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Effects of Renal Denervation on the Course of Cardiorenal Syndrome: Insight From Studies With Fawn-Hooded Hypertensive Rats

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Summary

Combination of chronic kidney disease (CKD) and heart failure (HF) results in extremely high morbidity and mortality. The current guideline-directed medical therapy is rarely effective and new therapeutic approaches are urgently needed. The study was designed to examine if renal denervation (RDN) will exhibit long-standing beneficial effects on the HF- and CKD-related morbidity and mortality. Fawn-hooded hypertensive rats (FHH) served as a genetic model of CKD and fawn-hooded low-pressure rats (FHL) without CKD served as controls. HF was induced by creation of aorto-caval fistula (ACF). RDN was performed 28 days after creation of ACF and the follow-up period was 70 days. ACF FHH subjected to sham-RDN had survival rate of 34 % i.e. significantly lower than 79 % observed in sham-denervated ACF FHL. RDN did not improve the condition and the final survival rate, both in ACF FHL and in ACF FHH. In FHH basal albuminuria was markedly higher than in FHL, and further increased throughout the study. RDN did not lower albuminuria and did not reduce renal glomerular damage in FHH. In these rats creation of ACF resulted in marked bilateral cardiac hypertrophy and alterations of cardiac connexin-43, however, RDN did not modify any of the cardiac parameters. Our present results further support the notion that kidney damage aggravates the HF-related morbidity and mortality. Moreover, it is clear that in the ACF FHH model of combined CKD and HF, RDN does not exhibit any important renoprotective or cardioprotective effects and does not reduce mortality.

Key words

Chronic kidney disease • Heart failure • Renal denervation • Fawn-hooded hypertensive rats

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Introduction

Heart failure (HF) is considered to be a global pandemic of the 21st century and represents an extreme burden to the public healthcare systems worldwide; epidemiological studies show that the life expectancy of HF patients is extremely poor [1]. Chronic kidney disease (CKD) is also a very serious public health problem with the incidence and prevalence increasing worldwide [2]. Moreover, CKD patients exhibit a dramatically reduced life expectancy as compared with those with normal kidney function [3,4]. It is now recognized that CKD must be considered one of the strongest risk factors for the development of cardiovascular disease, especially of HF [3,5,6]. The cardiorenal syndromes result from the complex bidirectional interaction between the heart and kidneys in acute and chronic circumstances. Five

syndrome types are described: type 5 refers to the situation when HF and CKD are combined [7,8]. Even though HF and CKD frequently coexist and patients with the combination represent probably the highest cardiovascular risk population [3,7,8], to date patients with CKD have largely been excluded from randomized control trials in HF. This results in paucity of evidence for the appropriate treatment of patients with combined HF and CKD [3,7,9-11] – a serious deontological failure, considering that the newest guidelines of the European Society of Cardiology had to admit that there is little direct evidence to support any of the proposed treatment options for these patients [12].

Thus, it is acknowledged that novel effective alternative therapies for patients with HF combined with CKD are urgently needed. Obviously, the prerequisite here is comprehensive understanding of the mechanism(s) underlying the progression of HF combined with CKD. This is still to come and appropriate focused experimental studies are needed.

Recent research focused on the role of sympathetic nervous system (SNS) and on the potential benefits of application of both afferent and efferent renal denervation (RDN). There is no doubt that SNS hyperactivity plays important role in the progression of HF, pathophysiology of CKD and hypertension [13-21]. Confirmation of the dogma of important role of SNS overactivity in HF and hypertension has come from application of guideline-directed medical therapy (GDMT) for the treatment of HF and hypertension [19,22]. However, despite positive outcomes of GDMT on the morbidity and mortality in patients with HF, CKD and hypertension, a growing patient population proved resistant to GDMT, and off-target side effects were reported with lifelong therapy. RDN as a one-time, minimally invasive, catheter-based procedure could also be beneficial in view of the common patients' non-adherence to GDMT. After initial controversies, RDN is now included as a therapeutic option for the treatment of hypertension in the latest European Society of Cardiology/European Society of Hypertension guidelines and position statements [23,24], and it is now considered as a promising tool for the treatment of HF and CKD [21,25]. The hope is growing that RDN could be a new non-pharmacological measure for the treatment of HF combined with CKD, particularly in originally hypertensive subjects. However, comprehensive, convincing preclinical studies are so far not available and clinical research cannot be initiated until such preclinical studies are completed.

The fawn-hooded hypertensive (FHH) rat represents a unique model of spontaneous hypertension with early development of CKD [26-30]. In our previous studies we demonstrated that even if FHH rats are only slightly hypertensive (systolic blood pressure 145-150 mm Hg), they develop early albuminuria and CKD in contrast to their control counterparts i.e. fawn-hooded low-pressure (FHL) rats [29,30].

The model of rat HF induced by volume overload which follows creation of aorto-caval fistula (ACF) was introduced 45 years ago [31]. As shown by various research groups, including ours, in many respects the model imitates the course of HF in untreated humans [30-37] and therefore was endorsed by the American Heart Association and the European Society of Cardiology for preclinical testing to identify new targets for HF treatment [38]. In our two previous studies we showed that ACF-induced HF in FHH rat seems to be an optimal experimental model to study the course and pathophysiology of concomitant HF and CKD [30,39]. In our latest study using the model, we found that in ACF FHH rats RDN delayed the rise in albuminuria but did not improve the survival rate [39], however, we did not examine the effects of RDN on cardiac function and structure. Since earlier studies employing canine models of HF induced by myocardial infarction (MI) or rapid heart pacing showed that RDN alleviated harmful cardiac remodeling [40-42], we focused here on the effects of the denervation on cardiac function and remodeling in ACF FHH rats. It will be noticed that the positive RDN effects on cardiac remodeling were observed only in short-range canine model studies and provided no data on the HF-related mortality. Moreover, these studies were not focused on HF and CKD coexistence. Therefore, we have decided to assess the long-standing effects of RDN on HF- and CKD-related mortality and morbidity in ACF FHH rats and to compare the effects with those in their normotensive counterparts without CKD (i.e. in ACF FHL).

Methods

Ethical approval

The studies were performed in agreement 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 *European Convention on Animal Protection and*

Guidelines on Research Animal Use and were approved by this committee and subsequently by the Ministry of Health of the Czech Republic (the decision number for this project is 14553/2019-4/OVZ).

Heart failure model, exclusion criteria

Male FHH and FHL rats, at the initial age of 12 weeks, derived from several litters, were randomly assigned to experimental groups. Rats were anesthetized with an intraperitoneal injection of ketamine/midazolam mixture (Calypsol, Gedeon Richter, Hungary, 160 mg/kg and Dormicum, Roche, France, 160 mg/kg) and HF was induced by volume overload caused by the ACF created using needle technique, as employed and validated by many investigators including our own group [31-37,39]. Sham-operated rats underwent an identical procedure but without creating ACF. If a technical error occurred during the ACF procedure or if pulsatile flow in the inferior vena cava could not be confirmed, suggesting incomplete ACF creation, the animals were excluded from the study.

RDN technique

Bilateral RDN or sham-RDN procedure was performed four weeks after ACF or sham surgery. Under ketamine/midazolam anesthesia both kidneys were approached through a midline abdominal incision, both renal arteries and veins were stripped of connective tissues and all visible nerves were separated from the arteries and cut close to the hilus. Thereafter, both renal arteries were painted with 10 % phenol solution in absolute alcohol to destroy any remaining nerve fibers. During application of phenol the adjacent tissues were carefully protected from the exposure to phenol, and disruption of the major lymphatic vessels in the area was avoided. The animals showing a spasm of the renal artery were excluded from the study. Control animals underwent a sham operation: laparotomy and retraction of the abdominal organs was done but the renal vessels were not isolated or painted with phenol solution. This procedure is now employed as a golden standard model of RDN and was repeatedly validated as fully effective [39, 43-47]. In our recent study with FHH rats [39], we demonstrated that in FHH rat and FHL rat as well as ACF FHH and ACF FHL this RDN technique was fully effective for 70 days: renal concentrations of norepinephrine (NE) (a golden standard marker for renal SNS activity) was reduced by about 90 % as compared to the level observed in sham-RDN procedure [39].

General analytic procedures and evaluation of cardiac function

For albumin excretion measurement, twenty-four-hour urine collection from the animals placed in individual metabolic cages (MC) was performed after appropriate habituation training. Urinary albumin was measured by a quantitative sandwich enzyme immunoassay technique, using the commercially available ELISA kit (ERA3201-1, AssayPro, MO, USA). This technique is routinely employed in our laboratory [39,48,49].

Cardiac function and structure evaluation was performed by echocardiography techniques as described in detail in our previous studies [48,49]. Briefly, prior the echocardiographic examination, the animals were anesthetized with 4 % isoflurane (IsoVet®, Piramal Healthcare, UK). During the image acquisition, the rats were maintained under isoflurane anesthesia (1.5-2 %, Combi-vet® system, Rothacher Medical GmbH, Heitenried, Switzerland), and fixed in the supine position. B-Mode and M-Mode images were recorded in parasternal long and short axis view and used for measurements of dimensions of left ventricle (LV) internal diameter, anterior and posterior walls. Echocardiographic examination was done using a 10 MHz transducer (Vivid System 7 Dimension, GE HealthCare, Illinois, USA).

Histological evaluation of kidney tissue

These were done to assess glomerular damage and tubulointerstitial injury. The kidneys were fixed in 4 % formaldehyde, dehydrated and embedded in paraffin. The sections stained with 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 to 50 % (moderate sclerosis); *grade 3*, sclerotic area 50 to 75 % (moderate-to-severe sclerosis); *grade 4*, sclerotic area 75 to 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 (TSI) was evaluated as defined by Nakano *et al.* [50], 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 TSI is 3. The values of GSI<0.5 and TSI<0.4 are considered as healthy renal tissue without sign of significant renal damage. This method is always employed in our studies evaluating the degree of kidney damage [29,30,39,49,51].

SDS-PAGE and Western blotting techniques in heart tissue

Frozen LV tissue was powdered in liquid nitrogen and homogenized in SB20 lysis buffer (20 % SDS, 10 mmol/l EDTA and 100 mmol/l Tris, pH 6.8). To analyze, the samples were prepared in Laemmli buffer without 2-mercaptoethanol and loaded onto gels without denaturation. For the abundance of proteins, the tissue lysate was diluted in the Laemmli sample buffer and boiled for 5 min, and an equal amount of protein was loaded in each well, followed by separation on SDS-PAGE 10 % bis-acrylamide gels at a constant voltage of 120 V (Mini-Protean TetraCell, Bio-Rad, Hercules, CA, USA), as previously described [51,52]. Subsequently, the separated proteins were transferred to a nitrocellulose membrane (0.2-µm pore size, Advantec, Tokyo, Japan) and blocked for 4 h with 5 % fat-free milk in Tris-buffered saline containing 0.1 % Tween 20 (TBST). The membrane was incubated overnight with the primary antibodies listed in Supplementary Table 1. The membrane was subsequently washed in TBST and incubated for 1 h with a horseradish peroxidase-linked secondary anti-rabbit antibody (1:2000, 7074S, Cell Signaling Technology, Denver, CO, USA) and anti-mouse antibody (1:2000, 7076C, Cell Signaling Technology, Denver, CO, USA). The enhanced luminol-based chemiluminescent was used for visualization of the proteins and the quantification of the relevant bands was assessed densitometrically using Carestream Molecular Imaging Software (version 5.0, Carestream Health, New Haven, CT, USA) and normalized to GAPDH.

Myocardial histology and enzyme histochemistry

Conventional hematoxylin-eosin and Van Gieson staining of heart left ventricular tissue sections was used for light microscopic examination of the myocardial structure (Zeiss Apotome 2 microscope: Carl Zeiss, Jena, Germany). For microscopical evaluation 10-µm-thick heart cryostat sections were used. Catalytic enzyme histochemistry was performed according to Lojda [53], to demonstrate the activities of capillary endothelium-related alkaline phosphatase (AP, E.C.3.1.3.1) with naphthol AS-MX phosphate as a substrate and dipeptidyl peptidase-4 (DPP4, E.C.3.4.15.4) with glycyl-L-proline-4-methoxy-beta naphthylamide as a substrate to detect functional arteriolar and venular capillary network. For quantification of collagen incidence (pink color) Van Gieson staining was used**.** For intensity of AP (blue color) and DPP4 (dark brown color) by histochemical reactions (corresponding to the enzyme activity), randomly selected areas (10 per heart) with colored reaction (pink, blue and dark brown) were analyzed by appropriate imaging system (Soft Imaging System, GmbH, Germany). The signal was expressed as a percentage of the total tissue area.

Noteworthy, assessment of the endothelial AP and DPP4 activity in myocardial cryosection via enzyme histochemistry was developed and validated by Lojda [53,54], and proved a very precise method. AP activity is specific for arterial part of capillary network and it is involved in transphosphorylation reactions that include adenosine metabolism. Lojda and Gutmann showed that alterations of endothelial AP activity indicate the functional changes of arterial part of capillaries [54]. DPP4 is a serine peptidase which removes N-terminal dipeptides from polypeptidase with a proline residue, and its activity is specific for the venous part of myocardial network [54]. DPP4 activity has been implicated in the collagen metabolism and synthesis of the endothelial coat, which is important for the binding of glycos-aminoglycans [53]; it was demonstrated that increased DPP4 activity was present in humans with persistent atrial fibrillation and it may play a role in the structural remodeling of fibrillating atria [55]. The method for evaluation of AP and DPP4 activity has been employed in our laboratory for the past 20 years and proved to be an accurate and specific methodology under various physiological and pathophysiological conditions [56,57].

Experimental design

The details of the design and timing of the experimental maneuvers are given in Figure 1. Animals underwent either sham-operation or ACF creation on the day labeled -28 and were left without any intervention for 28 days. Our previous studies showed that after this time period HF features are fully developed in FHL rat as well as FHH rat, but animals are still in the compensation phase of HF [30,39]. At this time point (day labeled 0) animals underwent either bilateral RDN or sham-RDN procedure and were assigned into the following experimental groups:

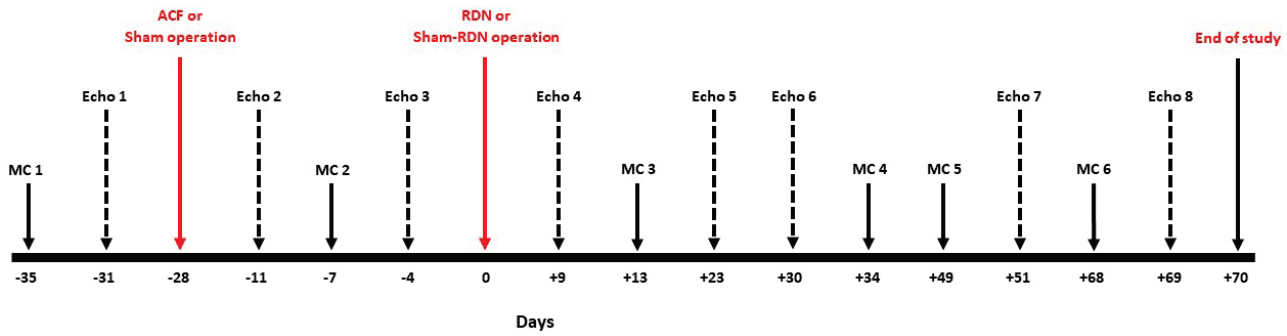

Fig. 1. The experimental design, especially the time sequence of experimental maneuvers for individual experimental groups. MC: placement into individual metabolic cage for 24-hour urine collection, Echo: echocardiography evaluation of cardiac structure and function, ACF: creation of aorto-caval fistula, RDN: bilateral renal denervation.

1. Sham-operated FHL rat + sham-RDN (initial n=8)
2. Sham-operated FHH rat + sham-RDN (initial n=8)
3. Sham-operated FHL rat + RDN (initial n=10)
4. Sham-operated FHH rat + RDN (initial n=10)
5. ACF FHL rat + sham-RDN (initial n=23)
6. ACF FHH rat + sham-RDN (initial n=18)
7. ACF FHL rat + RDN (initial n=20)
8. ACF FHH rat + RDN (initial n=28)

The follow-up period was until day +70. On the days labeled -35 (i.e. before ACF creation – basal values), -7 (i.e. before bilateral RDN), +13, +34, +49 and +68 (days after bilateral RDN), 24-hour urinary albuminuria was assessed.

On the days labeled -31 (i.e. before ACF creation – basal values), -11 and -4 (i.e. before bilateral RDN), +9, +23, +30, +51 and +69 (days after bilateral RDN) echocardiography evaluation of cardiac structure and function was performed. In order to obtain reliable data regarding the effects of RDN as a treatment regime on the survival rate, relatively high initial n values were employed (except for the sham-operated animals) to allow reliable comparison of the long-term survival rates. To define such required initial n values, statistical power analysis by method developed by Cohen [58] was used. At the end of the study, the survived animals were killed by an overdose of thiopental sodium and the organs were collected.

Statistical analysis

Statistical analysis of the data was performed using Graph-Pad Prism software (Graph Pad Software, San Diego, California, USA). 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 expressed as mean ± SEM. The values of P below 0.05 were considered statistically significant.

Results

Effects of RDN on the survival rate, albuminuria, kidney damage and organ weights (Figs 2 and 3 and Suppl. Fig. 1)

All sham-operated FHL and FHH (i.e. those without ACF), both after sham-RDN or RDN, survived until the end of experiment (data not shown).

As shown in Figure 2A, the ACF FHH after sham-RDN began markedly to die by day +10 (i.e. 38 days after creation of ACF) and the final survival rate was 34 %. In contrast, the ACF FHL after sham-RDN showed higher survival rate throughout the study and the final survival rate was 79 %, which was significantly higher than in ACF FHH after sham-RDN ($P<0.01$) (Fig. 2A).

RDN did not improve the course and the final survival rate in ACF FHL, even though the final survival rate in ACF FHL that underwent RDN was numerically higher than in those exposed to sham-RDN (90 % vs. 79 %) (not significant, Fig. 2B). Nor did RDN improve the course and the final survival rate in ACF FHH; the final survival rate was almost identical as that in ACF FHH that underwent only sham-RDN (34 % vs. 33 %) (Fig. 2C).

Albuminuria normalized to urinary creatinine excretion was in FHH before ACF creation (basal level) was about 300 fold higher than in FHL (Fig. 3B vs. 3A). In sham-operated FHL (no ACF) sham-RDN did not significantly change albuminuria throughout the study (Fig. 3A). In ACF FHL sham denervation did not consistently change albuminuria, only in MC 3 and MC 6 it was significantly increased (Fig. 3A), but was still dramatically lower (600 and 1000 fold, respectively) than

observed in sham-operated FHH after sham-RDN (Fig. 3B). However, albuminuria was not considerably increased compared with sham-operated FHL after RDN (Fig. 3A). RDN in ACF FHL did not noticeably alter the course of albuminuria as compared with sham-operated FHL as well as ACF FHL after sham-RDN (Fig. 3A).

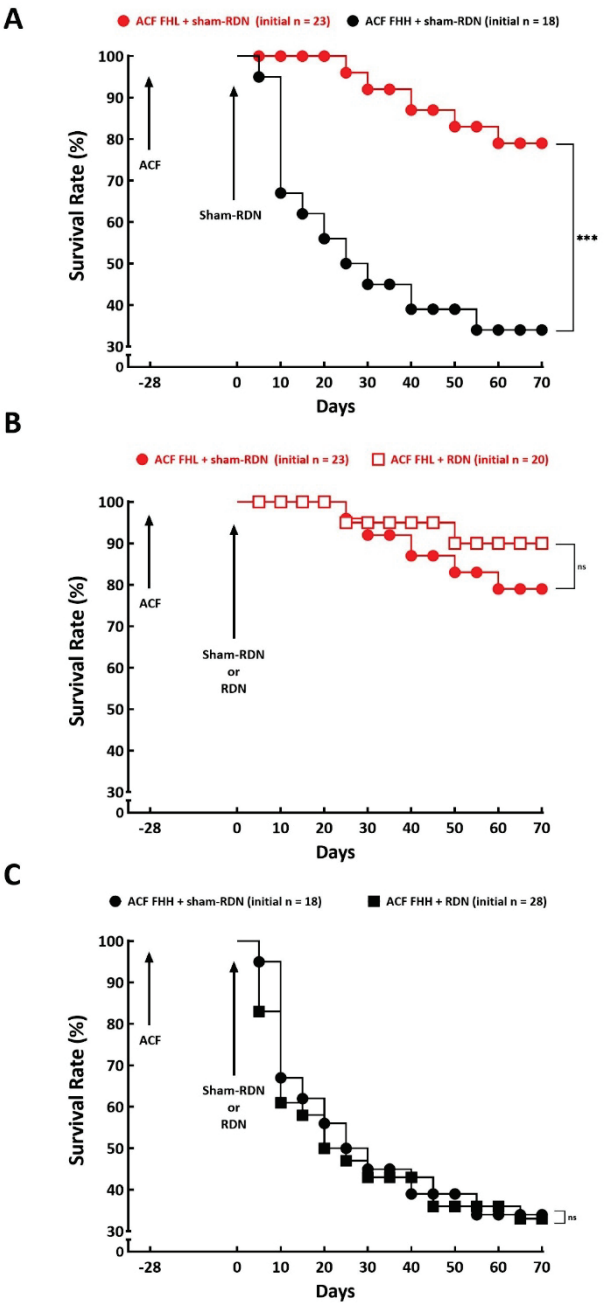

Fig. 2. (**A**) Survival rate in Fawn-hooded normotensive rats (FHL) (closed red circles) and fawn-hooded hypertensive rats (FHH) (closed black circles) with aorto-caval fistula (ACF) that underwent sham-renal denervation (RDN). (**B**) Survival rate in ACF FHL that underwent either sham-RDN (closed red circles) or bilateral RDN (open red squares). (**C**) Survival rate in ACF FHH that underwent either sham-RDN (closed black circles) or bilateral RDN (closed black squares). *** P<0.001 by long-rank Mantel-Cox test followed by Gehan-Breslow-Wilcoxon test.

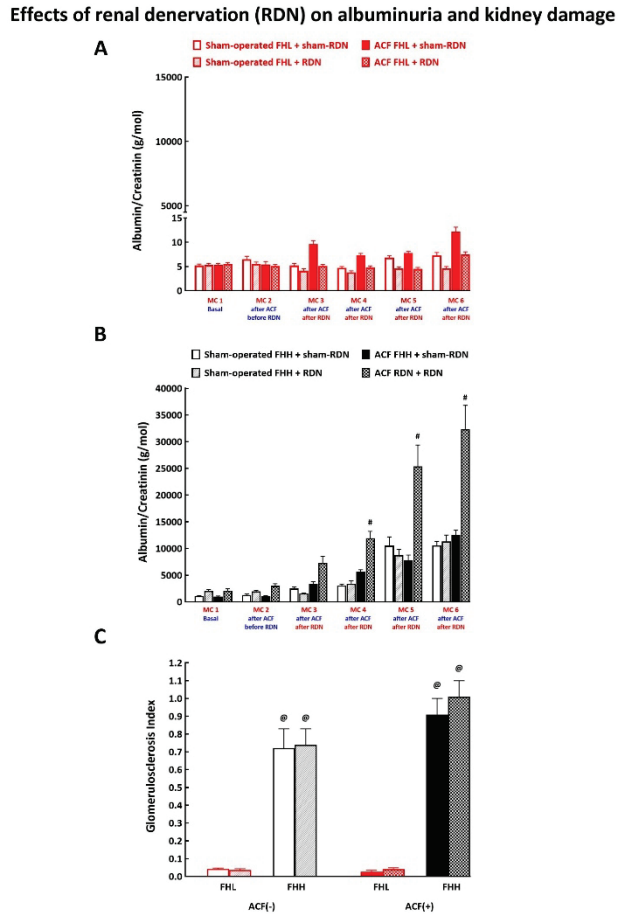

Fig. 3. Albumin to creatinine ratio in Fawn-hooded normotensive rats (FHL) (**A**) and Fawn-hooded hypertensive rats (FHH) (**B**) that underwent either sham-operation or creation of aorto-caval fistula (ACF) or were exposed to either sham-renal denervation (RDN) or bilateral RDN procedure. (**C**) Glomerulosclerosis index in FHL and FHH groups. MC, metabolic cage. ACF(-), experimental groups without ACF; ACF(+), experimental groups with ACF. # P<0.05 vs. all other groups at the same time point. @ P<0.05 vs. FHL groups.

As shown in Figure 3B, albuminuria in FHH increased throughout the study and the creation of ACF in FHH that subsequently underwent sham-RDN did not promote albuminuria in FHH. RDN in ACF FHH resulted in higher albuminuria in MC4, MC5 and MC6 (P<0.001) as compared with all the other FHH groups (Fig. 3B).

Figure 3C summarizes the data of glomerulosclerosis index (GSI) at the end of the study (in animals that survived until the end of experiment). Sham-operated FHH after sham-RDN displayed markedly higher GSI as compared with sham-operated FHL after sham-RDN. Creation of ACF as well as RDN did not alter GSI similarly in FHL or FHH. Kidney TSI studies showed the same pattern as did GSI and therefore the data are not shown.

Representative images of renal kidney sections of all FHL and FHH groups are shown in Supplementary

Figure 1. It is seen that all experimental groups of FHL exhibited normal renal morphology whereas FHH showed moderate renal parenchymal injury, with focal segmental and global glomerulosclerosis associated with small areas of tubular atrophy.

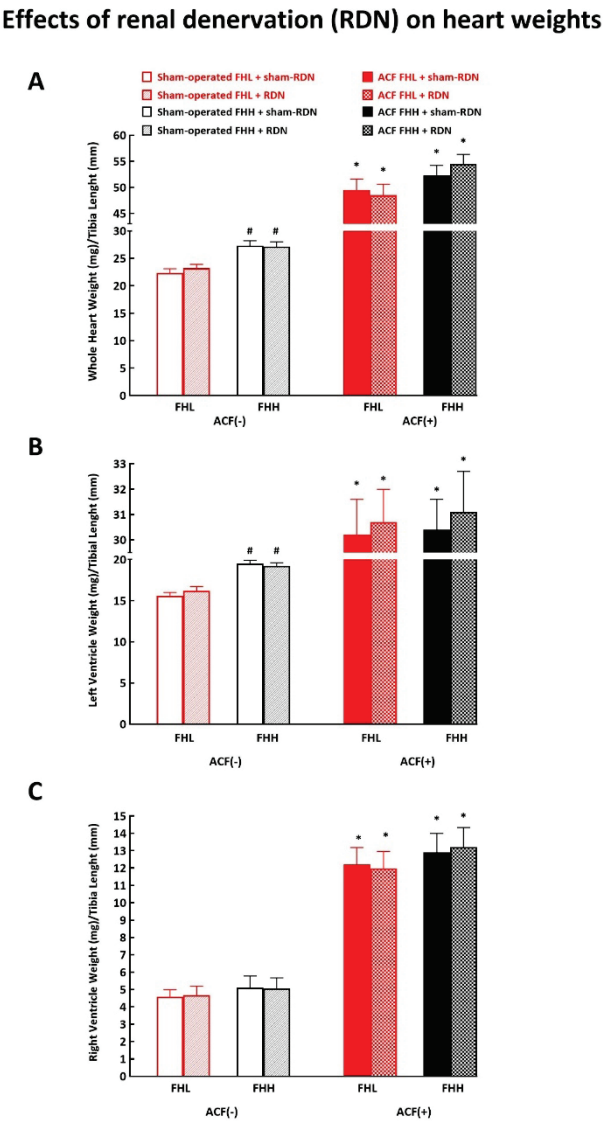

Fig. 4. Morphometric parameters measured in materials collected from rats that survived until the end of the observation in Fawn-hooded normotensive rats (FHL) and Fawn-hooded hypertensive rats (FHH) that either underwent sham-operation or creation of aorto-caval fistula (ACF) or were exposed to either sham-renal denervation (RDN) or bilateral RDN procedure. (**A**) Whole heart weight, (**B**) left ventricle weight, (**C**) right ventricle weight. ACF(-), experimental groups without ACF; ACF(+), experimental groups with ACF. * P<0.05 vs. sham-operated animals. # P<0.05 vs. FHL groups.

Effects of RDN on organ weights (Fig. 4 and Suppl. Fig. 2)

Figure 4 and Supplementary Figure 2 collect the values of organ weight at the end of the study. As shown in Figures 4A and 4B, sham-operated FHH (no ACF) after sham-RDN exhibited significant cardiac hypertrophy (expressed as whole heart weight normalized to tibia length), with significant left ventricle (LV) hypertrophy as compared with sham-operated FHL after RDN. There was no significant right ventricle (RV) hypertrophy (Fig. 4C). Creation of ACF in the FHL as well as in FHH rats, those that subsequently underwent sham-RDN, resulted in marked bilateral cardiac hypertrophy, as seen from whole heart weight, LV weight, and RV weight, as compared with their sham-operated (no ACF) counterparts after sham-RDN. Interestingly, the degree of bilateral cardiac hypertrophy in ACF FHL and ACF FHH was similar. Furthermore, the degree of RV ventricle hypertrophy in ACF FHL as well as in the FHH that subsequently underwent sham-RDN was higher than that of the LV, as seen from the increases of RV to LV ratio in those animals (Suppl. Fig. 2A). RDN did not alter any of the cardiac parameters in any group. As shown in Supplementary Figure 2B, there were no significant differences in lung weight between sham-operated FHL and sham-operated FHH when measured after sham-RDN. Creation of ACF elicited a similar degree of lung congestion (based on the lung weight) in FHL and FHH when measured after sham-RDN. RDN did not modify lung weight in any group (Suppl. Fig. 2B).

As shown in Supplementary Figure 2C, all groups of FHH showed higher kidney weight as compared with the groups of FHL rats and neither creation of ACF nor RDN changed this difference.

Effects of RDN on cardiac function and morphology assessed by echocardiography (Figs 5 and 6 and Suppl. Figs 3 and 4)

As shown in Figure 5 and Supplementary Figure 3, there were no significant differences in the basal values of stroke volume, cardiac output, heart rate, ejection fraction of the LV, fractional shortening of the LV and ejection fraction of the RV (measured as a change of fractional area) between FHL and FHH. As shown in Figures 5A and 5B, when measured after sham-RDN, creation of ACF in FHL as well as in FHH elicited an immediate striking increase in stroke volume and cardiac output (a consequence of the shunt). However, already in the second echocardiographic analysis after ACF creation (marked as Echo 3 in the figures, i.e. 24 days after ACF creation) the stroke volume and cardiac output reached a plateau at the levels that were

about twice higher than the basal values and this remained unchanged until the end of the study (Fig. 5A and B). Creation of ACF in FHL as well as in FHH when measured after sham-RDN did not significantly change ejection fraction, heart rate, and fractional shortening of the LV and ejection fraction of the RV throughout the experiment (Fig. 5C and Suppl. Fig. 3). RDN did not alter any of the cardiac function parameters in any group (Fig. 5 and Suppl. Fig. 3).

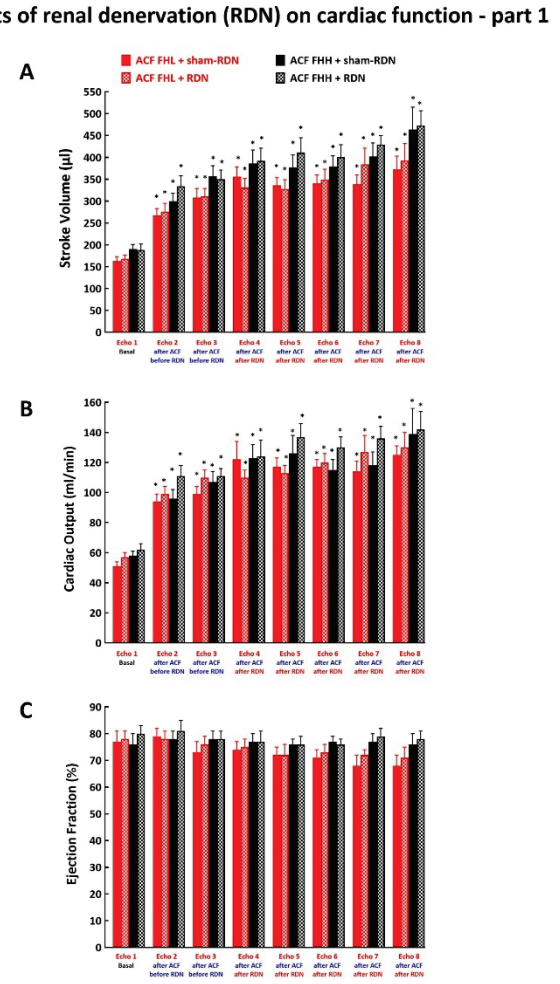

Fig. 5. Echocardiographic analyses of cardiac function throughout the study in Fawn-hooded normotensive rats (FHL) and Fawn-hooded hypertensive rats (FHH) that either underwent sham-operation or creation of aorto-caval fistula (ACF) or were exposed to either sham-renal denervation (RDN) or bilateral RDN procedure. (**A**) Stroke volume of the left ventricle, (**B**) cardiac output of the left ventricle, (**C**) ejection fraction of the left ventricle, * P<0.05 vs. basal values at the same group (i.e. vs. data obtained from Echo 1 measurements).

As shown in Supplementary Figure 4A, echocardiographic evaluation confirmed the data from the organ weight analysis: the baseline measurements showed LV hypertrophy in FHH when compared with FHL. Echocardiographic studies showed the dynamic of the process of LV hypertrophy in FHH as well as in FHL after creation of ACF: marked hypertrophy was seen in FHH as well as in FHL. Until the Echo analysis 4 (i.e. 37 days after ACF creation) the degree of LV hypertrophy was similar in ACF FHL and ACF FHH, but starting with Echo analysis 5 (i.e. 51 days after ACF creation) it became markedly greater in ACF FHH as compared with ACF FHL. RDN did not alter the course or the final level of LV hypertrophy in any group (Suppl. Fig. 4A).

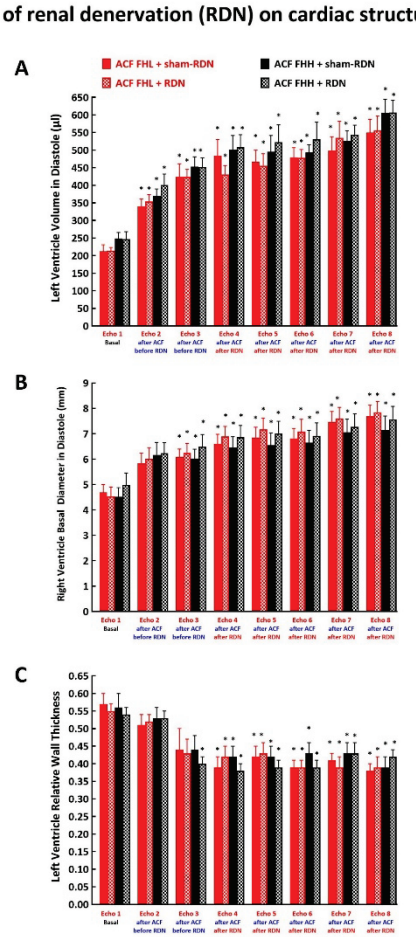

Fig. 6. Echocardiographic analyses of cardiac structure throughout the study in Fawn-hooded normotensive rats (FHL) and Fawn-hooded hypertensive rats (FHH) that either underwent sham-operation or creation of aorto-caval fistula (ACF) or were exposed to either sham-renal denervation (RDN) or bilateral RDN procedure. (**A**) Left ventricle volume in diastole, (**B**) Right ventricle basal diameter, (**C**) Relative wall thickness of the left ventricle. * P<0.05 vs. basal values at the same group (i.e. vs. data obtained from Echo 1 measurements).

As shown in Supplementary Figure 4C, even the basal body weight (BW) was in FHH significantly higher

than in FHL, despite the same initial age of the animals. Creation of ACF did not alter the difference in BW between FHH and FHL. BW gain in the course of the experiment was slightly but significantly greater in ACF FHH after sham-RDN than ACF FHL after sham-RDN (+24±2 % vs. 16±2 %, P<0.05). RDN did not modify BW changes in any group (Suppl. Fig. 4C).

As shown in Supplementary Figure 4B, there were no significant differences in the baseline LV anterior wall thickness between FHH and FHL, apart from a tendency for higher values in FHH. Creation of ACF resulted in gradual increases in LV anterior wall thickness in FHL as well as in FHH after sham-RDN. In the late phase of study, starting with Echo analysis 5 (i.e. 51 days after ACF creation) the increases were more pronounced in ACF FHH than in ACF FHL. RDN did not alter the dynamic or the final level of LV anterior wall thickness in any group (Suppl. Fig. 4B). LV posterior wall thickness showed the same pattern of changes (data not shown).

As shown in Figure 6A and B, there were no significant differences in the basal LV volume (the same was true for LV diameter – therefore data are not shown) and RV diameter between FHH and FHL. Creation of ACF elicited abrupt increases in the LV volumes as well as in RV diameters to similar levels in ACF FHH and ACF FHL. RDN did not change the course or the final values of LV volumes and RV diameters in any group (Fig. 6A and B).

As shown in Figure 6C, there were no significant differences in the baseline relative wall thickness of the LV between FHH and FHL. Creation of ACF resulted in a progressive decrease in the relative wall thickness of the LV, despite an increase in LV posterior wall thickness. This was so both in FHL and FHH when measured after sham-RDN. Throughout the study there were no significant differences in the relative wall thickness of the LV between ACF FHL and ACF FHH, which suggests that ACF FHL as well as ACF FHH developed eccentric cardiac hypertrophy. RDN did not alter the course of changes in the relative wall thickness in any group (Fig. 6C).

Effects of RDN on myocardial protein levels, collagen deposition, capillary density and myocardial histology staining (Fig. 7, Suppl. Figs 5,6,7,8 and 9)

As shown in Figure 7A and Supplementary Figure 5A, when measured after sham-RDN, protein abundance of total connexin 43 (Cx43) and its active phosphorylated variant was decreased in all FHH groups as compared with the sham-operated FHL. Creation of ACF resulted in FHL as well as in FHH in a significant reduction in both forms of Cx43 and RDN did not abolish the decrease in either form of Cx43. Moreover, after RDN both forms of Cx43 were surprisingly lower in sham-operated (no ACF) FHL.

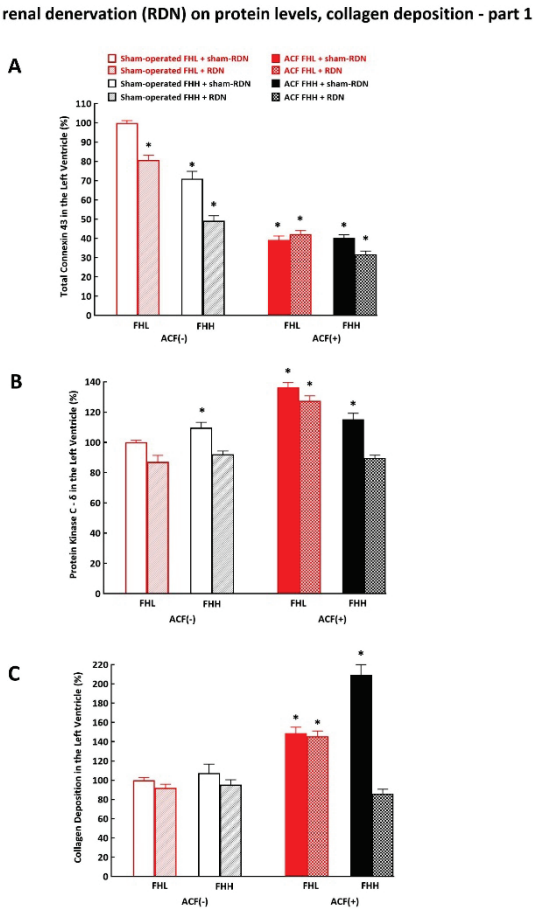

Fig. 7. Western blot demonstration of total connexin 43 (**A**), protein kinase C delta (δ) (**B**). Histological quantification of collagen deposition (**C**), in the left heart ventricle collected from rats that survived until the end of the study in Fawn-hooded normotensive group (FHL) and Fawn-hooded hypertensive rats (FHH) that either underwent sham-operation or creation of aorto-caval fistula (ACF) or were exposed to either sham-renal denervation (RDN) or bilateral RDN procedure. ACF(-), experimental groups without ACF; ACF(+), experimental groups with ACF. * P<0.05 vs. sham-operated FHL + sham-RDN.

As shown in Figure 7B, in sham-RDN group FHH showed significantly higher protein abundance of protein kinase C delta (δ) (pro-apoptic and profibrotic kinase) as compared with sham-denervated FHL. As shown in Supplementary Figure 5B, there were no significant differences in protein levels in protein kinase C epsilon (ε) (one of the protein kinases that can

phosphorylate Cx43 at serine-368) in any group. Creation of ACF caused a significant increase in protein levels of protein kinase C – δ in FHL as well as in FHH as seen in sham-RDN rats. RDN did not alter the protein abundance of protein kinase C δ either in FHL or ACF FHL, but reduced it in FHH as well as in ACF FHH (Fig. 7B).

As shown in Supplementary Figure 5C, there were no significant differences in the protein level of matrix metalloproteinase 2 between FHL and FHH and RDN did not alter it. Creation of ACF elicited a significant decrease in the protein levels of matrix metalloproteinase 2 in ACF FHL as well as in ACF FHH, and RDN abolished such decreases in both groups (Suppl. Fig. 5C).

As shown in Figure 7C, there were no significant differences in the collagen content between FHL and FHH and RDN did not alter it. Creation of ACF caused a significant increase in collagen content in FHL as well as FHH as seen in the sham-denervated group. RDN did not alter the collagen content in ACF FHL but significantly decreased it in ACF FHH.

As shown in Supplementary Figure 5D, AP activity (a marker of function of the arterial part of the capillary network) in FHH was significantly lower than in sham-denervated FHL. RDN did not alter AP activity in FHL, but increased it in FHH. Creation of ACF caused a significant decrease in AP activity in FHL and FHH as seen in the sham-denervated group and RDN did not alter these decreases (Suppl. Fig. 5D).

As shown in Supplementary Figure 5E, in FHH the DPP4 activity (a marker of function of the venous part of the capillary network) was in sham-denervated groups significantly lower than in FHL. RDN caused a significant decrease in DPP4 activity in FHL and did not change it in FHH. Creation of ACF did not alter DPP4 activity either in FHL or FHH, as seen both in sham-RDN and RDN groups but caused a significant decrease in DPP4 activity in ACF FHL as well as in ACF FHH (Suppl. Fig. 5E).

Supplementary Figure 6 shows representative images of collagen deposition in LV tissue of all experimental groups and the relevant quantification is summarized in Figure 7C.

Supplementary Figure 7 shows representative images of AP activity in LV tissue of all experimental groups and the relevant quantification is summarized in Supplementary Figure 5D.

Supplementary Figure 8 shows representative images of DPP4 activity in LV tissue of all experimental groups and the relevant quantification is summarized in Supplementary Figure 5E.

Supplementary Figure 9 shows representative microscopic images of hematoxylin/eosin-stained LV tissue in all experimental groups. There were no significant histological differences between experimental groups and there were no signs of myocardial necrosis or appearance of scar tissue in any experimental group.

Discussion

RDN is relatively a minimally invasive, catheter-based procedure included as a therapeutic option for the treatment of hypertension and it is now considered as a promising tool for the treatment of HF and CKD [21,23,24,25]. Over the recent years, since RDN was proposed as therapeutic measure, there were many controversies surrounding this procedure. Comprehensive clinical studies are still missing and cannot be initiated until preclinical studies are completed, especially in relation to cardiorenal syndromes. We aimed to evaluate the long-lasting effects of RDN on the HF- and CKD-related morbidity and mortality in the FHH rats, which underwent a creation of ACF. The most important, however very disappointing finding of our study is that the renal denervation did not exhibit any beneficial actions in this particular model of cardiorenal syndrome. However, we have provided several interesting findings, which can strengthen our understanding of cardiorenal syndromes and therefore could be used in future to provide relief to patients.

We found that ACF FHH when studied after sham-RDN exhibited substantially lower survival rate as compared with ACF FHL after sham-RDN, however, the degree of end-organ damage (see GSI) and bilateral cardiac hypertrophy was similar in ACF FHH and ACF FHL after sham-RDN at the end of study. However, it is emphasized that organ weights and end-organ damage were analyzed in the animals that survived until the end of experiment. Therefore, these results might be influenced by higher mortality in ACF FHH after sham-RDN: the animals with the most severe organ damage were likely dying throughout the study (mortality rate was 66 %), whereas in ACF FHL after sham-RDN the mortality rate was only 21 %; most probably, more animals with severe organ damage survived until the end of study. It should be emphasized that already at the age of 3 months FHH exhibited extraordinarily high albuminuria (300 fold times higher than observed in age-

matched FHL), even though they were only modestly hypertensive. This disparity between albuminuria and hypertension was documented by our former study employing radiotelemetry blood pressure (BP) measurements [29]. Our present and previous findings together with results reported by other investigators strongly suggest that the development of proteinuria, renal damage and subsequently CKD observed in FHH is the consequence of primary intrarenal alterations rather than hypertension-induced renal damage [26-30,39].

Therefore, the first interim conclusion of our present study is that kidney damage itself substantially increases the mortality in this model of "two-organ damage", in agreement with our recent findings [39,49] and with the recent view regarding the patients with combined HF and CKD (i.e. patients with so called cardiorenal syndrome) [7-11]. In addition, our present findings further support the recent opinion that albuminuria is an independent predictor of exacerbation of HF and increased risk of HF-related mortality [1,2,59].

Moreover, an important conclusion from our present and recent study is that ACF FHH represents a suitable model of combined CKD and HF, where CKD is primarily induced by intrarenal disorders [39]. The advantage of FHH as a model of CKD is that unlike the so far dominating approach involving renal mass reduction [60], it does not require surgical intervention. The model of 5/6 renal mass ablation (5/6 NX) [61,62], provided the majority of knowledge about the pathophysiology of CKD but the disadvantage is high mortality associated with the required surgical procedure. Moreover, the progression of glomerulosclerosis and CKD is primarily a consequence of hypertension (elicited by 5/6 NX and not by initial renal disease) [61-64].

One of the most valuable advantages of our present study is the evaluation of LV by histological methods and of the myocardial proteins by the western blot method, which was never performed in detail in this particular model of cardiorenal syndrome, *i.e.* ACF FHH and ACF FHL. The need for these assessments is obvious considering the knowledge that life-threatening arrhythmias in HF are associated with down-regulation of Cx43 and increased fibrosis in the LV [65,66]. Altered phenotype at the myocardial level was repeatedly confirmed in the ACF-induced HF model [51,52,66]. In addition, our recent study demonstrated that long-term treatment with either angiotensin-converting enzyme inhibitor or angiotensin II receptor antagonist, markedly attenuated the alterations in cardiac Cx43 and extracellular matrix in ACF-induced model of HF [52]. We found that ACF FHH showed a decline of Cx43 protein, which is consistent with previous findings in the HF model [66,67]. In addition, in ACF FHH we found a decrease of Cx43 variant phosphorylated at serine 368, which is of special interest: the presence of this variant was associated with anti-arrhythmic phenotype [68]. However, our studies did not show any protective effect of RDN on the alterations in cardiac Cx43 in ACF FHH, but the denervation did reduce to some extent the augmented collagen content in the LV of ACF FHH. Nevertheless, our present data do not support the notion that RDN exhibits important cardioprotective (e.g. anti-arrhythmic) effects in this particular form of HF induced by chronic volume overload and combined with CKD.

The main goal of the current study was to evaluate the long-term effects of RDN on morbidity and mortality in ACF FHH. The rationale for employing RDN as a new treatment of combined CKD and HF is based on studies showing that in CKD as well as HF, the SNS activity and particularly renal sympathetic nerve activity (RSNA) is augmented: if so, the removal of inappropriately high RSNA should be beneficial [7,13-15,18-21]. The notion about the beneficial effects of RDN on the mortality and morbidity in the combined CKD and HF was also supported by our recent studies showing that RDN substantially attenuated HF-dependent mortality in the ACF-induced HF model in Ren-2 transgenic hypertensive rats (TGR). Such beneficial effects were mediated by improvement of autoregulatory capacity of the renal hemodynamics and of the pressure-natriuresis relationship when measured at the stage of HF decompensation [47,69]. Moreover, previous studies demonstrated that FHH exhibit increased SNS activity as compared with FHL [26,29,39]. Importantly, we showed recently that RSNA is markedly increased in ACF FHH as compared with sham-operated FHH as well as ACF FHL [39].

The most disappointing and unanticipated finding of our present study is that in ACF FHH renal denervation did not exhibit any beneficial actions on the course of CKD and HF-related morbidity and mortality. Specifically, RDN did not affect albuminuria, renal glomerular and tubulointerstitial injury, lung congestion, bilateral cardiac hypertrophy and eccentric chamber remodeling nor did it alter the course and the final level of mortality. Surprisingly, at the end of study albuminuria was even higher than in FHH exposed only to sham-RDN procedure.

We cannot provide satisfactory explanation for these disappointing findings. There is ample encouraging, even though sometimes indirect, evidence, also from our own studies, suggesting that RDN should exhibit beneficial actions under the circumstances when CKD and HF are combined. Evidently, the present study failed to confirm the hope: CKD- and HF-related morbidity and mortality was not attenuated by RDN. We do not have any solid explanation of these disappointing findings, however, some hints for the potential reasons will be presented below in the section "Limitations and strengths of the study".

In summary, our study demonstrates that ACF FHH is a suitable model to explore the pathophysiology of combined CKD and HF (so called cardiorenal syndrome) and for pre-clinical evaluation of the effectiveness of new therapeutic strategies. This is important because the current therapies applied in this type of cardiorenal syndrome are ineffective. In addition, our present findings further support the notion that kidney disease dramatically aggravates the HF-related morbidity and mortality. On the other hand, the findings clearly show that in ACF FHH, the experimental model that combines CKD and HF, renal denervation does not exhibit any reno- and cardioprotective effects and does not attenuate the mortality.

Limitations and strengths of the study

The reliability of the classical RDN method used here presents problems related to the lack of a biomarker that would allow noninvasive and continuous evaluation of the effectiveness of denervation procedure [70]. The common and robust indicator of RSNA activity and effectiveness of RDN procedure is the renal tissue NE concentration [25,44-46]. However, to be the most reliable, renal NE should be measured in samples obtained from conscious decapitated animals [39,47,71], which was not feasible in our experiments. Reinnervation is known to occur 9-12 weeks after RDN [46,72]. In our previous study using sham-operated as well as ACF FHL and FHH we found that renal NE was substantially reduced (>90 %) 70 days after RDN [39]. Admittedly, the testing was done in separate groups of animals and it can be disputed whether all the animals throughout the study showed no activity of RSN. Thus, our assumption of the effectiveness of RDN in the present experiments is based on our previous relevant data. On the other hand, in the long-term study employing ACF-induced HF model we demonstrated that 84 days after RDN the kidney NE concentration was reduced by only about 40 %, indicating substantial reinnervation [47]. This limits the possibility to perform studies evaluating the effects of RDN on morbidity and mortality. In the animals without comorbidities (e.g. in ACF FHL), a considerably longer follow-up period, at least 200 days, is required, as, was, indeed, practiced in our studies of the value of new pharmacological therapies in the HF-related mortality in the ACF-induced HF model [34,35,37,48,49]. To achieve such long follow-up periods, repeating the RDN procedure (e.g. even 3 to 5 times) would be necessary. Since RDN surgery under general anesthesia is highly invasive, a dramatic negative impact on the outcomes of the study should be expected.

The strength of the present work is that it is a complex *in vivo* study evaluating long-term mortality in a suitable model of combined CKD and HF. Repeated measurements disclosed dynamic changes in cardiac function and the development of kidney damage, and the effects were examined of RDN on all these parameters. In the last decades we are witnessing an incredible progress in molecular biological techniques which appears to overshadow the merit of the biomedical research using *in vivo* biological models. However, as recently emphasized in a special compendium published in Circulation Research [69], basic animal models with precisely defined pathophysiological features are irreplaceable for the understanding of the pathophysiology of cardiovascular disease and in the search for new therapeutic targets in cardio-renal diseases.

Another strength of our present study is that it was performed using a relatively new model of cardiorenal syndrome. As such, it follows the recommendation by Claude Bernard, the founder of modern experimental medicine, that before generalization of scientific findings into universal claim, results should be validated by employing various experimental models [74].

In this context, it is important to briefly address the differences in the RDN techniques employed in experimental settings and in the clinical practice. In experimental studies employing rodents the above described RDN techniques combining surgical and chemical approach is used. The advantage of this RDN method is that it is highly effective [39,43-47], the disadvantage is that it is evidently invasive and virtually impossible to use in the clinical practice. Therefore, for clinical purposes RDN techniques employing a percu-

taneous catheter-based procedure have been developed. Radiofrequency, ultrasound, and alcohol-based devices have been developed [75-77] and tested in several randomized trials and analyzed in comprehensive meta-analysis studies [78-81]. This analysis documented the safety and finally also efficiency of radiofrequency and ultrasound-based RDN. In November 2024 Food and Drug Administration (FDA) approved them as adjunctive treatment to be applied when lifestyle changes and medication have failed to adequately control BP. This view has also been adopted in the hypertension guidelines of the European Society of Cardiology [23,24].

Nevertheless, several topics of interest remain and have to be clarified. For instance, a disadvantage of the energy-based RDN is that the efficiency is limited by the fine balance between the nerve ablation depth and the degree of renal artery wall injury. Thus, under some conditions the RDN does not have to be complete. This concern was also raised by two experimental studies in sheep performed lately in our laboratory, the study that evaluated the efficiency of RDN elicited by single-point and multi-point radiofrequency ablation devices. It was shown that if a safe energy delivery in accordance with manufacturer's instructions was done, both systems provided only incomplete renal nerve ablation in sheep [82,83]. Therefore, catheter-based, alcohol-mediated RDN employing perivascular alcohol infusion has been developed and the newest study confirmed the safety and high efficiency of this technique [77,80]. However, this RDN approach has not been so far approved by FDA as a standard therapeutical method and it is limited only to clinical trials. Nevertheless, the current topic of interest in RDN in human medicine is no longer safety and efficiency of employed techniques, but mainly durability of RDN: it is recognized that sympathetic nerve regeneration counteracts the effects of RDN [72]. Another concern is appropriate identification of responders: since RDN is an invasive therapeutical approach, the patients that would most profit should be selected by teams of specialists that can appropriately balance pro and cons of this therapy.

Translation perspectives

RDN is nowadays being promoted as a new therapeutic approach to the treatment of hypertension and cardiorenal diseases; it is even proposed that RDN could exhibit organ-protective effects unrelated to the effect exhibited on BP ("BP-independent organ-protective effects") [70,84]. It is thought that such BP-independent protection against end-organs damage could be particularly beneficial when renal injury is concomitant with cardiovascular disease. The information obtained in our recent [39] and the present preclinical studies should be considered before initiating comprehensive clinical studies, to moderate excessive hope that RDN may become a miraculous approach to the treatment of cardiorenal diseases.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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