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Short communication

Effect of sacubitril/valsartan on inflammation and oxidative stress in doxorubicin-induced heart failure model in rabbits

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Our study evaluates the effects of sacubitril/valsartan (SAC/VAL) in the rabbit model of doxorubicin-induced heart failure. Twenty rabbits (5 per group) were administered with doxorubicin (DOX, 1.5 mg kg^{-1}, *i.v.*) to induce heart failure. Specific biomarkers such as BNP, CnT, CRP and ROMs were determined. The cardiac enzymatic antioxidant systems were recorded with their electrographic profiles. HR, SBP, DBP and MAP were restored at 5 or 10 mg kg^{-1} (*p.o.*) of SAC/VAL compared to DOX, followed by reduced levels of creatinine and BNP ($p < 0.001$). Significant improvements ($p < 0.05$) compared to DOX were also noticed in CAT, SOD and LPO with the same doses of SAC/VAL. Specific biomarkers such as BNP, CnT, CRP and ROMs descended significantly ($p < 0.001$) with treatment when compared to their baseline values. Our findings implied that SAC/VAL treatment reduced the inflammation and oxidative stress to improve the cardiac function.

Keywords: sacubitril, valsartan, doxorubicin-heart failure, inflammation, oxidative stress, natriuretic peptide

Heart diseases are present all over the world (1). The characteristics of heart failure include reduced myocardial function with reduced stroke volume and cardiac output (2). The reduction in myocardial activity may be attributed to many causes, such as cardiomyopathy, ageing and free radical degradation (3–5). Of the several reasons for the reduced stroke volume and cardiac output, coronary artery endothelium inflammation and increased oxidative stress contribute mainly to heart failure (6, 7). Doxorubicin causes heart failure with ventricular wall thinning accompanied by left ventricular damage resulting in increased dilation. Doxorubicin-induced cardiomyopathy involves various underlying mechanisms that result in mitochondrial injury, myocardial cell intracellular dysfunction and increased calcium ion flux with reduced ejection output. Changes in myocardial vulnerability may include elevation of biochemical biomarkers and endothelial injury with increased levels of enzymes such as catalase, oxidase, *etc.*, resulting in inflammation and oxidative stress with reduced free radical scavenging (5).

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The treatment of heart failure includes cardiotonics, angiotensin-converting enzyme inhibitors, beta-blockers, selective angiotensin-II receptor inhibitors, followed by many others (8). Although treatment for heart failure involves the use of several standard therapeutic drugs, inflammation and oxidative stress interventions are often ignored, resulting in a significant increase in morbidity, mortality and health care costs (9). Sacubitril (SAC) is a natriuretic peptide inhibitor and valsartan (VAL) is the angiotensin-II peptide inhibitor (10) used in the treatment of heart failure, hypertension, myocardial infarction with left ventricular dysfunction and coronary heart disease (11). They act primarily by inhibiting the natriuretic peptide system and by binding to the angiotensin-II receptors, resp. (12). Several studies (12, 13) documented their therapeutic use in inflammation and oxidative stress by inhibiting nitric oxide release and the cascade of inflammatory mediators such as cytokines.

The latest US FDA's Entresto (SAC/VAL) approval was a significant step in the management of heart failure with reduced ejection fraction (HFrEF) due to improved clinical outcomes (14). Eventually, a study by Trivedi *et al*. (13) published evaluating the SAC/VAL effects in a rat model of HFrEF, demonstrated an endothelial function improvement with an increase in bioavailability of nitric oxide and a decrease in arterial strength and stiffness. Additionally, in an ischemic model of HFrEF in streptozotocin-induced diabetic mice, the antifibrotic ability of SAC/VAL was greater than that of VAL alone, possibly by reducing the transforming growth factor-β (15). Until today, no study has reported the effectiveness of sacubitril/valsartan in doxorubicin-induced cardiac failure in rabbits. Therefore, our research aims to investigate the effects of SAC/VAL in doxorubicin-induced heart failure in rabbits and to validate their role in inflammation and oxidative stress.

EXPERIMENTAL

Chemicals and drugs

The drugs and chemical constituents required, such as sacubitril/valsartan (SAC/VAL, Entresto 49/51 tablets, equivalent to 48.6 mg sacubitril and 51.4 mg valsartan, a sodium salt complex) (Novartis, Switzerland) and doxorubicin hydrochloride (≥ 95 % purity, DOX) were obtained from Novartis Pharma AG (Switzerland) as gift samples. The substances such as ketamine hydrochloride, heparin, phenylephrine, sodium nitroprusside, acetylcholine, inorganic salts, dextrose and ethylenediaminetetraacetic acid were procured from Sigma-Aldrich (USA).

Animals

Twenty male adult New Zealand white rabbits (age 12 weeks, weighing about 2–3 kg) were obtained from the Institute of Laboratory Animal Sciences, Jilin (China) for this study. They were housed in metal cages (humidity 20 %) at room temperature (25 °C) in a 12-h dark and 12-h light cycle, providing a free supply of food, water, and other necessities. The study protocols were previously approved by the Institutional Ethics of Animal Committee, and all experimental studies were carried out in accordance with the Animal Ethical Guidelines rules and regulations.

Experimental design

The induction of heart failure was done following the methods described by Romão *et al*. (12) with minor alterations. Doxorubicin (1.5 mg kg^{-1}, *i.v.*) was given once a week for five weeks. For each week of doxorubicin administration, the rabbits were classified into four groups with five animals in each group – 1 – one naïve or control group (containing the animals who received distilled water only), 2 – DOX group (where rabbits received doxorubicin only), 3 – SAC/VAL 5 mg kg^{-1} and 4 – SAC/VAL 10 mg kg^{-1}. The combined dose of SAC/VAL was given orally once a day for four weeks, beginning from the second week of doxorubicin administration. SAC/VAL doses were based on the drug composition with a 1:1 mass ratio of sacubitril to valsartan described in the PARADIGM-HF trial and the highest dose of VAL reported in rabbits was 10 mg kg^{-1} day^{-1} (16, 17). Hence, we selected 5 or 10 mg kg^{-1} SAC/VAL doses (approximately, 2.45 mg SAC+ 2.55 mg VAL in case of 5 mg, and 4.9 mg SAC+ 5.1 mg VAL in case of 10 mg). Each of SAC/VAL (Entresto) was suspended in 0.5 % carboxymethyl cellulose and administered *via* oral gavage in a volume of 2 mL kg^{-1} body mass.

In a separate pilot study, the optimal dose of SAC/VAL (2.5, 5 or 10 mg kg^{-1}) was determined in adult health male New Zealand Wister rabbits (3 per group). Measured pharmacodynamics effects involved hemodynamic parameters and atrial natriuretic peptide (ANP) test to assess the inhibition of neprilysin following treatment with SAC/VAL (5 or 10 mg kg^{-1}), followed by cGMP analysis after injecting atrial natriuretic peptide (40 mg kg^{-1} *i.v.*). We selected 5 and 10 mg kg^{-1} doses of SAC/VAL based on the hemodynamic responses and significant escalation in cGMP plasma levels after the ANP test.

No animal deaths were recorded in any of the groups during daily recording. At the end of the study period (36^{th} day), the animals were given diazepam (10 mg kg^{-1}) and ketamine (115 mg kg^{-1}) to induce anesthesia and to alleviate discomfort to carry out cannulation and collection of blood samples from the left carotid artery.

Electrocardiography and assessment of blood pressure

The rabbits were anesthetized as described above prior to the positioning of electrodes on elbows and knees (RL, LL, LA and RA). The electrocardiogram was recorded using an ECG machine (BPL Cardiart 108T ECG Machine, China), with electrodes V1, V2, V3, V4, and V5.

The animals were given heparin (45 IU kg^{-1}) subcutaneously. The left carotid artery was exposed and cannulated, then attached to the blood pressure recording system (PowerLab®) through the pressure transducer (Blood pressure transducer, China). The changes in heart rate (HR, beats min^{-1}), mean arterial pressure (MAP), systolic (SBP) and diastolic blood pressure (DBP) were recorded for 15 minutes.

Biochemical parameters

Blood samples obtained from the left carotid artery were centrifuged at 2000×*g* for 15 min to separate serum. The parameters such as alanine aminotransferase (ALT), albumin, aspartate aminotransferase (AST), γ-glutamyl transpeptidase (GGT), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), indirect, direct and total bilirubin, alkaline

phosphatase (ALP), creatinine, triglycerides, urea, uric acid and the levels of Na^+ and K^+ were determined using a biochemical analyzer (Bioline Technologies, China). Enzyme-linked immunosorbent assay (ELISA) (Bioline Technologies, India) determined the levels of brain natriuretic peptide (BNP) and troponin.

Cardiac enzymes responsible for antioxidant activity

After the completion of blood sample collection, electrocardiogram and blood pressure measurements, the heart tissues were immediately collected after euthanasia by intravenous administration of 20 % (*m*/*V*) potassium chloride and were homogenized in phosphate buffer (pH 6.8). Activities of enzymes such as catalase (CAT), superoxide dismutase (SOD), and lipid hydroxyperoxidase (LPO) were determined according to Romão *et al*. (12) and expressed as mmol min^{-1} g^{-1} tissue for CAT activity, IU g^{-1} of tissue for SOD activity and mmol hydroperoxide g^{-1} of tissue for LPO levels.

Analysis of specific biomarkers

Centrifugation of blood samples was done instantly at 1500×*g* for 15 min at room temperature, and the separated serum was stored at –4 °C until analyzed. The serum C-reactive protein was determined by nephelometer (Behring Nephelometer BN II analyser, Siemens Healthineers, Germany) using monoclonal anti-CRP antibodies. Consequently, reactive oxygen metabolites (ROM) were measured based on Fenton radical reaction by photometer at 535 nm. This measurement involves the estimation of serum hydroperoxides formed from free radicals, thus quantifying the amount of ROMs produced.

Statistical analysis

The results were represented as mean ± SEM. The variations among groups were estimated using analysis of variance (ANOVA), followed by Dunnett's *t*-test. The skewed variations among the values of serum cardiac troponin (CnT), BNP (brain-type natriuretic peptide), CRP (C-reactive protein), and reactive oxygen metabolites (ROM) were transformed into logarithms for analysis. The variations between groups, determined using *t*-tests (paired) were used for continuous variables to compare intra- and inter-group variations.

RESULTS AND DISCUSSION

Effects of SAC/VAL on blood pressure

Heart rate, systolic blood pressure, diastolic blood pressure and mean arterial pressure were recorded for 15 min. The heart rate was found to increase significantly in the group receiving 5 or 10 mg kg^{-1} SAC/VAL ($p < 0.01$); however, SBP and DBP decreased in both the treatment groups ($p = 0.002$) when compared to the DOX group. MAP was 71.67 ± 4.60 mm Hg in the DOX group and descended to 44.1 ± 2.48 and 46.77 ± 2.52 mm Hg after treatment with 5 and 10 mg kg^{-1} SAC/VAL, resp. (Fig. 1). No significant differences were found when treatment groups were compared with the naive group.

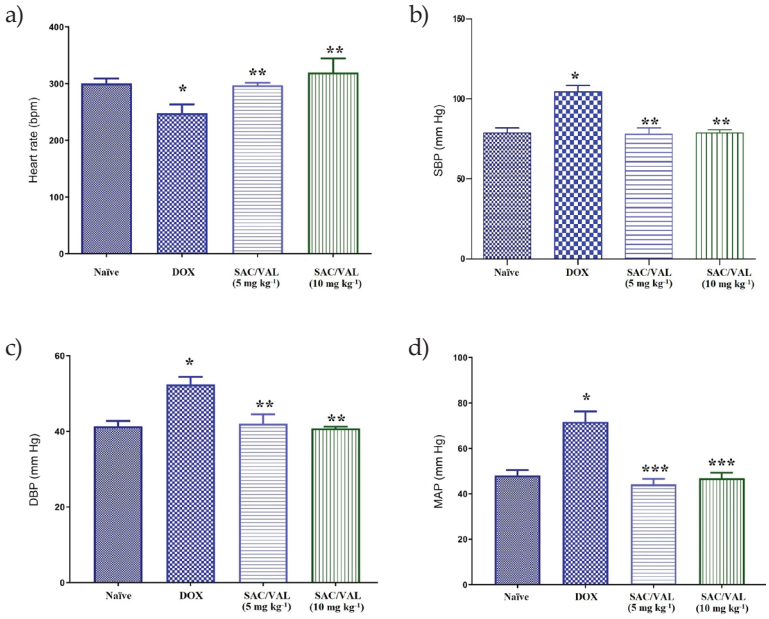

Fig. 1. Graphical representation of the changes in the heart rate, blood pressure in naïve, DOX and SAC/VAL (5 mg kg^{-1} or 10 mg kg^{-1}) groups. Values are expressed as mean ± SEM (n = 5). Significant difference: *versus* naïve group *$p < 0.05$; *versus* DOX group: **$p < 0.05$ and ***$p < 0.001$ (using one-way ANOVA, including Dunnett's test).

Effects on the electrocardiography

The prolongation of the PR segment was significantly greater in the DOX group (p = 0.0166) relative to naïve rabbits but no difference was observed in 5 or 10 mg kg^{-1} SAC/VAL groups Such significant changes were due to the effect of doxorubicin altering cardiac potassium channels. The down-regulation of these channels by doxorubicin activates the caspase activity which changes the heart's electrical activity (18). There was a substantial decrease in QTC segment prolongation and QT interval prolongation ($p < 0.001$) in SAC/VAL (5 and 10 mg kg^{-1}) groups as compared to the DOX group. Q-wave amplitude was similar to the naïve group in 5 and 10 mg kg^{-1} SAC/VAL groups but it significantly increased ($p < 0.001$) in the DOX group (Fig. 2). Changes in repolarization in the myocardium are due to altered QT-segment and Q-wave (19).

Effects of SA/VAL on biochemical parameters

The AST levels increased in 5 and 10 mg kg^{-1} SAC/VAL groups when compared with the DOX group. The ALT levels remained identical in all groups (Table I) with no significant changes compared to the naïve group. The creatinine levels of the DOX group were observed to be significantly ($p < 0.001$) higher than in the naïve and treatment groups. Increased serum creatinine levels in the DOX group indicate shifts in renal clearance

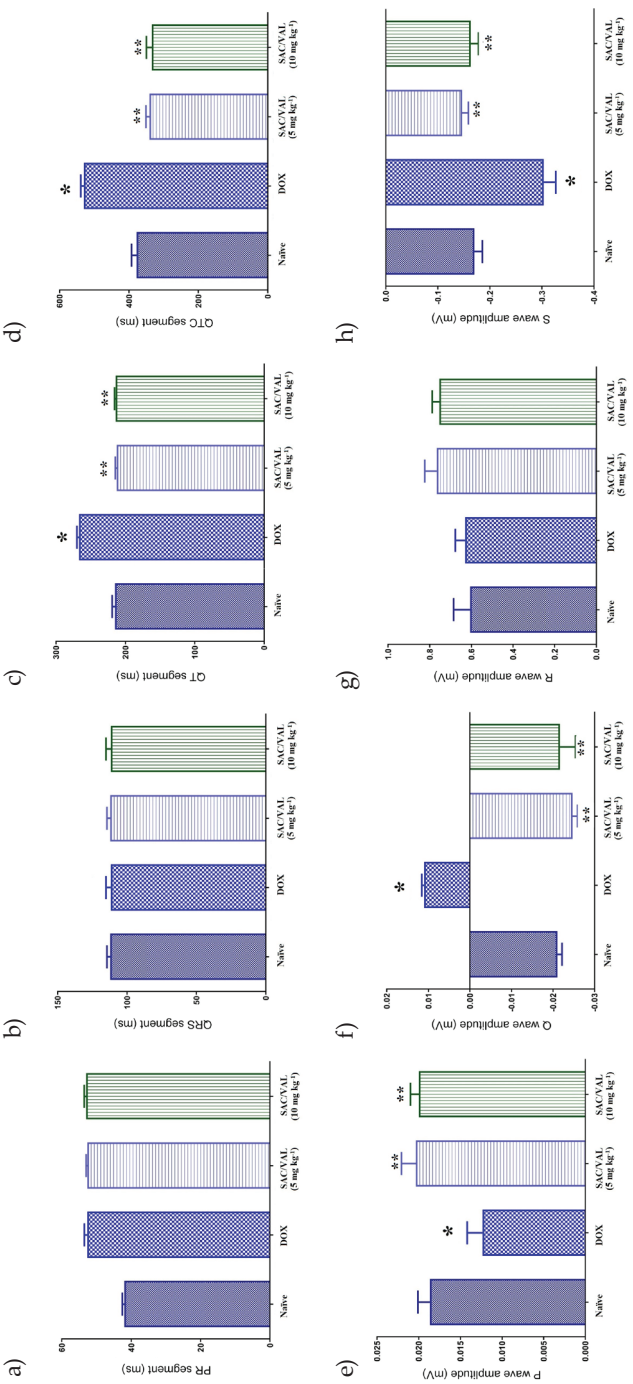

Fig. 2. Electrographic data of naïve, DOX and SAC/VAL (5 mg kg^{-1} or 10 mg kg^{-1}) groups. Values are expressed as mean ± SEM (n = 5). Significant difference: *versus* naïve group *$p < 0.05$; *versus* DOX group **$p < 0.05$ (using one-way ANOVA, including Dunnett's test).

Table I. Serum analysis of biomarkers and biochemical parameters of naïv, DOX or SAL/VAL rabbits

Parameter	Naïve	DOX (1.5 mg kg^{-1}, *i.v.*)	SAL/VAL (5 mg kg^{-1}, *per os*)	SAL/VAL (10 mg kg^{-1}, *per os*)
Albumin (g per 100 mL)	2.84 ± 0.20	2.95 ± 0.15	2.77 ± 0.22	2.99 ± 0.09
AST (U L^{-1})	32.5 ± 1.7	25.9 ± 1.5*	31.37 ± 1.01	30.13 ± 1.04
ALT (U L^{-1})	19.47 ± 1.05	20.33 ± 1.47	19.80 ± 0.81	18.83 ± 1.16
Total bilirubin (mg per 100 mL)	0.066 ± 0.009	0.063 ± 0.009	0.066 ± 0.012	0.067 ± 0.012
Creatinine (mg per 100 mL)	1.00 ± 0.10	2.67 ± 0.26*	0.87 ± 0.06	0.89 ± 0.06
Urea (mg per 100 mL)	26.43 ± 1.20	28.70 ± 2.02	27.53 ± 1.86	27.30 ± 0.53
Uric acid (mg per 100 mL)	0.247 ± 0.009	0.233 ± 0.019	0.233 ± 0.014	0.227 ± 0.019
Triglycerides (mg per 100 mL)	92.27 ± 1.70	87.87 ± 0.97	90.20 ± 0.61	91.53 ± 0.50
Total cholesterol (mg per 100 mL)	42.53 ± 0.65	45.43 ± 1.24	43.13 ± 1.30	43.03 ± 1.30
HDL-C (mg per 100 mL)	24.03 ± 1.49	22.50 ± 0.44	24.40 ± 0.91	23.33 ± 1.75
GGT (U L^{-1})	4.89 ± 0.13	5.04 ± 0.07	4.86 ± 0.08	4.44 ± 0.50
ALP (U L^{-1})	24.03 ± 1.69	26.07 ± 0.54	24.8 ± 0.89	26.3 ± 1.56
Na^+ (mmol L^{-1})	111.2 ± 4.7	103.2 ± 3.7	108.5 ± 1.6	103.1 ± 3.4
K^+ (mmol L^{-1})	4.64 ± 0.27	4.57 ± 0.25	5.01 ± 0.03	4.36 ± 0.34
Cardiac troponin (ng mL^{-1})	0.02 ± 0.00_5	0.025 ± 0.007	0.02 ± 0.00_5	0.04 ± 0.00_5
BNP (ng L^{-1})	89.37 ± 1.9	406.5 ± 4.23*	87.6 ± 1.47**	83.97 ± 1.96**

ALT – alanine aminotransferase, AST – aspartate aminotransferase, ALP – alkaline phosphatase, BNP – serum *N*-terminal pro-brain type natriuretic peptide, GGT – gamma-glutamyl transpeptidase, HDL-C – high-density lipoprotein cholesterol

Data are represented as mean ± SEM ($n = 5$).

Statistically significant: *$p < 0.05$ against naïve; **$p < 0.001$ against DOX group (using one-way ANOVA, including Dunnett's test).

which are partly caused by the deterioration of kidney cells or a gradual reduction in cardiac output that is caused by cardiac failure. Additionally, we found a significant increase ($p < 0.001$) in BNP levels in the DOX group as compared to naïve and treatment groups (Table I).

Effect on the cardiac antioxidant defense system

SOD levels in 5 and 10 mg kg^{-1} SAC/VAL groups increased significantly compared with the DOX group. The CAT level in the DOX group increased significantly ($p = 0.0027$) after treatment with 5 or 10 mg kg^{-1} SAC/VAL, thus restoring the normal value of the naïve group. These results indicate that DOX resulted in the development of free radicals in the heart tissue which reduced ROS detoxification. The levels of LPO increased significantly ($p < 0.05$) in 5 and 10 mg kg^{-1} SAC/VAL groups when compared to the naïve group and decreased significantly ($p < 0.001$) when compared to the DOX group. Increased levels of

Table II. Oxidative enzymes of naïve, DOX or SAL/VAL rabbits

Parameter	Naïve	DOX (1.5 mg kg^{-1}, *i.v.*)	SAC/VAL (5 mg kg^{-1}, *per os*)	SAC/VAL (10 mg kg^{-1}, *per os*)
SOD (IU g^{-1} tissue)	40.50 ± 0.57	17.77 ± 0.81*	35.3 ± 1.5***	38.13 ± 2.77***
Catalase (mmol min^{-1} g^{-1} tissue)	4.50 ± 0.34	2.567 ± 0.176*	2.467 ± 0.200**	3.467 ± 0.210**
Lipid peroxidation (nmol H_2O_2 g^{-1} tissue)	334.3 ± 4.6	1857.4 ± 32.6*	703.5 ± 35.8***	799.5 ± 20.1***

SOD – superoxide dismutase.

Data are represented as mean ± SEM ($n = 5$).

Statistically significant: *$p < 0.05$ against naïve; **$p < 0.01$, ***$p < 0.001$ against DOX group (using one-way ANOVA, including Dunnett's test).

Table III. Changes in specific biomarker levels between SAC/VAL groups

Parameter	SAC/VAL (5 mg kg^{-1}, *per os*)		SAC/VAL (10 mg kg^{-1}, *per os*)	
	Before	After	Before	After
Serum *N*-terminal pro-brain type natriuretic peptide (BNP) (log pg mL^{-1})	3.73 ± 0.12	2.33 ± 0.14**	3.70 ± 0.11	2.03 ± 0.07*,**
Serum high-sensitivity cardiac troponin (CnT) (log ng mL^{-1})	1.83 ± 0.03	1.53 ± 0.03**	$1.8 \pm 0.0_6$	$1.1 \pm 0.0_5$*,**
Serum high-sensitivity C-reactive protein (CRP) (log ng mL^{-1})	$3.7 \pm 0.0_6$	$2.8 \pm 0.0_5$**	3.667 ± 0.089	2.103 ± 0.054*,**
Reactive oxygen metabolites (ROMs) (U.CARR.)[a]	410 ± 6	340 ± 10**	300 ±131	326 ± 18*,**

Data are expressed as mean ± SEM ($n = 5$).

Statistical significance: *$p < 0.05$ between both SAC/VAL groups after treatment; **$p < 0.001$ between before and after treatments of SAC/VAL (5 mg kg^{-1} or 10 mg kg^{-1}) employing paired *t*-test.

[a] One U.CARR. corresponds to 0.8 mg L^{-1} hydrogen peroxide.

LPO confirm the presence of oxidative stress. Cardiac myopathy may be a resultant factor of ROS produced by doxorubicin (20, 21). Therefore, SAC/VAL treatment was able to restore the enzymatic levels in heart failure (22) (Table II).

Effects on the specific biomarkers

The levels of serum natriuretic peptide, cardiac troponin and C-reactive protein showed a significant ($p < 0.001$) decrease in 5 and 10 mg kg^{-1} SAC/VAL treated groups when compared to DOX values before the treatment (Figs. 3, 4). Likewise, there was a significant decrease of reactive oxygen metabolites (ROMs) in both SAC/VAL groups when compared to the DOX group (Figs. 3, 4 and Table III). Inflammatory biomarkers such as CRP, CnT, BNP, and oxidative stress markers such as ROM increased after treatment with SAC/VAL (both doses) indicating their anti-inflammatory and antioxidant effects in agreement with the study of Angelis *et al*. (23) where cardiac failure was a result of reduced activity of antioxidant enzymes and specific biomarkers (hsCRP, BNP, and CT) (24, 25).

Our findings showed that the highest dose of SAC/VAL (10 mg kg^{-1}) showed a significant antioxidant effect, with reduced lipid proliferation, thus acting as a free radical scavenger in accordance with the study reporting that oxidative stress was involved in causing cardiotoxicity in patients (12, 13, 23, 26). Our results are consistent with previous studies (27) concerning the antioxidant activity of SAC/VAL with reduced hsTnT and NT-ProBNP. Another research (12) found an increase in myocardial permeability promoting the release of troponin, suggesting that inflammation triggers the release of cardiac troponin in DOX-induced heart failure and thus supports our study findings. Additionally, Jing *et al*. (27) suggested that SAC/VAL suppressed the expression of proinflammatory cytokines in rat/rabbit cardiac failure models indicating inflammatory actions of SAC/VAL. This was further supported by our findings in which CRP and cardiac troponin levels were reduced significantly ($p < 0.001$) after treatment with 5 and 10 mg kg^{-1} SAC/VAL in DOX-induced heart failure.

Limitations of the study

Histological (cellular) and molecular-level analysis may be needed to determine the role of SAC/VAL treatment in heart failure caused by doxorubicin. The biochemical criteria used in the current study have shortcomings in correlating the cardiac performance and measures of renal or hepatic dysfunction in doxorubicin-induced heart failure.

CONCLUSIONS

Our findings suggest the restorative effect of SAC/VAL in doxorubicin-induced heart failure, which is due to a decrease in the systolic and diastolic blood pressure and mean arterial pressure, followed by an increase in heart rate. Our study also found that SAC/VAL prevented heart failure induced by doxorubicin, by reducing serum BNP, cardiac troponin, and CRP levels, indicating suppressed inflammatory action. Due to SAC/VAL treatment, ROMs and various enzymatic activities were reduced as well, which improved the cardiac function offering cardiac protection in heart failure. However, the exact mechanism of

SAC/VAL in reducing the inflammation and oxidative stress is yet to be understood, increasing the need for further studies.

Abbreviations, acronyms, symbols. – ALT – alanine aminotransferase, ANP – atrial natriuretic peptide, AST – aspartate aminotransferase, BNP – brain natriuretic peptide, CAT – catalase, cGMP – cyclic guanosine monophosphate, CnT – cardiac troponin, CRP – C-reactive protein, DBP – diastolic blood pressure, DOX – doxorubicin, ECG – electrocardiogram, ELISA – enzyme-linked immunosorbent assay, GGT – gamma-glutamyl transferase, HFrEF – heart failure with reduced ejection fraction, HDL-c – high-density lipoprotein cholesterol, HR – heart rate, hsCRP – high sensitivity C-reactive protein, hsTnT – high sensitivity troponin T, LPO – lipid hydroxyl peroxidase, MAP – mean arterial pressure, NPS – natriuretic peptide system, NT-ProBNP – *N*-terminal pro-brain natriuretic peptide, ROM – reactive oxygen metabolites, SAC – sacubitril, SBP – systolic blood pressure, SOD – superoxide dismutase, TC – total cholesterol, VAL – valsartan.

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