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Original articles

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CARDIOVASCULAR EFFECTS OF THE COMBINATION OF LEVOSIMENDAN AND VALSARTAN IN HYPERTENSIVE DAHL/RAPP RATS

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Hypertension is the main risk factor for left ventricular hypertrophy and development of diastolic heart failure. There is no yet treatment, which can effectively reduce mortality in patients suffering from heart failure with preserved systolic function. We tested whether the calcium sensitizer levosimendan and the AT1-receptor antagonist valsartan could protect from salt-induced hypertension, cardiovascular mortality and heart failure in Dahl/Rapp salt-sensitive rats fed for 7 weeks with a high salt diet (8% NaCl). Levosimendan (1 mg/kg/day *via* drinking water) and valsartan (30 mg/kg in the food) monotherapies and their combination prevented mortality in Dahl/Rapp rats. The drug combination evoked an additive effect on blood pressure, cardiac hypertrophy, cardiomyocyte cross-sectional area, target organ damage and myocardial ANP mRNA expression. There was a close correlation between systolic blood pressure and cardiac hypertrophy, cardiac and renal damage. As compared to Dahl/Rapp controls kept on low-salt diet (NaCl 0.3%). The high salt rats exhibited impaired diastolic relaxation as assessed by isovolumic relaxation time. Levosimendan alone and in combination with valsartan, improved diastolic relaxation without significantly improving systolic function. Our findings are evidence for an additive effect between levosimendan and valsartan on blood pressure and a blood pressure-dependent protection against the development of salt-induced target organ damage. The present study also demonstrates that levosimendan, alone or in combination with valsartan, can correct diastolic dysfunction induced by salt-dependent hypertension.

Key words: *AT1 receptor antagonists, calcium sensitizers, heart failure, hypertension, hypertrophy*

INTRODUCTION

Diastolic heart failure appears initially as a result of left ventricular pressure overload, being followed by myocyte hypertrophy, fibrosis, cardiac dysfunction, and ultimately the transition into systolic heart failure (1, 2). Complications of heart failure are further aggravated by chronic renal failure which is one of the strongest risk factors for mortality in patients suffering from heart failure (3). Since it is not only common but also a lethal condition, patients with heart failure would benefit from novel therapies that would reduce both morbidity and mortality (4). Calcium sensitizers represent a new class of drugs that provide hemodynamic and symptomatic relief without significantly increasing cAMP and intracellular calcium concentrations (5). These properties are advantages in comparison to the classical inotropic agents such as β_1-adrenergic agonists and phosphodiesterase III (PDE III) inhibitors which act *via* mechanisms linked to elevated intracellular calcium levels, increased myocardial oxygen consumption and thus, to a risk of arrhythmias and cardiovascular mortality (6-7). Levosimendan, a calcium sensitizer, and its long-lasting active metabolite OR-1896, improve cardiac contractility by stabilizing the calcium-bound conformation of troponin C, allowing a prolonged interaction between actin and myosin, and hence an improvement in cardiac muscle force generation, without increasing oxygen requirements or elevating intracellular calcium concentrations (7, 8). It is also known to confer vasodilatating properties and better tissue perfusion due to opening sarcolemmal ATP-sensitive potassium channel (K^+_{ATP}), while opening of mitochondrial K^+_{ATP}-channels results in protection from cardiac myocyte apoptosis (9-11). Levosimendan has been also shown to inhibit PDE III in cardiac myocytes, however the clinical significance of this finding is uncertain (5).

It has been previously claimed that calcium sensitizers may impair diastolic function as they could impair diastolic relaxation by reducing the ventricular filling rate (5). Levosimendan, however, has been shown to enhance diastolic function and coronary blood flow in pre-clinical and clinical studies (12-14). Diastolic dysfunction is also encountered in infiltrative cardiomyopathies *e.g.* conditions where excessive lysosomal material accumulation gives rise to diastolic dysfunction which can eventually progress to overt systolic heart failure (15). The accumulation of lysosomal aggregates is seen in senescent cells losing their viability, but to the extent to which this is a result of an enhanced production of misfolded proteins or disrupted clearing mechanisms, remains unclear. In the healthy heart, autophagy, a major mechanism for clearing toxic proteins and organelles (16), serves as a homeostatic mechanism, maintaining cardiomyocyte size, structure and function (16-18).

Cardiomyocyte autophagy has been linked to cardiac remodeling in patients with terminal heart failure (16, 19). Induction of cardiomyocyte autophagy in left ventricular hypertrophy and in heart failure is thought to be an adaptive response to protect the cells from hemodynamic stress and accumulation of misfolded proteins (17, 19). Renin-angiotensin-aldosterone system (RAAS) plays a major role in the regulation of blood pressure and sodium homeostasis, vascular tone, aldosterone secretion and sympathetic activity (20). Ang II is also produced locally. Local RAAS in tissues such as kidney, heart, vasculature and brain amplifies the actions of circulating Ang II, and thus contributes to the pathophysiology of cardiovascular diseases (21). The detrimental actions of local RAAS in the heart include AT_1 receptor mediated inflammation, fibrosis, and cardiac remodeling, eventually leading to cardiac dysfunction (22). Valsartan is a highly selective, orally active, non-peptide angiotensin II type 1 (AT_1) receptor antagonist, which has no affinity for the angiotensin II AT_2 receptor (23, 24). Valsartan has been shown to be safe, effective, and tolerated in large-scale studies in hypertension, heart failure and post-myocardial infarction (25). Previous studies have revealed that valsartan prevents the transition from compensatory cardiac hypertrophy to heart failure in Dahl salt-sensitive rats fed a high salt diet *via* protein kinase C-mediated pathways (26). Levosimendan and valsartan, are used separately in the treatment of heart failure (22, 27). Levosimendan is commonly used in hospitalized patients for the treatment of acute decompensated heart failure, whereas AT_1 receptor blockers represent the "gold standard" for the treatment of essential hypertension and congestive heart failure (28, 29).

The present study was conducted with Dahl/Rapp salt-sensitive rats, a widely used animal model of severe hypertension, cardiac hypertrophy and diastolic heart failure (30, 31). Levosimendan, valsartan and their combination were tested to reveal any possible additional effects on lifespan, development of salt-dependent hypertension as well as their influence on cardiac function, hypertrophy and target organ damage.

MATERIALS AND METHODS

Experimental animals, blood pressure measurement and sample preparation

Sixty nine 7-week-old male Dahl/Rapp salt sensitive rats (SS/JrHsd) purchased from Harlan (Harlan, Indianapolis, Indiana, USA) were divided into 5 groups to receive the different dietary and drug regimens for 8 weeks: 1) Dahl/Rapp SS controls on high salt diet (NaCl 8%, n=20); 2) Dahl/Rapp SS rats on high salt diet+levosimendan (1 mg/kg, n=15); 3) Dahl/Rapp SS rats on high salt diet+valsartan (30 mg/kg, n=12); 4) Dahl/Rapp SS rats on high salt diet+levosimendan+valsartan (n=12); and 5) Dahl/Rapp SS rats on low salt diet (NaCl 0.3%, n=10). The development and characteristics of the strain of Dahl/Rapp salt-sensitive rats have been described in detail elsewhere (31, 32). The rats were housed four to five to a cage in a standard experimental animal laboratory (illuminated from 7.00 a.m. to 7.00 p.m., temperature 22±2°C, humidity 55±15%). The protocols were approved by the Animal Experimentation Committee of the University of Helsinki, Finland, and the Provincial State Office of Southern Finland (approval number STU 1187 A), whose approval criteria correspond to those of the American Physiological Society. The levosimendan and valsartan dosages used in the present study were chosen based on our previous studies (21, 33). A high salt diet was produced by adding NaCl (Riedel-de Haen AG, Seelze, Germany) to commercial low salt diet (Na 0.3%, K 0.8%, Mg 0.2%; Harlan, Indianapolis, Indiana, USA). Levosimendan (Orion Pharma, Espoo, Finland) was given *via* the drinking fluid at the concentration of 3 mg/L^{-1} using daily-prepared water solutions to produce an approximate daily dosage of 1 mg/kg^{-1}, and valsartan (Orion Pharma, Espoo, Finland) was mixed with the food at the concentration of 350 mg/kg to produce an approximate daily dosage of 30 mg/kg^{-1}. The average food and water consumptions were recorded at cage level on daily basis. Rats had free access to chow and drinking water.

Systolic blood pressure was measured from pretrained rats every second week (post-natal week 7, 9, 11, 13) using a tail cuff blood pressure analyzer (Apollo-2AB Blood Pressure Analyzer, Model 179-2AB, IITC Life Science, Woodland Hills, CA, USA). At the age of 15 weeks, rats were anesthetized with CO2/O2 (AGA, Riihimaki, Finland), decapitated, and terminal blood samples were collected. The hearts and kidneys were excised, washed with ice-cold saline, blotted dry and weighed. Tissue samples were snap-frozen in liquid nitrogen and stored at -80°C until assayed. Samples for histology were fixed in 10% formalin and processed to paraffin with routine methodology.

Echocardiography

Transthoracic echocardiography (Toshiba Ultrasound, Tokyo, Japan) was performed on all rats under isoflurane anesthesia (AGA, Riihimaki, Finland) at week 14 and 15 in a blinded fashion by the same technician during the last study week as described previously (34). Parameters needed for the calculation of cardiac function and cardiac dimensions were measured from three systole-diastole cycles. A short-axis view of the left ventricle at the level of the papillary muscles was obtained by a two-dimensional imaging method (Gibson method), using a 15- MHz linear transducer. Two-dimensionally guided M-mode recording through the anterior and posterior walls of the left ventricle was used to measure the left ventricle (LV) end-systolic (LVESD), and end-diastolic (LVEDD) dimensions. Interventricular septum (IVS) and posterior wall (PW) thickness were also measured. Fractional shortening (FS) and ejection fraction (EF) were calculated from the M-mode LV dimensions using the following equations:

FS (%)={(LVEDD-LVESD)/LVEDD} x 100
EF=SV/EDV
SV=EDV–ESV
EDV=0.52x(0.98x(LVIDD/10)+5.90)x(LVIDD/10)2
ESV=0.52x(1.14x(LVIDS/10)+4.18)x(LVIDS/10)2
LVEDD=diameter of the short-axis left ventricle in end diastole
LVEDS=diameter of the short-axis left ventricle in end systole
SV=stroke volume
EDV=end diastolic volume

We assessed diastolic dysfunction by measuring the isovolumic relaxation time (IVRT) using color Doppler imaging. IVRT was measured as the interval between the aortic closure click and the start of mitral flow.

Tissue morphology and cardiomyocyte cross- sectional area

Tissue morphology was evaluated from hematoxylin and eosin (H&E) stained cardiac and renal sections in a blinded fashion. The severity of observed lesions was graded with numerical values denoting to the degree of damage at the whole tissue level. The following system of severity grading was used to evaluate coronary and myocardial damage, as well as kidney arterial, glomerular and tubular damage: 0- no abnormalities detected; 1- minimal; 2- mild; 3- moderate; 4- marked; or 5- severe (35). Conventional light microscopy at x400 magnification was used to determine cardiomyocyte cross-sectional area. Fifteen to 17 random fields were studied, and in

each field, the cell borders were measured from myocytes cut in the short axis with a visible nucleus. An average of 40 cardiomyocytes per animal was studied from each animal in the group. The cross-sectional area was evaluated and analyzed using ISIimaging software (Image Solutions Inc., Whippany, New Jersey, USA) (3).

Cardiac mRNA expression analysis by quantative real-time RT-PCR

Quantititative real-time RT-PCR was performed using the LightCycler® instrument (Roche diagnostics, Neuilly sur Seine, France) for detection of atrial natriuretic peptide (ANP) and ribosomal 18S mRNA as described elsewhere (36, 37). Briefly, total RNA from the rat hearts was collected with Trizol® (Gibco, Invitrogen, Carlsbad, CA, USA), treated with DNAse 1 (Deoxyribonuclease 1, Sigma Chemicals Co., St Louis, MO, USA) and reverse transcribed to cDNA by reverse transcription enzyme (Im-Prom-II reverse transcription system, Promega, Madison, WI, USA). One µl of cDNA was subjected to quantitative real time polymerase chain reaction for detection of ANP, and ribosomal 18S mRNA. The following primers were used: ANP forward CCGATAGATTCTGCCCTCTTGAA, reverse CCCGAAGCAGCTTGATCTTC; 18S forward CATCCAAGGAAGGCAGCAG, reverse TTTTCGTCACTACC TCCCCG. The samples were amplified using FastStart DNA Master SYBR Green 1 (Roche diagnostics) according to the protocol of the manufacturer. The quantities of the PCR products were quantified with an external standard curve amplified from purified PCR product.

Western blotting

Cardiac samples from the left ventricle were electrophoretically separated by 8% SDS-PAGE for binding immunoglobulin protein (BiP) protein and 14% SDS-PAGE for Light chain 3 isoform B (LC3B) (24 µg and 60 µg total protein respectively of the whole cell lysate per lane). Each lane corresponded to one rat and all 5 groups were run on one gel. Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Immobilon-P®, Millipore, Bedford, MA, USA) and blocked in 5% non-fat milk-TBS-0.01% Tween-20® buffer. The membranes were probed with the following primary antibodies; anti-LC3B, 1/500 (Cell Signalling Technology); anti-BiP, 1/1000 (Cell Signalling Technology); tubulin was used as the loading control (Antialpha tubulin, 1/3000; Abcam). Horseradish peroxidase-conjugated anti-rabbit secondary antibody (Chemicon, Temecula, CA, USA) was subjected to enhanced chemiluminescence solution (ECLplus, Amersham Biosciences, Buckinghamshire, UK). The relative protein expressions in separate samples from the membranes were quantified with Fluorescent Image Analyzer (FUJIFILM Corp, Tokyo, Japan). Samples were measured as triplicates.

Biochemical determinations

Serum creatinine and electrolytes and liver enzymes were measured by routine laboratory techniques. Plasma samples were analyzed for levosimendan and OR-1896 concentration by liquid chromatography-tandem mass spectrometry (38).

Statistical analysis

Data are presented as the mean±S.E.M. Statistically significant differences in mean values were tested by analysis of variance (ANOVA) and the Newman-Keul's post-hoc test. The differences were considered significant when $P<0.05$. The Kaplan-Meier test was used for survival analysis. The Pearson correlation coefficients were calculated to measure the correlation between two variables. Linear regression curves were obtained by the partial least squares method.

RESULTS

Survival rate in Dahl/Rapp rats

Cardiovascular mortality of untreated Dahl/Rapp salt-sensitive rats on a high-salt diet at postnatal week 13 was 30% (6/20) (*Fig. 1a*). All rats from valsartan, levosimendan, and the combination group as well as from low-salt group survived the whole follow-up period. There was one death in the levosimendan treated Dahl/Rapp rats receiving the high-salt diet (*Fig. 1a*).

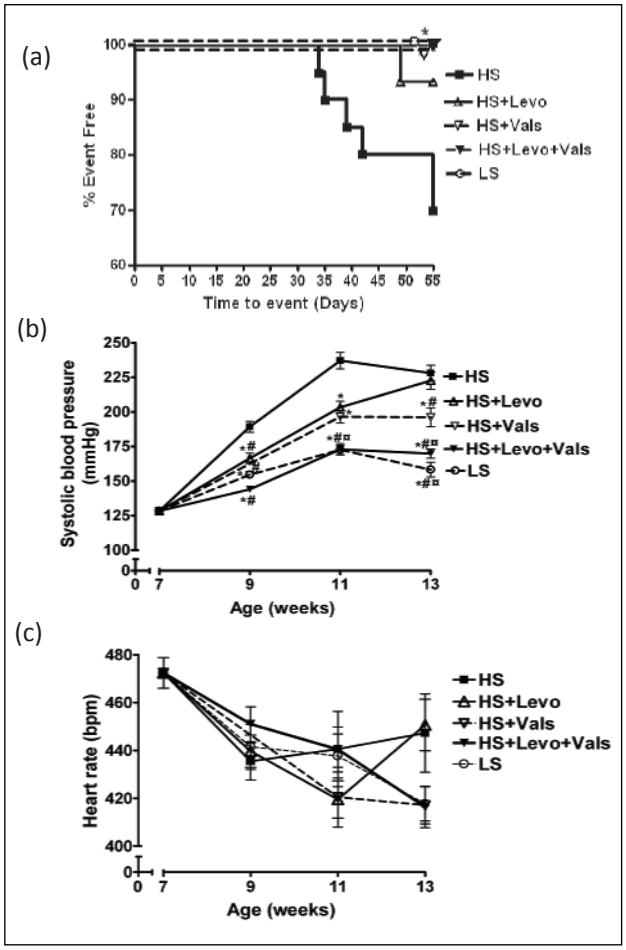

Fig. 1. Bar graphs showing the effects of levosimendan, valsartan and the drug combination on (a) survival curve during the 7-week experimental period of Dahl/Rapp rats on a high-salt diet, and their low-salt Dahl/Rapp rats normotensive controls; (b) systolic blood pressure; (c) heart rate. The log rank test was used to compare the Kaplan-Meier survival curves to each other. The concentration of levosimendan in drinking water (given *ad libitum*) was 2 mg/L corresponding to 1 mg/kg. HS denotes Dahl salt-sensitive rats on high salt diet; HS+Levo–Dahl salt sensitive rats on high salt diet treated with levosimendan; HS+Vals–Dahl salt-sensitive rats on high salt diet treated with valsartan; LS–Dahl salt-sensitive rats on low salt diet. Means±S.E.M. are given, n=10-20 in each group. *P<0.05 compared to HS; #P<0.05 compared to HS+Levo; ¤P<0.05 compared to HS+Vals; §P<0.05 compared to HS+Levo+Vals.

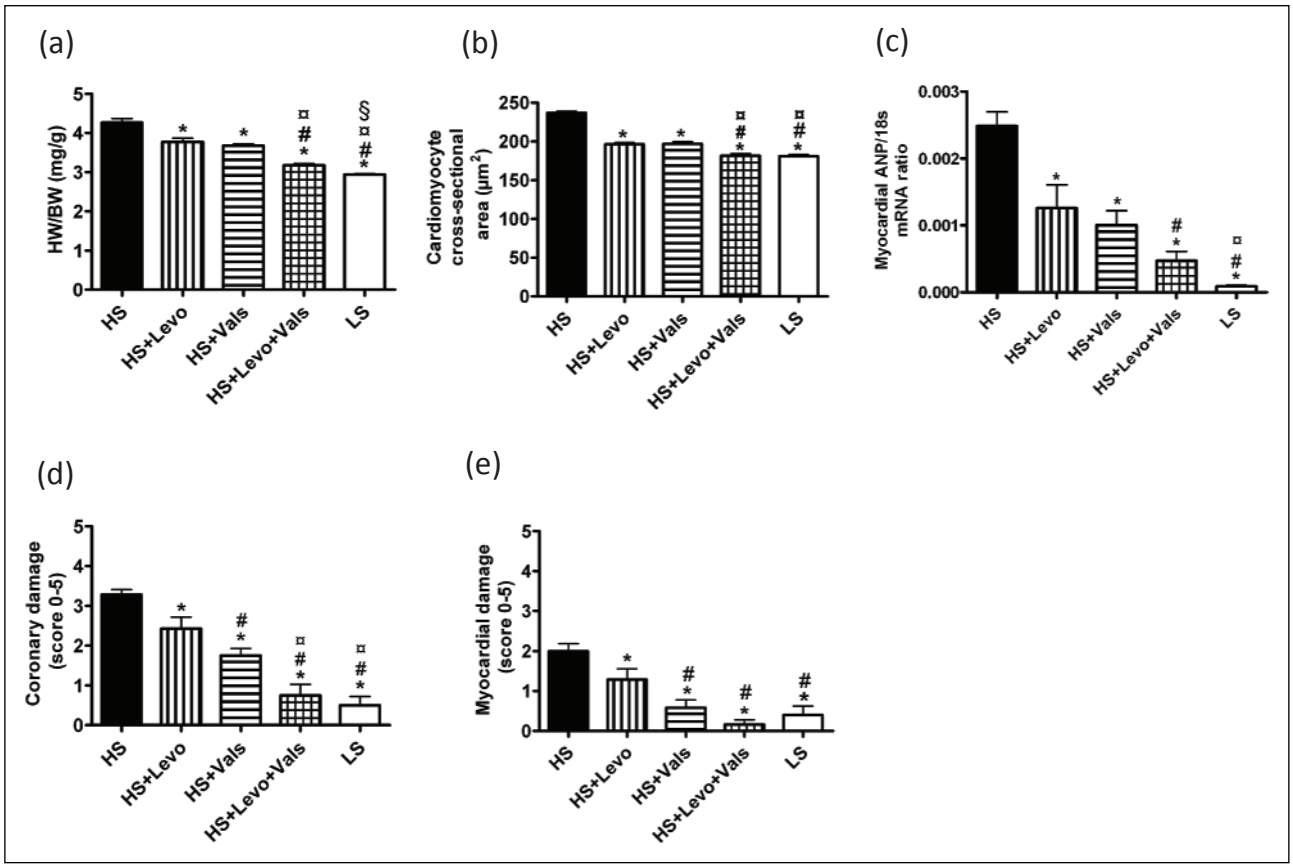

Fig. 2. Bar graphs showing the effects of levosimendan, valsartan and the drug combination on cardiac hypertrophy calculated as (a) heart weight to body weight ratio (HW/BW) and (b) cardiomyocyte cross-sectional areas; (c) mRNA ANP expression; (d) coronary damage; (e) myocardial damage.

Blood pressure, heart rate, cardiac hypertrophy and body weight

Systolic blood pressure in untreated Dahl Rapp rats on high-salt diet increased progressively, and leveled 228±6 mmHg at week 13 (*Fig. 1b*). Levosimendan alone did not decrease systolic blood pressure, whereas valsartan reduced it moderately (*Fig. 1b*). Levosimendan combined with valsartan significantly reduced the development of hypertension so that it achieved the level found in LS Dahl rats. At week 13, heart rate was not affected by treatments over the 7- week follow-up period (*Fig. 1c*).

At postnatal week 13 Dahl Rapp rats on high-salt diet exhibited significant decrease in body weight when compared to LS Dahl rats (289.1±9.817 g *vs*. 395.3±5.333 g). Both, levosimendan and valsartan treated HS Dahl rats weighed significantly more than untreated HS (350.9±7.222 g *vs*. 366.5±3.263 g) and the combination group body weight was comparable to the level of LS Dahl rats (380.5±5.263 g).

Dahl Rapp rats on high-salt diet demonstrated pronounced cardiac hypertrophy expressed as heart weight-to-body weight ratio (*Fig. 2a*) and cardiomyocyte cross sectional area (*Fig. 2b*). There was a close correlation between systolic blood pressure and cardiac hypertrophy (*Fig. 7a*). The combination of levosimendan and valsartan prevented cardiac hypertrophy more effectively than the drugs administered as monotherapies (*Fig. 2a* and *2b*).

Cardiac function and cardiac dimensions

Ejection fraction (EF) and fractional shortening (FS), two indicators of systolic function which are calculated from the parameters given in *Table 1*, were not statistically influenced by high salt intake (*Fig. 4a* and *4b*). While valsartan decreased EF and FS variables, treatment with levosimendan or the combination did not significantly increase cardiac function at systole (*Fig. 4a* and *4b*). Diastolic function as assessed as isovolumic relaxation time (IVRT) was prolonged in the high-salt group indicating diastolic dysfunction. Both levosimendan and the combination of the drugs significantly shortened IVRT comparably to the situation in low-salt rats. Valsartan did not correct diastolic dysfunction (*Fig. 4c*). The early diastolic velocity to late diastolic velocity (E/A ratio) remained unchanged (*Fig. 4d*). All drug treatments ameliorated cardiac hypertrophy measured as interventricular septum thickness and posterior wall thickness (*Table 1*). None of the drug treatments influenced heart rate (*Table 1*).

Tissue morphology and cardiac ANP mRNA expression

The severity of cardiac damage was graded as coronary artery and myocardial damage. The observed lesions in the coronary arteries ranged from minimally thickened media and a slight increase of connective tissue around the arteries up to severe hyperplasia of intimal/medial layer and necrosis of the arterial wall with perivascular inflammatory cell infiltration (*Fig. 3*). The lesions in the myocardium ranged from a focal increase of slender connective tissuebundles to necrotic foci in the myocardium with inflammation (*Fig. 3*). Valsartan provided superior cardioprotection as compared to levosimendan (*Fig. 2d-2e*). The combination therapy provided an additive effect.

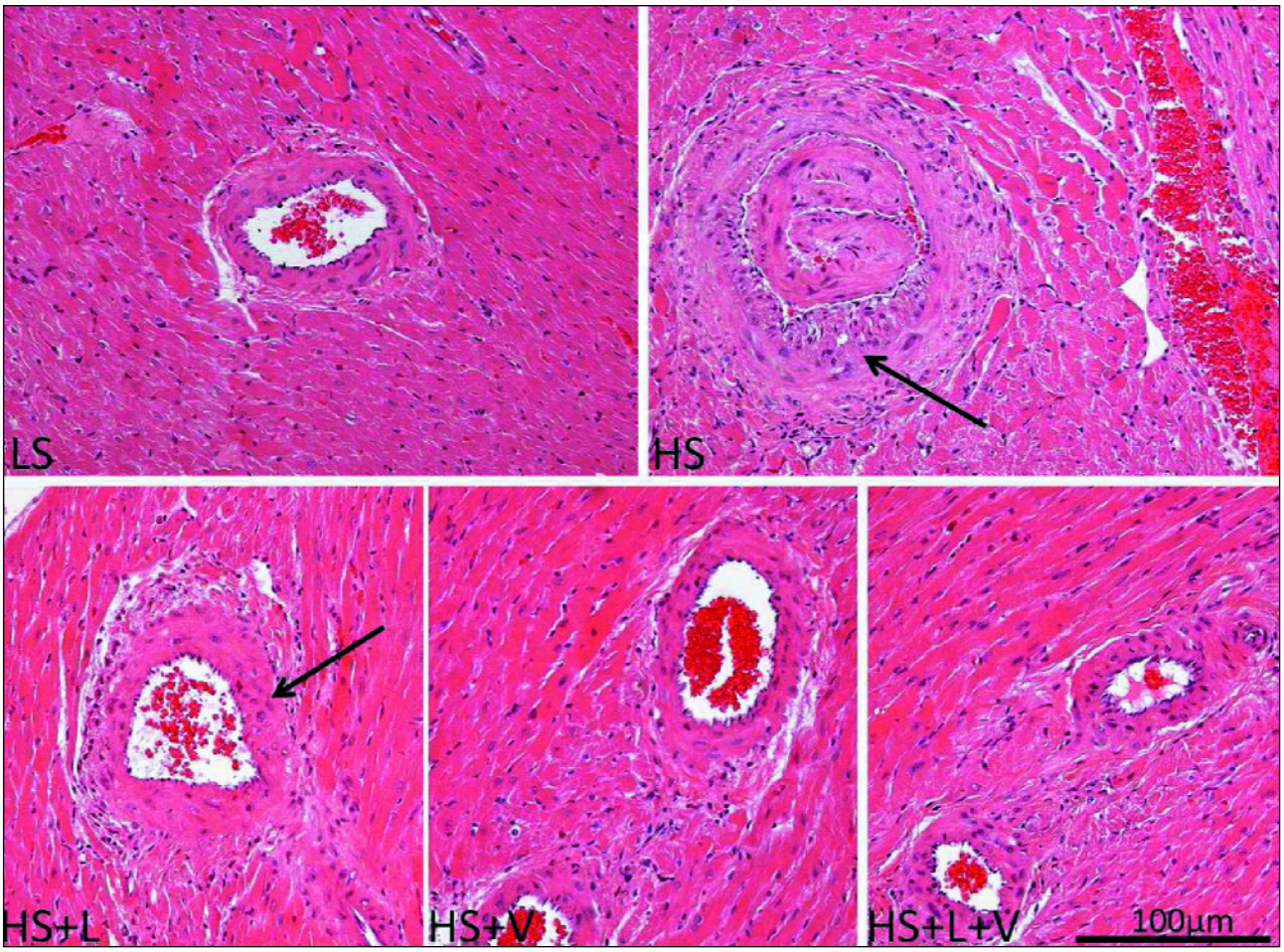

Fig. 3. Representative photomicrographs of cardiac morphology from Dahl/Rapp rats on high salt and low salt diet. High salt diet induced thickening of the arterial medial layers (arrows), which was variably prevented by different drug regimens (H&E staining of 4 µm thick paraffin embedded sections. Scale bar 100 µm).HS denotes Dahl salt-sensitive rats on high salt diet; HS+L–Dahl salt sensitive rats on high salt diet treated with levosimendan; HS+V–Dahl salt-sensitive rats on high salt diet treated with valsartan; HS+L+V–Dahl salt-sensitive rats on high salt diet treated with levosimendan and valsartan; LS–Dahl salt-sensitive rats on low salt diet.

Table 1. Effects of 7-week-drug (levosimendan and/or valsartan) treatment on cardiac function measured by echocardiography in HS and LS Dahl/Rapp rats. Means±S.E.M. are given, n=10-15 in each group. * denotes p<0.05 *vs*. HS; # denotes p<0.05 vs HS+Levo; ¤ denotes p<0.05 *vs*. HS+Vals; § denotes p<0.05 *vs*. HS+Levo+Vals.

Variable	HS (n=15)	HS+Levo (n=14-15)	HS+Vals (n=12)	HS+Levo+Vals (n=11-12)	LS (n=10)	ANOVA (P-value)
LVESD, mm	4.04±0.23	4.31±0.14	5.29±0.18*#§	4.56±0.20	5.05±0.27*#	P<0.0003
LVEDD, mm	7.09±0.23	8.00±0.11*	8.27±0.11*	8.00±0.17*	8.11±0.21*	P<0.0001
IVS (d), mm	2.39±0.09	2.4±0.6*	2.11±0.04*	1.92±0.06*	1.79±0.06*#¤	P<0.0001
PW (d), mm	2.61±0.1	2.35±0.06*	2.36±0.05*	2.17±0.06*	1.94±0.06*#¤§	P<0.0001
EDV, ml	0.83±0.06	1.3±0.04*	1.23±0.05*	1.13±0.06*	1.18±0.08*	P<0.0001
ESV, ml	0.19±0.03	0.21±0.02	0.37±0.04*#§	0.25±0.03	0.33±0.06*#	p= 0.0002
HR, bpm	241.3±6.47	256.3±6.07	239.5±4.74	242.5±5.61	290.6±7.15*#¤§	P<0.0001

The renal samples were graded according to the lesions observed in the arteries, glomerules and tubules (*Fig. 6*). The lesions manifested as variable degrees of arterial and glomerular necrosis, tubular atrophy and dilatation containing proteinaceous casts with diffuse inflammatory infiltrate affecting the parenchyma. The renoprotective effect of valsartan was greater compared to levosimendan (*Fig. 6a-c*). An additive renoprotective effect was found by the drug combination. Both cardiac and renal damage correlated very closely with systolic blood pressure (*Fig. 7d-f*). Representative photomicrographs of cardiac and renal morphology are given in *Fig. 3* and *Fig. 6*).

Myocardial ANP mRNA expression closely correlated with systolic blood pressure (r=0.71; p<0.05) and was increased by 25-fold by consumption of a high-salt diet (*Fig. 2c*). Levosimendan, valsartan and in particular, the drugs combination decreased cardiac ANP mRNA expression (*Fig. 2c*).

Table 2. Effects of 7-week- drug (levosimendan and/or valsartan) treatment on serum electrolytes and liver enzymes in HS and LS Dahl/Rapp rats. Means±S.E.M. are given, n=9-14 in each group. ALT denotes alanine aminotransferase, AST aspartate aminotransferase, and ALP alkaline phosphatase, Iphos inorganic phosphorus. * denotes p<0.05 *vs*. HS, § denotes P<0.05 *vs*. HS+Levo+Vals.

Variable	HS (n=14)	HS+Levo (n=14)	HS+Vals (n=11)	HS+Levo+Vals (n=12)	LS (n=9)	ANOVA (P-value)
S-K (mmol/l)	6.89±0.23	6.321±0.3	6.155±0.24	6.07±0.26	6.27±0.14	p=0.13
S-Na (mmol/l)	149.8±0.78	145.5±1.5*	148.2±1.21	148.9±0.97	144.0±1.03*§	p=0.005
S-Cl (mmol/l)	113.5±1.17	102.7±1.32*	105.4±0.96*	105.9±0.83*	102.3±0.9*	P<0.0001
S-Creatinine (µmol/l)	52.97±1.7	49.72±1.99	49.24±1.23	48.17±1.45	50.57±0.83	p=0.26
s-ALT (U/l)	67.21±5.66	71.07±4.9	69.1±4.16	84.5±9.19	58.89±10.99	p=0.18
s-AST (IU/l)	283.2±23.59	225.6±28.57	265.1±27.12	280.8±31.61	321.1±80.0	p=0.52
s-ALP (U/l)	102.1±7.42	110.3±4.33	112.4±3.61	120.3±3.72	104.3±2.14	p=0.10

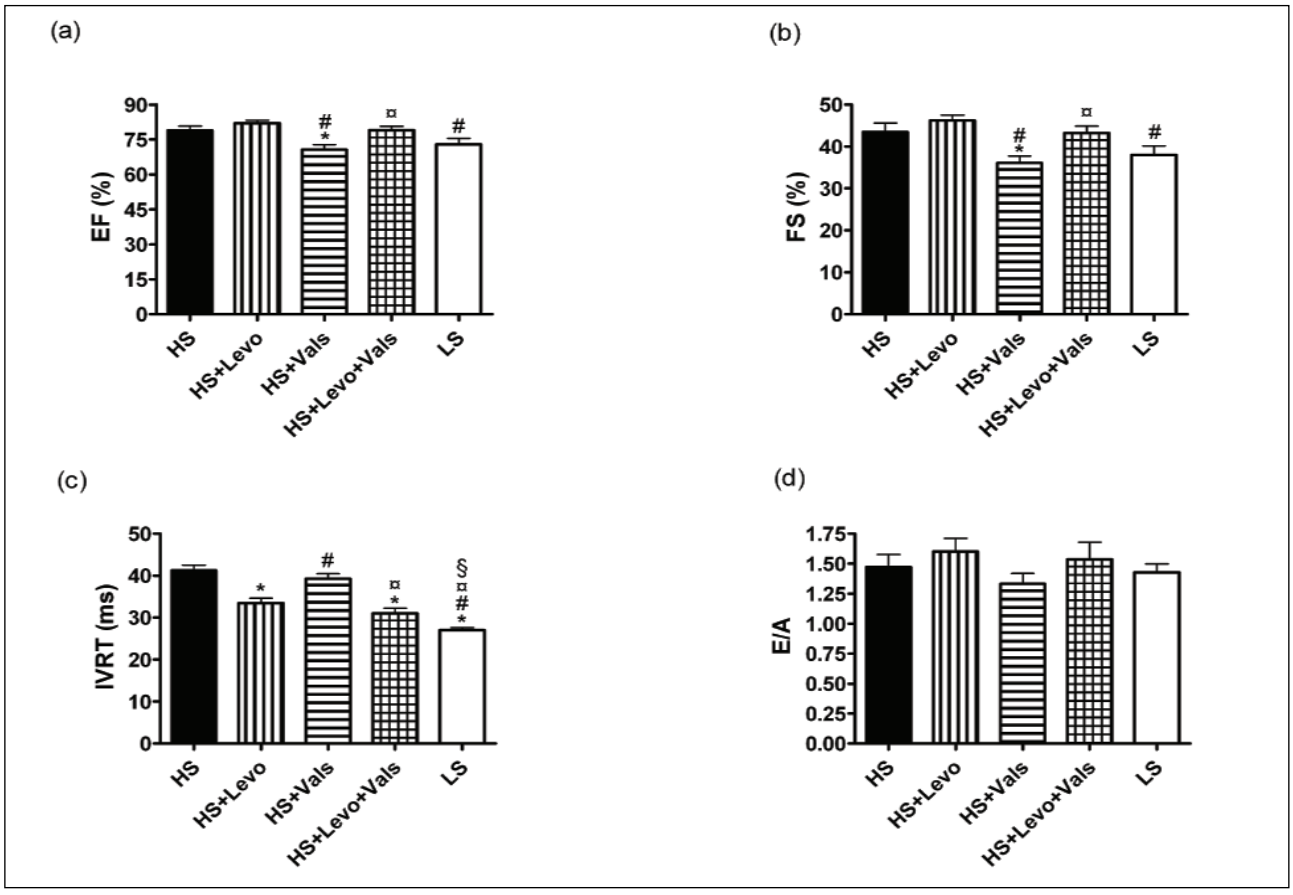

Fig. 4. Bar graphs showing the effects of levosimendan, valsartan and the drug combination on heart functions (a) ejection fraction, EF; (b) fractional shortening (FS); (c) isovolumetric relaxation time (IVRT) and (d) passive filling of the ventricle-active filling with atrial systole ratio (E/A). For abbreviations see *Fig. 1*. Means±S.E.M. are given, n=10-15 in each group. *P<0.05 compared to HS; #P<0.05 compared to HS+Levo; ¤P<0.05 compared to HS+Vals; §P<0.05 compared to HS+Levo+Vals.

Cardiomyocyte autophagy and endoplasmic reticulum stress

High salt diet did not induce cardiomyocyte autophagy measured as LC3B or LC3B 14 kDa/16 kDa ratio (*Fig. 8a-b*). Neither levosimendan nor valsartan treatment affected cardiomyocyte autophagy, whereas the drug combination tended to increase cardiac autophagy (*Fig. 8a-b*). The diet and drug regimens did not influence cardiac BiP expression (*Fig. 8c*).

Serum electrolytes, creatinine and liver enzymes

There was no difference between the treatment groups and in serum levels of potassium, creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST), or alkaline phosphatase (AFOS). Serum sodium and chloride concentrations were increased by consumption of the high salt diet (*Table 2*).

Levosimendan dosage and fluid intake

The average daily dose of levosimendan was 0.69±0.03mg/kg (range 0.31-0.72 mg/kg) and in the drug combination group it was 0.52±0.03 mg/kg (range 0.3-0.57 mg/kg). The terminal plasma concentrations of levosimendan and its stable metabolite OR-1896 were slightly lower in the drug combination group (6.91±1.42 ng/ml and 4.15±0.38

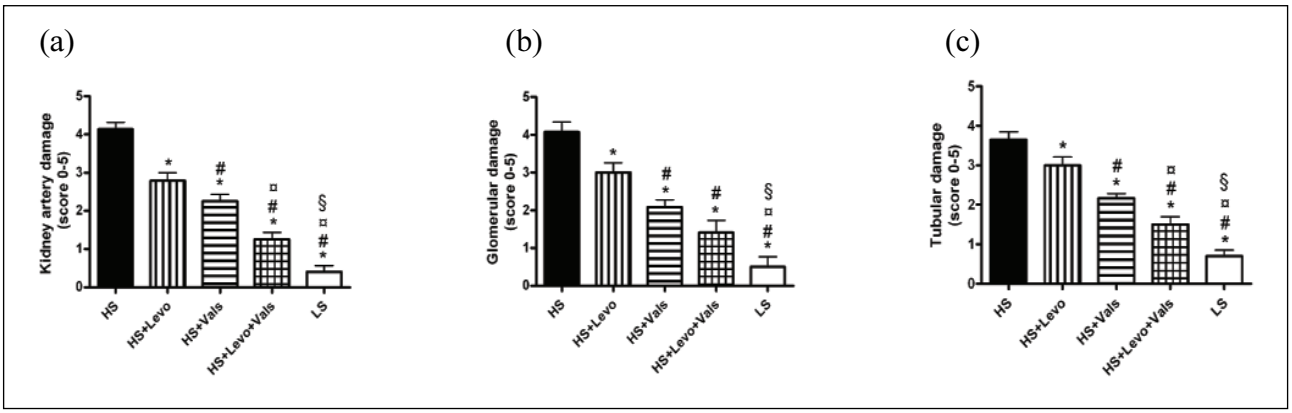

Fig. 5. Bar graphs showing the effects of levosimendan, valsartan, and the drug combination on (a) kidney artery damage; (b) glomerular damage and (c) tubular damage.

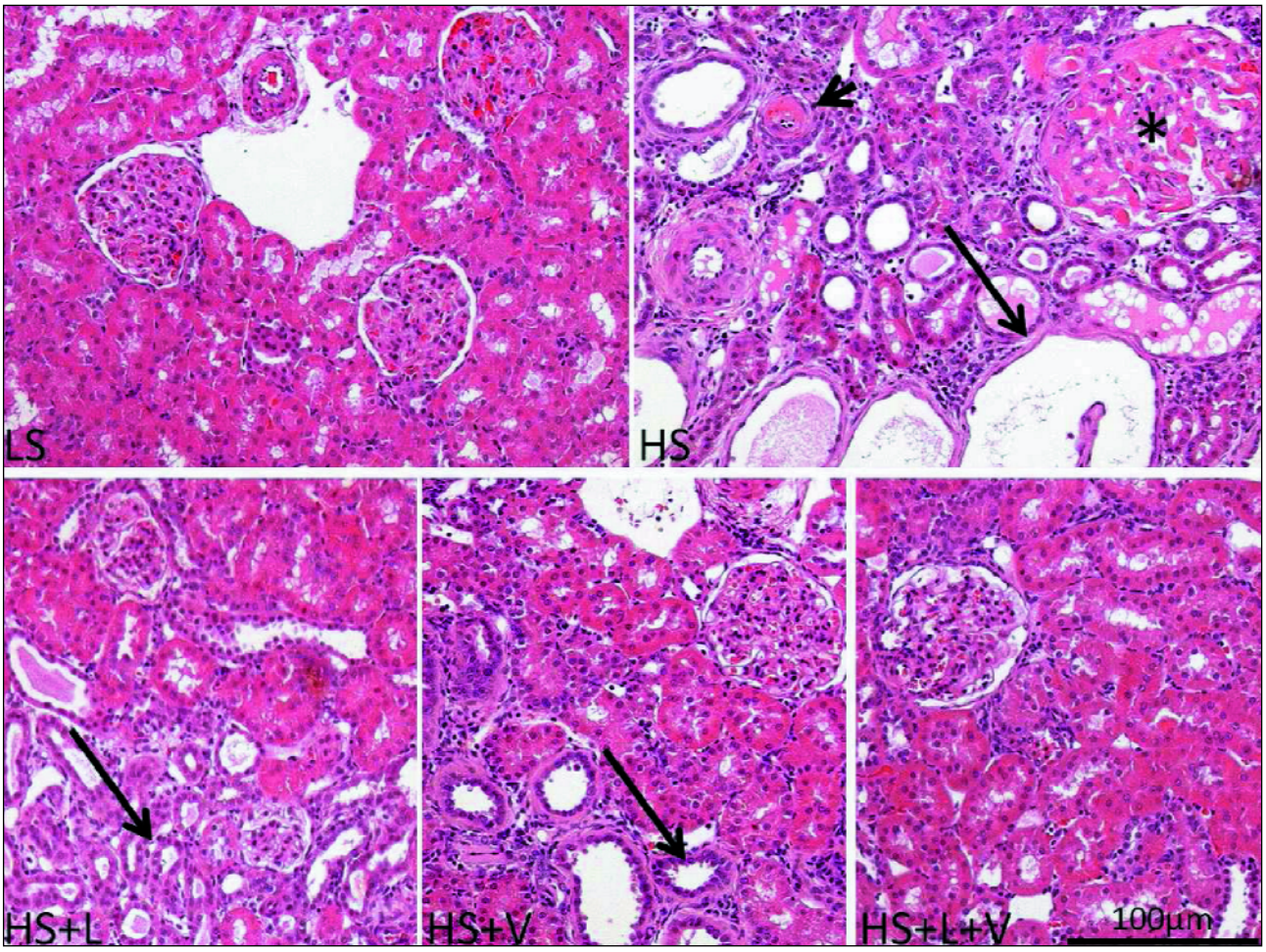

Fig. 6. Representative photomicrographs of renal morphology from Dahl/Rapp rats on high salt and low salt diet. High salt diet induced arterial thickening and necrosis (arrowhead), tubular atrophy/regeneration (arrows) and glomerular necrosis (asterisk). The lesions were variably prevented by different drug regimens (H&E staining of 4 µm thick paraffin embedded sections. Scale bar 100 µm). For abbreviations see *Fig. 3*.

ng/ml, respectively) as compared to levosimendan monotherapy (16.65±3.25 ng/ml and 17.36±1.73 ng/ml, respectively).

Levosimendan did not influence daily fluid intake in Dahl/Rapp rats kept on high salt diet (24.6±2.9 ml/100g body weight in levosimendan group *vs*. 22.9±2.0 ml/100g body weight in controls). Valsartan alone (21.7±1.3 ml/100g), and in combination with levosimendan (19.4±1.0 ml/100g), modestly decreased the average daily fluid intake ($p<0.05$) in Dahl/Rapp rats on high salt diet. Dahl/Rapp rats fed with LS diet had the lowest daily fluid intake (5.7±0.9 ml/100g) ($p<0.05$ compared to all groups).

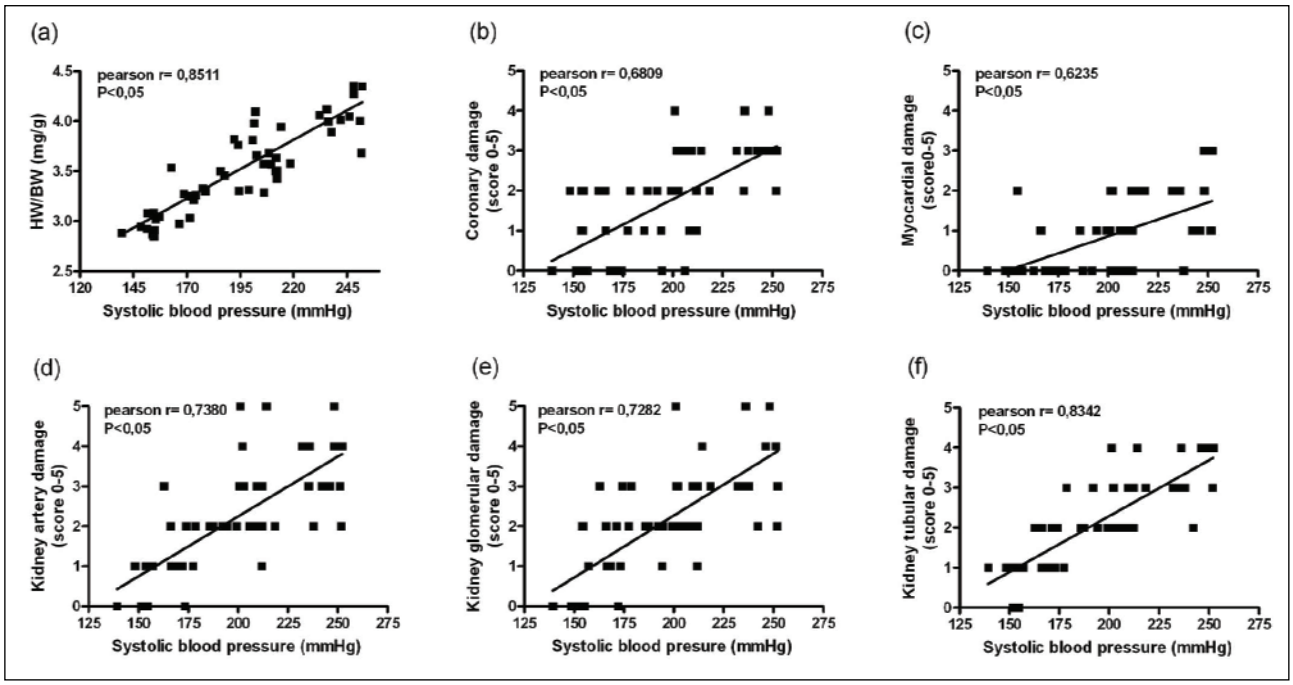

Fig. 7. Bar graphs showing systolic blood pressure dependency of (a) HW/BW ratio; (b) coronary damage; (c) myocardial damage; (d) kidney artery damage; (e) kidney glomerular damage; (f) kidney tubular damage. For abbreviations see *Fig. 1*. Pearsson correlation coefficients and statistical significances are given.

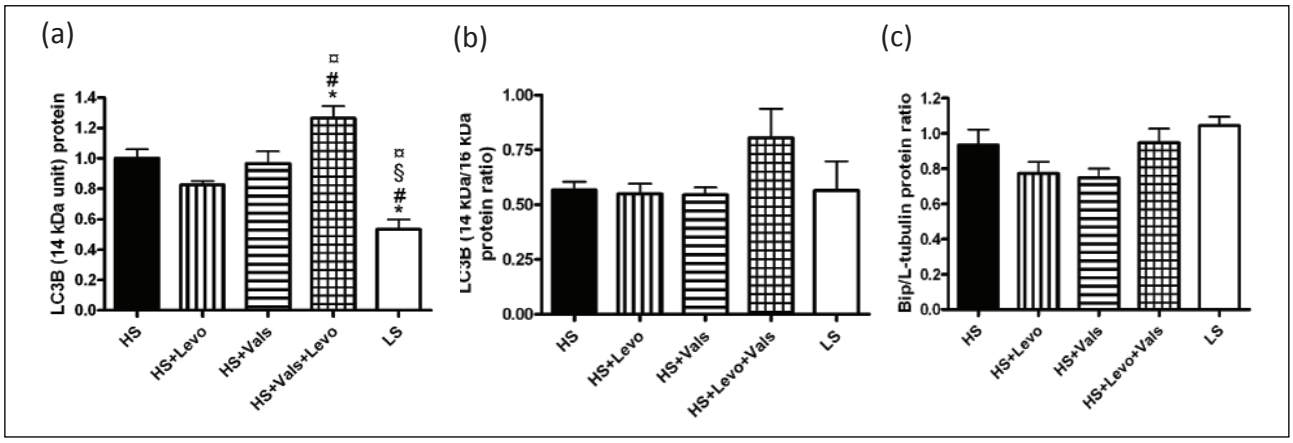

Fig. 8. Bar graphs showing the effects of levosimendan, valsartan, and the drug combination on cardiac autophagy measured as (a) LC3B (14 kDa unit) protein expression; (b) LC3B 14 kDa/16 kDa protein ratio; and on ER stress measured as (c) BiP/L-tubulin protein ratio. Each lane corresponded to a specimen from one rat, and all 5 of the groups were run on 1 gel. For abbreviations see *Fig. 1*. Means±S.E.M. from triplicate runs are given, n=6-8 in each group. *P<0.05 compared to HS; #P<0.05 compared to HS+Levo; ¤P<0.05 compared to HS+Vals; §P<0.05 compared to HS+Levo+Vals.

DISCUSSION

To date there is no satisfactory hypertensive heart failure treatment that can effectively reduce morbidity and mortality. In the present study, Dahl/Rapp salt sensitive rats, which develop severe hypertension and end organ damage when kept on high salt diet, were used to test the cardioprotective effects of the combination of a Ca^{2+} sensitizer and an AT_1 receptor antagonist. We found a very close correlation between blood pressure as well as cardiac hypertrophy and organ damage, indicating that the beneficial effects of the drug combination were largely due to their pronounced anti-hypertensive actions.

High systolic blood pressure is a major risk factor for cardiovascular diseases, such as left ventricular hypertrophy, myocardial infarction, stroke, congestive heart failure, and nephrosclerosis, and the correlation between mortality and high blood pressure is generally recognized (39). In the present study, levosimendan and valsartan displayed additive effects on blood pressure and against hypertension- induced target organ damage during high salt intake. We found a very close correlation between blood pressure as well as cardiac hypertrophy and organ damage, indicating that the beneficial effects of the drug combination were largely due to their pronounced anti-hypertensive actions.

Chronic renal dysfunction, including hypertensive nephrosclerosis, is one of the strongest risk factors for mortality in patients with heart failure. In fact, the worsening of renal function can be considered as a prognostic marker in patients

with heart failure (3). The renal damage in salt-sensitive Dahl/Rapp rats exhibited the features typical for hypertensive nephrosclerosis and was strongly dependent on blood pressure. Previous studies have demonstrated that renal damage in Dahl rats involves oxidative stress, inflammation and glomerular podocyte injury mediated by Ang II and aldosterone (40, 41). In the present study, the salt-induced renal damage was markedly attenuated by monotherapies and an additive effect was seen in the combination group. While the Dahl/Rapp salt-sensitive rat on the high salt diet has traditionally been considered as a low renin, volume- expanded model due to suppression of circulating RAAS by high salt intake (42, 43), later experiments have demonstrated activation of local RAAS in the heart, kidney and brain (43-47). It is therefore likely that the beneficial effects of valsartan on salt-induced hypertension and target organ damage are due to, at least in part to blockade of local RAAS. Although the protection against conferred the histopathological renal damage with levosimendan was significant in this study, it was less efficient than that obtained with valsartan. Previous studies have revealed that levosimendan can improve renal function (48). The mechanisms underlying the renoprotective effects of levosimendan have been shown to include an improvement in general hemodynamics, increased blood flow to renal medulla, decreased renal medullary/cortical vascular resistance due to K^{+}_{ATP} channel opening, anti-inflammatory properties, and a reversal of Ang II-mediated mesangial cell contraction (48). This is believed to lead to an improvement of the podocyte function, which is compromised in the Dahl/Rapp rats (49, 50). Moreover aldosterone antagonism has been shown to prevent the glomerular injury blood pressure-dependently in Dahl salt-sensitive rats. We have reported recently that although the high salt intake did not influence serum aldosterone level, levosimendan and its active metabolite, OR-1896, could reduce the serum aldosterone level by 50% thus providing further evidence for a renoprotective role of levosimendan (32, 51). Concomitantly we have noted a fourfold increase of plasma renin activity in levosimendan - treated Dahl/Rapp rats (32, 51). This could be linked to the possible diuretic effect of levosimendan encountered also in clinical settings (48) thus providing a possible explanation for the additive protective effect of the combination therapy in the current study.

In the present study, Dahl/Rapp rats on high sodium diet expressed cardiac hypertrophy and histological cardiac damage as evidenced by increased cardiac weight to body weight, cardiomyocyte cross- sectional area and elevated cardiac damage scores. Diastolic function was also compromised as reflected in the in the prolonged cardiac isovolumic relaxation time (IVRT). Cardiac hypertrophy and histological damage were partially prevented by monotherapies. However, combination therapy, however, conferred almost complete protection against the detrimental cardiac changes. Dahl rats fed a high salt diet have previously been shown to develop diastolic dysfunction which is partially preventable with valsartan in a blood-pressure dependent manner (52). We demonstrate here for the first time that only oral levosimendan as monotherapy and in combination but not valsartan alone could correct the diastolic dysfunction encountered in Dahl/Rapp rats. Our finding is in line with a previous clinical study showing that levosimendan could improve diastolic function and shorten IVRT in patients with acutely decompensated heart failure (53, 54). It is noteworthy that the correction of IVRT found in the present study was mediated largely *via* blood pressure-dependent mechanisms. Levosimendan has been shown to increase cardiac sarco/endoplasmic reticulum Ca^{2+}-ATPase 2a (SERCA2) expression in Dahl/Rapp rats (32). Levosimendan has also been shown to increase coronary blood flow in the absence of changes in cardiac function and exert cardiac anti-stunning effects (55). Furthermore, levosimendan induces vasodilation through opening of the ATP-sensitive potassium channels suggesting that the beneficial effects of levosimendan on diastolic dysfunction are largely due to decreased afterload. However, it should also be underlined that the principal mechanism of action mediating levosimendan-induced inotropic effect is calcium sensitization; Levosimendan binds to calcium-saturated N-terminal domain of troponin C and stabilizes the troponin molecule with subsequent prolongation of its effect on the contractile proteins (56). Furthermore, it has been shown that levosimendan did not impair the relaxation of cardiac muscle, but may actually even enhance diastolic function (14). Therefore it is likely that the beneficial effects of levosimendan on diastolic dysfunction found in the present study are mediated both *via* levosimendan's direct action on cardiac excitation-contraction coupling and *via* vasodilatation-induced decrease in afterload. However, it should be emphasized that as the combination therapy provided the most efficient cardioprotection, the relationship between blood pressure and diastolic dysfunction remain obvious in this setting.

Since a disturbance in endoplasmic reticulum and autophagic functions have been linked to cardiac remodeling in patients with heart failure (18) and an isolated diastolic dysfunction has been noted in various infiltrative cardiomyopathies (15), we examined the effects of levosimendan and valsartan on cardiac endoplasmic reticulum stress and autophagy. Tang *et al*. (2008) demonstrated recently that AT_1 -blockade could attenuate cardiomyocyte apoptosis, and the extent of cardiac hypertrophy through reduced ER stress (57). LC3 is an ubiquitin-like protein which is widely used as reliable autophagosome marker for monitoring autophagy. Typically autophagy is measured by tracking the level of conversion of LC3-I to LC3-II which provides an indicator of autophagic activity. In particular the levels of LC3-II correlate with autophagosome formation as LC3-II associates with the autophagosome membrane. In the present study, high salt intake did not induce cardiomyocyte autophagy. Although levosimendan has been shown previously to induce type II form of LC3B (LC3II) expression and autophagy in pig myocardium (55), neither levosimendan nor valsartan monotherapy altered LC3II expression in the present study. It is interesting that the drug combination exhibited a tendency towards activation of cardiac autophagy and thus towards an enhanced cellular clearance mechanism, which could partially explain the cardioprotective effects of the drug combination in our animal model of salt-induced hypertension.

In the present study, valsartan alone and in combination with levosimendan reduced fluid intake, pointing to drug- induced suppression of the dipsogenic action of Ang II in the central nervous system. This effect on water intake slightly decreased the levosimendan dosage in the combination group. However, even the smaller dose of levosimendan produced a powerful anti-hypertensive and tissue protecting effect when combined with valsartan. It is also noteworthy that, in the present study, systolic blood pressure increased in salt-sensitive Dahl/Rapp rats even when the animals were kept on low-salt diet. Our study thus confirms the seminal findings by Rapp and Dene (31) and Kurtz and Morris (58) on salt-independent increase in blood pressure in Dahl salt-sensitive rats. The precise molecular mechanisms leading to salt-independent increase in blood pressure still remain unclear, however, it is likely that salt-independent impairment of renal function found in these animals (31) is involved in the pathogenesis.

Levosimendan is a novel calcium sensitizer used as pharmacological inotropic support in acute decompensated heart failure as an intravenous infusion. Levosimendan has a volume of distribution of 0.2 l/kg, binds strongly (97-98%) to plasma proteins, mainly albumin, and has a clearance of 3.0 ml/min/kg

(59). Levosimendan is rapidly metabolized with elimination half-life of approximately 1 hour in humans and 0.7 hours in rats. The circulating active metabolites OR-1855 and OR-1896 are formed slowly. In rats, OR-1855 is very extensively and rapidly transformed into OR-1896. OR-1986 has a longer elimination half-life than the parent compound levosimendan (about 75-85 hours in humans and 6.5 hours in rats), but exhibits hemodynamic effects similar to levosimendan. Levosimendan is well absorbed from the intestinal tract after oral ingestion. Currently there are only limited data available on the safety and efficacy of chronic treatment with oral form of levosimendan in patients with heart failure. The randomized, double-blind, placebo-controlled PERSIST study (60) did not reveal any differences between oral levosimendan and placebo in repeated subjective symptom assessments, worsening heart failure events or all-cause mortality. However, oral levosimendan exerted some encouraging effects as it improved quality of life score, renal functions and reduced plasma NT-proBNP levels (60) . On the other hand, it should be underlined that oral levosimendan produced a modest but persistent increase in heart rate in PERSIST study (60). Very recently, Jalanko *et al*. (61) reported in the subgroup analysis of PERSIST study that oral levosimendan produced a persistent improvement in hemodynamic function in chronic heart failure. Finally, if is of great interest, that levosimendan has been shown to exert anti-inflammatory and anti-apoptotic properties in clinical studies (62, 63) which could explain, at least in part, the beneficial effects of oral levosimendan on cardiac function and structure. Further studies on the safety and efficacy of oral levosimendan are thus warranted in future.

In conclusion, the present study found evidence for an additive anti-hypertensive effect of levosimendan and valsartan. The drug combination provides blood pressure- dependent protection against target organ damage in the heart and kidneys. Levosimendan, alone and in combination with valsartan, can correct diastolic dysfunction induced by salt-dependent hypertension.

Abbreviations: RAAS, renin-angiotensin- aldosterone system; Ang II, angiotensin II; ANP, atrial natriuretic peptide; K^{ATP}, ATP-dependent K^+ channel; PDE III, phosphodiesterase III.

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