk factor for the progression of CHF. Furthermore, the present findings and long-term mortality and morbidity studies [2,4,5,7,8] indicate that 5/6 NX + ACF HanSD rats present an optimal model to study the pathophysiology and perform preclinical testing aimed to identify new targets for the treatment of combined CKD and CHF.

The albuminuria observed in the sham-operated and untreated rats also deserves some comment. The progressive increase in albuminuria in the normotensive rats without any intervention is a natural phenomenon of aging and age-related end-organ damage and in hypertensive rats, it is in addition a generally acknowledged marker of age- and hypertension-related end-organ damage. We have seen this age-dependent progression in sham-operated normotensive, i.e. fully healthy animals and also in two models of hypertensive rats, i.e. in healthy animals with "hypertension only" in our previous studies [12,29,30,49,50]. In this context, it is important to acknowledge that even if the albuminuria in sham-operated HanSD rats increased with age, it was still considerably lower than that observed in the sham-operated hypertensive rats, as we recently observed in the sham-operated Fawn-hooded hypertensive rats, a genetic model of spontaneous hypertension associated with CKD [12].

The second set of findings helps to evaluate the effects of atrasentan, a highly selective ET_A receptor antagonist, on the course of combined CKD and CHF. We found here a considerable improvement of survival rate in 5/6 NX + ACF HanSD rats, to the same level as observed in the

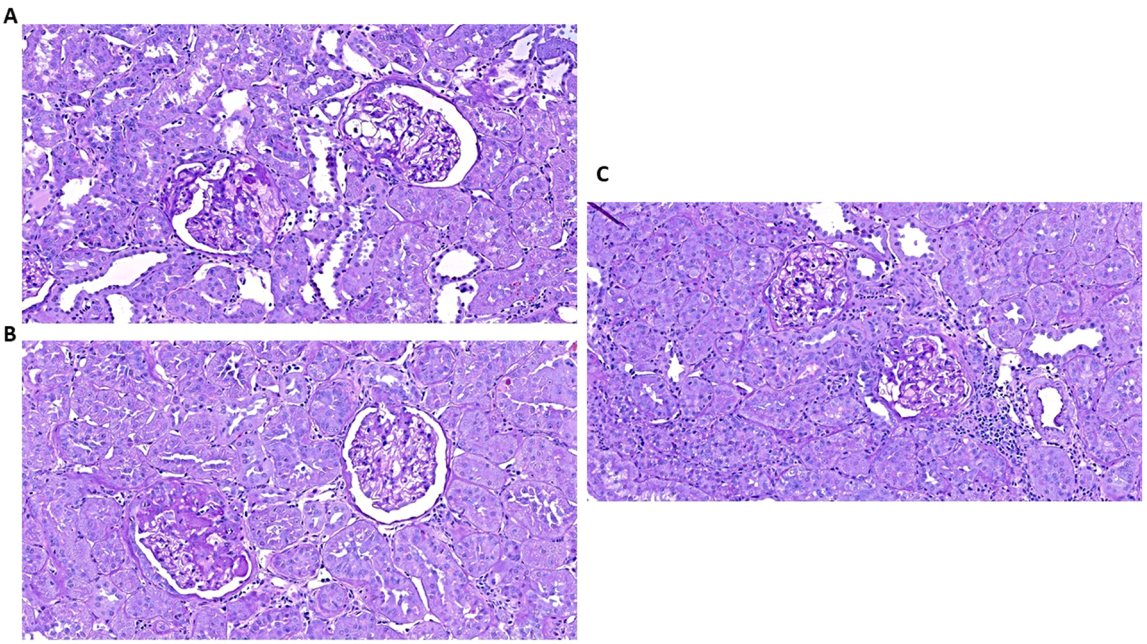

Fig. 7. Representative histological images of the renal cortex from the end of series 1, i.e. after long-term treatment (18 weeks) and 24 weeks after 5/6 renal mass ablation (5/6 NX) and 20 weeks after creation of the aorto-caval fistula (ACF) in 5/6 NX + ACF HanSD rats treated with endothelin type A (ET_A) receptor antagonist alone (A), or with angiotensin-converting enzyme inhibitor (ACEi) alone (B), or with combination of ET_A receptor antagonist and ACEi (C).

animals treated with ACEi alone. In addition, ET_A antagonist alone attenuated the progressive rise in albuminuria as compared with untreated 5/6 NX + ACF HanSD rats even though, admittedly, at some phase of the study ACEi alone was more effective. Moreover, at the end of the study the animals treated with ET_A alone did not show any significant attenuation of renal glomerular damage, bilateral cardiac hypertrophy and lung congestion, and showed only slight amelioration of the kidney tubulointerstitial injury. Nevertheless, approximately the same levels of albuminuria and end-organ damage were found in 5/6 NX + ACF HanSD rats treated with ACEi alone. This requires consideration for several reasons.

First, our data support the evidence that in the advanced stage of CKD as well as CHF organ-protection afforded by RAS blockade is far from complete [3,4,6,8,9,15,28,29,49], especially when both diseases are combined. Second, our present findings emphasize the difference between organ-protective effects of treatment regimens when initiated immediately after the onset of the damaging insult, and when some degree of CKD and CHF has already beenestablished. For instance, with RAS blockade initiated directly after 5/6 NX ("early treatment protocol"), the renoprotective actions are considerably stronger than with some postponement of such therapy ("late treatment protocol") [15,51,52]. Therefore, new pharmacological strategies should be evaluated (as in the present study) in late treatment protocols, the experimental setting more relevant to patients with established CKD or CHF and particularly in patients with combined diseases.

Our first conclusion is that the ET system via activation of ET_A importantly contributes to the progression of CHF coexisting with CKD. Very probably, selective inhibition of ET_A receptor mediated the beneficial actions by: (i) preventing direct vasoconstrictor actions of ET-1, (ii) preventing ET-1 induced glomerular large-pore hyperpermeability and glomerular barrier (podocyte) damage, (iii) unmasking ET_B receptor-mediated vasodilatory and natriuretic actions of ET-1, in the light of the current knowledge about the pathobiology of ET-1 in the kidney [18–20,53]. Remarkably, previous studies using less selective ET_A blockade failed to improve the course of CHF and CKD. Admittedly, our conclusion is only tentative due to the complex interplay of ET-1 with ET_A and ET_B receptors [54] but there is no doubt that ET_A receptor blockade attenuates the progression of CHF combined with CKD.

How to interpret the effects of combined treatment with ET_A receptor antagonist and ACEi on the progression of CHF occurring in tandem with CKD? Since we found that ET_A blockade alone and ACEi alone considerably attenuated mortality in 5/6 NX + ACF HanSD rats, and both treatment regimens affect different neurohormonal system, we expected additive beneficial effects with the combined treatment. However, this hope has not been fulfilled: the protection against CKD- and CHF-related mortality was not improved.

Nevertheless, the combined treatment displayed marked additional beneficial actions on albuminuria and important renoprotective effects, such as alleviation of the renal glomerular and cortical tubulointerstitial injury. Increased albuminuria is a strong and independent predictor for all-cause mortality in CKD as well as in CHF [3–5,7,8,53,55], hence with the prolonged treatment the additive beneficial actions on the course of CKD- and CHF-related mortality could occur. Such reasoning is supported by the favourable actions on renal glomerular and tubulointerstitial morphology: 5/6 + ACF HanSD rats treated within the late treatment protocol with either ET_A antagonist alone or ACEi alone showed marked renal glomerular damage, similar as observed in untreated animals. In contrast, the rats receiving combined therapy displayed only slight renal glomerular and tubulointerstitial injury, similar to that seen in sham-operated animals of the same age. To finally confirm such conclusions, 60-week follow-up studies would be necessary, and for reliable analysis of the survival curves, the initial n values should be at least 42 per group, to fulfil the power analysis requirements [31]. Such demanding thorough studies, admittedly long-standing, time consuming and costly will be needed in future.

In this context, our findings from the second series of experiments when the effects of 8-weeks´ treatment on renal glomerular and tubulointerstitial morphology and organ weights were evaluated are of particular importance. At this time point, i.e. 14 weeks after 5/6 NX creation and 10 weeks after ACF creation, untreated 5/6 NX + ACF HanSD rats showed an onset of high mortality whereas all the treated groups showed an almost complete survival (rates 92%, 84% and 88%, respectively). Our results show that at that time point, untreated 5/6 NX + ACF HanSD rats displayed remarkably high degree of renal glomerular and tubulointerstitial injury: both indices of kidney injury reached almost the arithmetically possible maximum (e.g. for GSI such

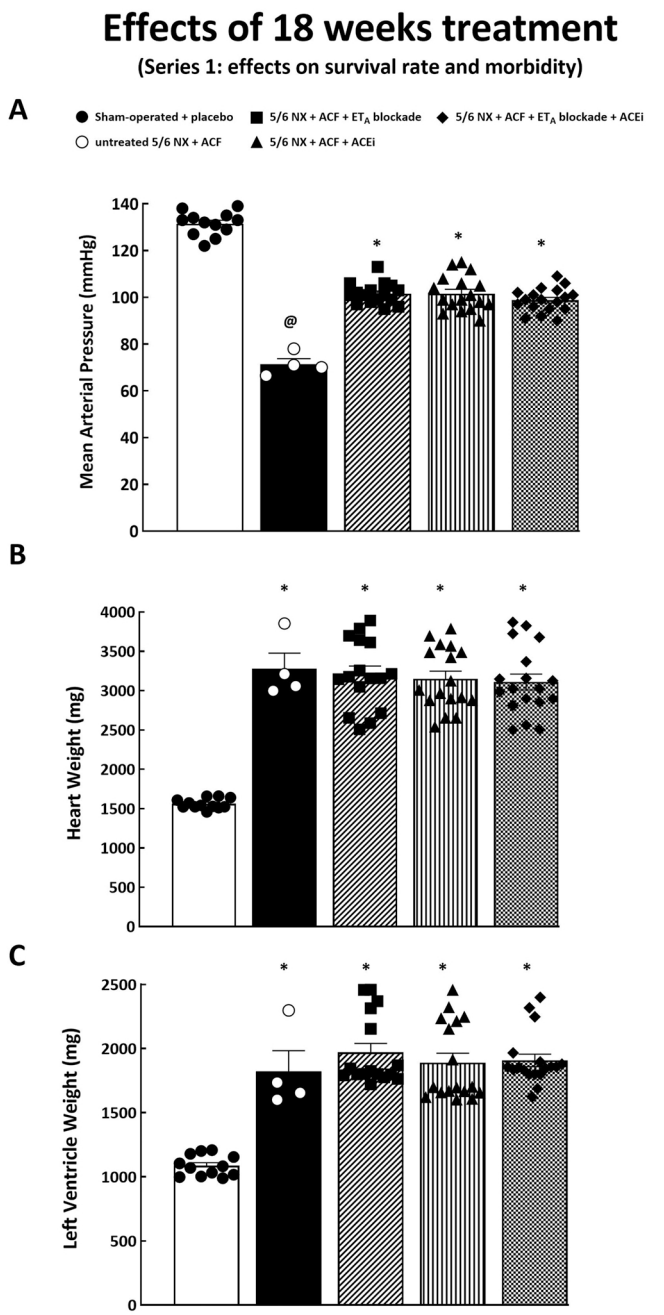

Fig. 8. Series 1: mean arterial pressure (A), whole heart weight (B) and left ventricle weight (C) 24 weeks after 5/6 renal mass reduction (5/6 NX) and 20 weeks after aorto-caval fistula (ACF) creation or sham operation, and after 18 weeks' treatment with endothelin type A (ET_A) receptor antagonist, or with angiotensin-converting enzyme inhibitor (ACEi), alone or combined. * $P < 0.05$ sham-operated HanSD rats. @ $P < 0.05$ versus all other groups.

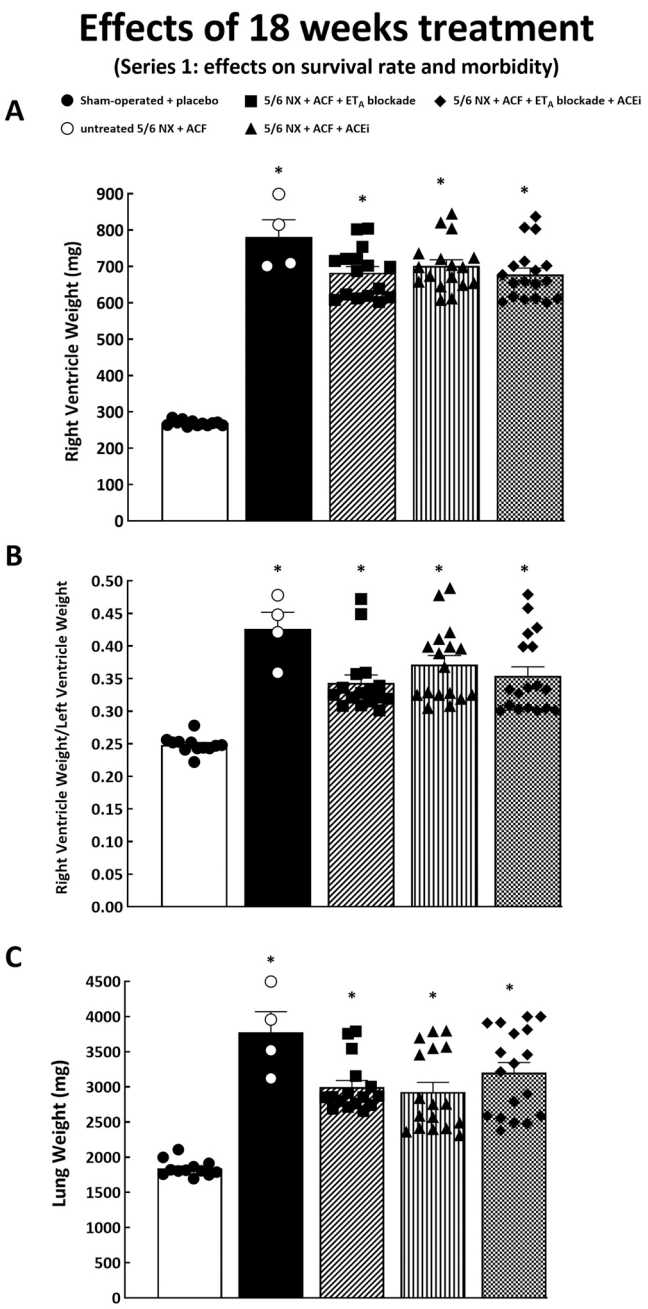

Fig. 9. Series 1: right ventricle weight (A), ratio of right ventricle weight to left ventricle weight (B) and lung weight (C) 24 weeks after 5/6 renal mass reduction (5/6 NX) and 20 weeks after aorto-caval fistula (ACF) creation or sham operation, and after 18 weeks' treatment with endothelin type A (ET_A) receptor antagonist, or with angiotensin-converting enzyme inhibitor (ACEi), alone or combined. * $P < 0.05$ sham-operated HanSD rats.

maximum equals 4 and in the animals that survived until the stated time point GSI was 3.16 ± 0.18). The treatment with ET_A receptor alone as well as ACEi alone substantially reduced renal glomerular and tubulointerstitial injury and, importantly, the animals receiving combined therapy showed only minimal degree of renal glomerular and tubulointerstitial injury. The indices at this time point were similar as observed in sham-operated HanSD rats at the end of series 1 experiment (the animals that served as healthy controls). Moreover, the combined treatment was the only regimen that reduced left ventricle as well as right ventricle hypertrophy as compared with untreated 5/6 NX + ACF HanSD rats. Since albuminuria and cardiac hypertrophy are regarded as independent risk factors for cardiovascular morbidity and mortality (including CKD- and CHF-related mortality) [2–4,55–58], greater beneficial effects on albuminuria, renal morphology and bilateral cardiac hypertrophy further support the notion that the addition of ET_A receptor antagonist to the treatment with ACEi exhibits supplementary protective effects.

Our present findings indicating additional protective effects of the combined treatment using the ET_A receptor antagonist and ACEi (as compared with standard pharmacological ACE inhibition alone) are in accordance with the preliminary results of two ongoing clinical trials evaluating the effects of the dual ET_A receptor plus angiotensin II

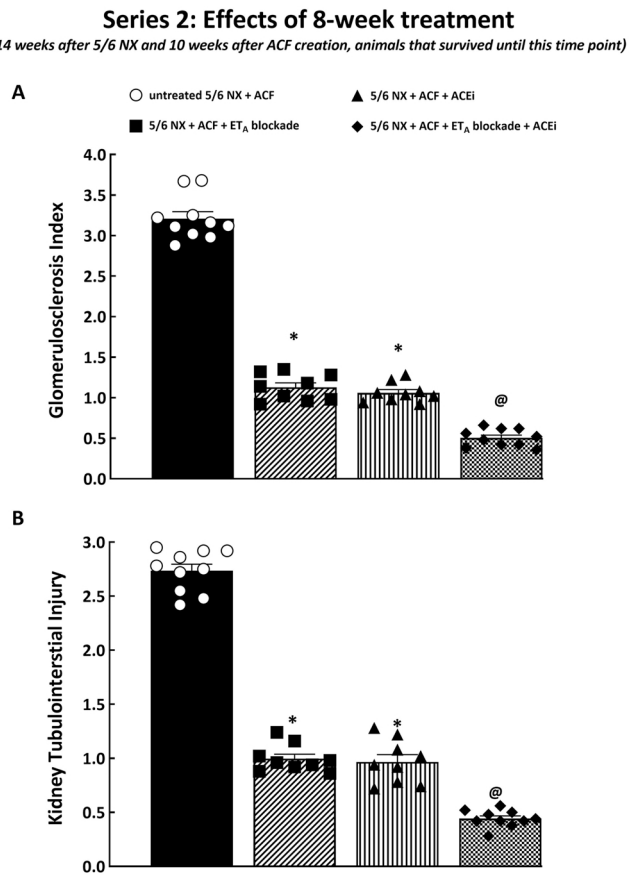

Fig. 10. Series 2: glomerulosclerosis index (A) and kidney tubulointerstitial injury (B) 14 weeks after 5/6 renal mass reduction (5/6 NX) and 10 weeks after aorto-caval fistula (ACF) creation or sham operation, and after 8 weeks' treatment with endothelin type A (ET_A) receptor antagonist, or with angiotensin-converting enzyme inhibitor (ACEi), alone or combined in untreated Hannover-Sprague Dawley (HanSD) undergoing combination of 5/6 NX and creation of the ACF, in 5/6 NX + ACF HanSD rats treated with endothelin type A (ET_A) receptor antagonist, or with angiotensin-converting enzyme inhibitor (ACEi), alone or combined. * $P < 0.05$ versus untreated 5/6 NX + ACF HanSD rats. @ $P < 0.05$ versus all other groups.

receptor type 1 (AT_1) antagonist (sparsentan) versus AT_1 receptor antagonist alone (irbesartan), which were obtained in individuals with either IgA nephropathy or with focal segmental glomerulosclerosis. Thus, both studies demonstrate better renoprotective actions of the dual blockade [59,60].

5. Limitations and strengths of the study

In addition to the uncertainties already discussed above, several important limitations should be here mentioned.

The first is that additional markers of kidney injury were not determined, such as urinary excretion of kidney injury molecule-1, which is recognized as a sensitive and specific biomarker for proximal tubular injury [61], and neutrophil gelatinase-associated lipocalin, another established marker for renal injury [62].

The second limitation relates to the absence of histological examination of the lungs and hearts, which would be especially relevant in the second series of experiments (evaluation of the effects of 8-week treatment), because here beneficial effects of the treatment on the cardiac hypertrophy were found, particularly in animals receiving combined therapy. Evidently, the evaluation of additional biomarkers of renal injury and the complex histological examination of the organs is needed to a further support of our conclusions.

The first strength of our work is that it is a complex in vivo study. Evidently, we are witnessing at present an explosion of tools, resources and publicly available data, which reflects an incredible progress of the molecular biological techniques. This appears to obscure the value of in vivo biological models in the biomedical research. However, as recently emphasized [63], basic animal models with strictly defined pathophysiological alterations are still irreplaceable in the search for new therapeutic targets in cardiovascular diseases. Let it be recalled that Claude Bernard, a founder of modern experimental medicine, stated more than 150 years ago that before generalization of scientific findings, results should by validated by employing various experimental models [64]. Fortunately, this view is increasingly accepted by the cardiovascular community in the development of new therapeutic measures for CHF [13,63].

Another strength of our present study is that it addresses the issue of the treatment with ET_A receptor antagonist when CHF is combined with CHF, the situation which was neglected until recently, particularly due to the concern that administration of ET_A receptor antagonist in patients with CKD, who are permanently in the status of subclinical volume overload, would cause excessive fluid retention, thereby increasing the risk of CHF decompensation. However, the recent post hoc analyses of the SONAR trial have also shown that the treatment with ET_A receptor antagonist could have some protective effects in patients with CHF and CKD [65]. Therefore, more information is needed regarding mechanisms underlying potential beneficial actions of ET_A receptor blockade in the situation when CHF is combined with CKD; and our present study offers such insight from in vivo experiments.

6. Translational perspective

In general, our present results demonstrated that the combined blockade of the ET system and the RAS should be considered in attempts to develop new pharmacological strategies for the treatment of combined CKD and CHF.

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CRediT authorship contribution statement

All authors conceived and designed the study. All authors have read and approved the final version of the manuscript.

P.K.: *Conceptualization, Methodology, Software, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Writing - Review & Editing, Project administration, Funding acquisition;* **Z.V.**: *Investigation, Visualization;* **P.S.**: *Investigation;* **E.K-J.**: *Writing - Review & Editing;* **J.S.**: Writing - *Review & Editing;* **A.W.**: *Writing - Review & Editing;* **J.V.**: *Validation, Writing - Review & Editing;* **M.T.**: *Validation, Resources, Writing - Review & Editing, Funding acquisition;* **H.M.**: *Software, Visualization;* **I.V.**: *Writing - Review & Editing*: **L.C.:***Conceptualization, Methodology, Resources, Data Curation, Writing - Original Draft, Writing - Review & Editing, Supervision, Funding acquisition.*

Declaration of Competing Interest

The authors of this manuscript have nothing to declare.

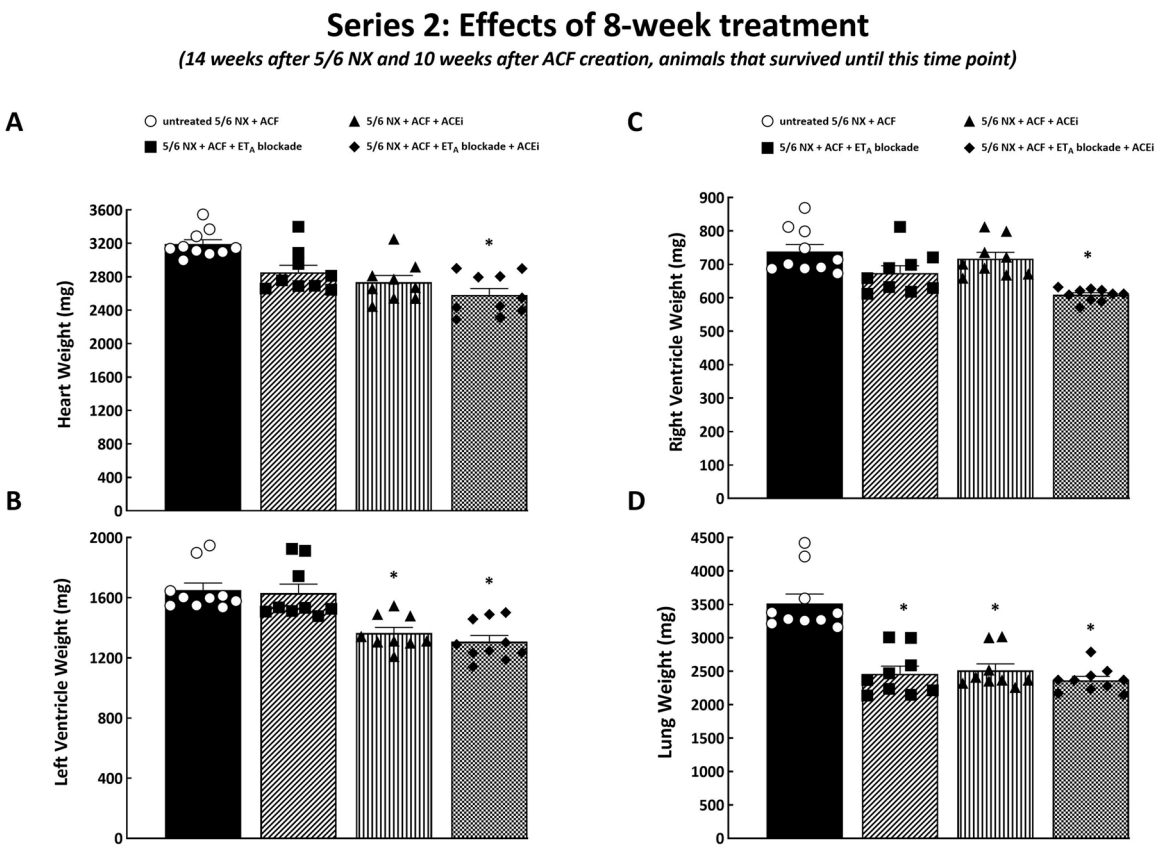

Fig. 11. Series 2: whole heart weight (A), left ventricle weight (B), right ventricle weight (C) and lung weight (D) 14 weeks after 5/6 renal mass reduction (5/6 NX) and 10 weeks after aorto-caval fistula (ACF) creation or sham operation, and after 8 weeks' treatment with endothelin type A (ET_A) receptor antagonist, or with angiotensin-converting enzyme inhibitor (ACEi), alone or combined in untreated Hannover-Sprague Dawley (HanSD) undergoing combination of 5/6 NX and creation of the ACF, in 5/6 NX + ACF HanSD rats treated with endothelin type A (ET_A) receptor antagonist, or with angiotensin-converting enzyme inhibitor (ACEi), alone or combined. * $P < 0.05$ versus untreated 5/6 NX + ACF HanSD rats.

Data availability

Data will be made available on request.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.biopha.2022.114157.

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