ORIGINAL ARTICLE

VALIDATION AND CHARACTERIZATION OF A HEART FAILURE ANIMAL MODEL

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Abstract

The major objective of the present study was to induce left ventricular hypertrophy (LVH) and subsequently heart failure (HF) by aortic banding in rats. Furthermore, with the use of different markers, we aimed to validate an animal model and to characterize the evolution of the cardiac function from normal to LVH and to HF. We used two study groups: abdominal aortic banding (AAB) (n = 20) and sham (n = 10), four echocardiography time-points (baseline, 8, 18 and 24 weeks post-operation - PO) and two plasma and tissue analysis time-points (18 and 24 weeks PO). Echocardiography parameters such as LVH parameters (heart weight-to-body weight ratio, LV mass, LV mass-index, anterior, posterior and relative wall thickness), LV dimension parameters, LV performance parameters (fractional shortening - FS%; ejection fraction - EF%), histopathology, as well as plasmatic markers such as malondyaldehyde and superoxide dismutase can be used to distinguish between LVH and HF in this animal model. The validated animal model can be used to study the pharmacology of drugs that may prevent or treat LVH and HF.

Rezumat

Obiectivul principal al studiului de faţă a fost inducerea hipertrofiei ventriculului stâng (HVS) şi ulterior a insuficienţei cardiace (IC) prin ligatură aortică la şobolan. De asemenea, prin utilizarea diferiţilor markeri am urmărit validarea modelului experimental şi caracterizarea evoluţiei funcţiei cardiace de la starea normală la HVS şi la IC. Am utilizat şobolani ligaturaţi (n = 20) şi şobolani martori (n = 10), patru determinări ecocardiografice (imediat după operaţie, la 8, 18 şi 24 de săptămâni post-operator - PO), două determinări din plasmă şi ţesut (la 18 şi 24 de săptămâni PO). Parametri ecocardiografici pentru HVS (raportul greutăţii inimii-corporale, masa VS, grosimea pereţilor VS), dimensiunile VS, performanţa VS (fracţia de scurtare, fracţia de ejecţie), analiza histopatologică, markeri plasmatici (malondialdehida, superoxid dismutaza) pot fi utilizate pentru a face distincţia dintre HVS şi IC în cazul acestui model animal. Modelul animal validat poate fi utilizat pentru a studia farmacologia medicamentelor care pot preveni sau trata HVS şi IC.

Keywords: **b**iomarkers, **a**nimal model, **l**eft ventricular hypertrophy, heart failure, aortic banding

Introduction

Heart failure (HF) is a disease with an unfavourable prognosis. Currently, HF has an estimated prevalence of 38 million patients worldwide and a mortality rate of 50%, within five years of diagnosis [2]. Despite progress in research, the intricate mechanisms responsible for HF are not yet completely elucidated [10]. In order to elucidate the pathophysiology of HF, several animal models of left ventricular (LV) systolic dysfunction have been developed over the years [26]. The pressure overload-hypertrophy model has been used to study hypertension-induced ventricular hypertrophy, but not HF [14]. Therefore, we aimed at modifying an animal model of pressure-overload hypertrophy in order to induce LVH, but also HF and to characterize the evolution of heart function from normal to hypertrophy and finally to HF. As such, we used transthoracic echocardiography, histology analysis and biochemistry markers to quantify changes in cardiac geometry and function. In addition, we determined the specific time-points for

this particular animal model, in which the hypertrophy is obvious and the dilation of the left ventricle is produced, by the use of echocardiography. Finally, we assessed plasma markers that could help pinpoint the transition from compensated to decompensated HF.

Materials and Methods

Animals and experimental design

Four weeks old male Wistar rats (n = 30; weighing 130 - 170 g) were provided by the Experimental Medicine and Practical Skills Centre of "Iuliu Hatieganu" University of Medicine and Pharmacy, Cluj-Napoca, Romania. All rats were housed five per cage, under standard laboratory conditions (controlled temperature around 22°C, 12 h light/dark cycle) and received water and standard pellet food *ad libitum*. All experiments and procedures were performed in conformance with the Guide for the Care and Use of Laboratory Animals, as published by the US National Institute of Health [19] and with the Universities Federation for Animal Welfare guidelines. The experimental protocol was approved by the Ethical Committee of our University.

Abdominal aortic banding (AAB) was performed under anaesthesia (50 mg/kgbw ketamine and 10 mg/kgbw xylazine, i.m.). Briefly, the descending abdominal aorta and the left and right renal arteries were exposed. A suture line (3 - 0 silk) was placed above the left and right renal arteries. A 23G (0.6 mm diameter) blunt needle was positioned along the isolated aorta and the suture line was tightly tied around the aorta and the overlying needle. The needle was then removed, leaving the vessel constricted to the diameter of the needle. Finally the abdomen cavity was closed.

In total, twenty rats were subjected to aortic constriction and ten rats underwent the same procedures without constriction of the aorta (sham group). Pain was controlled with the administration of diclofenac (25 mg/kgbw; twice *per* day for 4 days, i.m.) and infection prophylaxis was provided by penicillin G (100.000 units daily for three days, i.m.).

At baseline, 8, 18 and 24 weeks post-operation (PO) rats were weighed and evaluated by echocardiography.

At 18 and 24 weeks, respectively, rats (AAB = 10 and sham = 5) were weighed, anesthetized (50 mg/kgbw ketamine and 10 mg/kgbw xylazine, i.m.). Blood was sampled from the retro-orbital sinus and the heart tissue was removed, weighed, washed with saline solution and deposited in 10% neutral formalin for further analysis.

Echocardiography measurements

After shaving the rat chest, transthoracic echocardiography was performed in the supine or left lateral position. Two-dimensional echocardiography images at the mid-papillary muscle level were acquired, using a commercially available echocardiograph equipped with a 7.5 MHz electric transducer (Ultrasonix, Boston, Massachusetts, USA). Rats were anesthetized (30 mg/kgbw ketamine and 0.5 mg/kgbw xylazine, i.m.) using a minimal dose to prevent respiratory and cardiovascular suppression during echocardiography. All structures were manually measured by the same observer, using the leading-edge method of the American Society of Echocardiography [13]. Left ventricular anterior wall thickness (AWT), left ventricular posterior wall thickness (PWT) and LV end-diastolic and end-systolic diameters (LVDd and LVDs) were measured from a short axis view. End-systole was defined as the moment when the left ventricular dimension was minimal and end-diastole as the time point when the left ventricle was maximal. The results represent the mean of consecutive cycles.

The following parameters were derived from our measurements:

1. Percent LV fractional shortening (FS%) was calculated as an index of LV systolic function [24]:

$$\boldsymbol{FS}\% = \frac{LVDd - LVDs}{LVDd} X\ 100;\ \text{FS\%} = \frac{\text{LVEDd-LVEDs}}{\text{LVEDd}} \times 100$$

2. LV mass and LV mass index were calculated for the determination of LV hypertrophy as follows [15]:

$$\boldsymbol{LV\ mass} = \left\{[(LVDd + AWTd + PWTd)^3 - LVDd^3] \times \frac{\pi}{3}\right\} \times 0.8 + 0.14$$

$$\boldsymbol{LV\ mass - index} = \frac{LV\ mass}{Body\ weight}$$

3. Relative wall thickness (RWT) was calculated as follows [24]:

$$\boldsymbol{RWT} = \frac{AWTd + PWTd}{LVDd}$$

4. Left ventricular systolic and diastolic volumes were estimated considering an ellipsoid model as follows [4]:

$$\boldsymbol{LV\ volume} = LVD^3 \times \frac{\pi}{3}$$

5. Percent LV ejection fraction (EF%) was calculated as follows [4]:

$$\boldsymbol{EF}\% = \frac{LV\ volume_{\ diastole} - LV\ volume_{\ systole}}{LV\ volume_{\ diastole}} \times 100$$

Histology

Hearts were harvested after the final *in vivo* measurements. The cardiac tissue was fixed with neutral 10% formalin and embedded in paraffin. Next, the heart tissue was sectioned transversely (cross section at the papillary muscle level of the left ventricle; thickness 5 μm) and stained with haematoxylin-eosin for evaluation of cardiomyocyte hypertrophy and with trichrome solution for interstitial fibrosis evaluation.

Assessment of oxidative stress plasma biomarkers

Blood was collected on heparin from the retro-orbital sinus. Plasma was separated by centrifugation (2000 rpm, RT, 6 min) and samples were stored at -80°C until use. Plasma levels of superoxide dismutase (SOD) were measured using commercial kits (R&D systems, Minneapolis, Minnesota, USA). Absorbance was detected at 550 nm by the use of a spectrophotometer. Total plasma malondialdehyde (MDA) levels were measured by HPLC, as previously shown [11].

Statistical analysis

Data were compared between groups using Mann-Whitney U test (non-normally distributed data) and Student's t-test (normally distributed data). Differences with p value less than 0.05 were considered statistically significant. All data are presented as mean ± SD and were analysed in R environment for statistical computing and graphics version 3.1.0.

Results and Discussion

Characterization of the animal model

Evolution of body weight and heart weight

Rats were initially chosen on the basis of their age (weanlings - 4 weeks) and body weight, which at baseline was similar between the groups (AAB = 150 ± 13.48; sham = 150.75 ± 17.73; p = 0.95). As depicted in Figure 1A, all rats registered an increase in body weight over time. However, AAB rats presented with a significantly reduced growth rate at 8, 18 and 24 weeks, respectively, in comparison to sham rats. At 18 and 24 weeks respectively, AAB rats displayed an increase in heart weight and heart weight-to-body weight ratio in comparison to sham rats (Figures 1B and 1C and Table I).

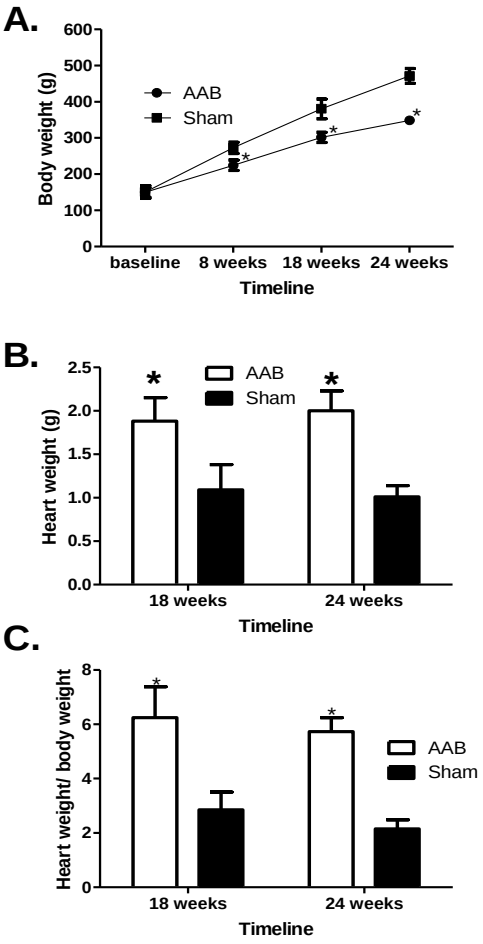

Figure 1.

Evolution of **A.** body weight (g); **B.** heart weight; **C.** heart weight/body weight ratio at the two time-points of euthanasia 18 weeks and 24 weeks, respectively

*p < 0.05 (AAB *vs.* sham)

Table I

Evolution of echocardiographic parameters throughout the experiment

Assessed parameter	Baseline		8 weeks		18 weeks		24 weeks	
	AAB	Sham	AAB	Sham	AAB	Sham	AAB	Sham
AWTd, mm	1.31 ± 0.13	1.32 ± 0.07	1.77 ± 0.16	1.45 ± 0.08	2.08 ± 0.18	1.47 ± 0.07	2.05 ± 0.26	1.46 ± 0.16
PWTd, mm	1.32 ± 0.14	1.31 ± 0.08	1.76 ± 0.16	1.43 ± 0.08	2.05 ± 0.16	1.45 ± 0.09	2.04 ± 0.12	1.43 ± 0.11
RWT	0.35 ± 0.04	0.34 ± 0.03	0.44 ± 0.04	0.36 ± 0.03	0.50 ± 0.04	0.36 ± 0.03	0.42 ± 0.03	0.36 ± 0.03
LVDd, mm	7.55 ± 0.32	7.47 ± 0.39	8.03 ± 0.21	7.90 ± 0.30	8.27 ± 0.16	7.95 ± 0.30	9.82 ± 0.74	8.02 ± 0.32
LVDs, mm	4.10 ± 0.19	4.09 ± 0.16	4.41 ± 0.17	4.24 ± 0.10	4.86 ± 0.12	4.36 ± 0.08	8.21 ± 0.53	4.36 ± 0.16
LV mass	0.52 ± 0.07	0.51 ± 0.05	0.86 ± 0.11	0.63 ± 0.06	1.12 ± 0.13	0.65 ± 0.05	1.47 ± 0.28	0.65 ± 0.09
LV mass-index	3.50 ± 0.56	3.50 ± 0.43	3.85 ± 0.57	2.38 ± 0.23	3.85 ± 0.45	1.74 ± 0.23	4.21 ± 0.79	1.38 ± 0.18
FS, %	45.65 ± 3.41	45.1 ± 3.94	45.1 ± 2.36	46.26 ± 2.29	41.17 ± 2.06	45.13 ± 2.61	16.23 ± 4.34	45.52 ± 2.92
EF, %	83.77 ± 3.06	83.23 ± 3.48	83.36 ± 2.1	84.41 ± 1.99	79.57 ± 2.09	83.39 ± 2.32	40.8 ± 8.98	83.73 ± 2.58

LV = left ventricular; AAB = Abdominal aortic banding; AWT = Left ventricular anterior wall thickness; PWT = left ventricular posterior wall thickness; LVDd = LV end-diastolic; LVDs = end-systolic diameters; FS% = Percent LV fractional shortening; RWT = Relative wall thickness; EF% = Percent LV ejection fraction; AWTd = Diastolic AWT; PWTd = Diastolic PWT;

Evolution of echo-cardiographic parameters

Parameters of left ventricular hypertrophy

Diastolic AWT (AWTd) and PWT (PWTd) were similar between the two groups at baseline (p = 0.86 AAB *vs.* sham; p = 0.93 AAB *vs.* sham). Throughout the experiment, AWTd and PWTd increased significantly in the AAB group in comparison to the sham group. Also, in the AAB group, there was a significant increase in AWTd and PWTd between baseline, 8, 18 and 24 weeks follow-up. However, there was no significant difference between the AWTd and PWTd values at 18 and 24 weeks (AWTd p = 0.34; PWTd p = 0.83) within the AAB group (Figure 2). The relative wall thickness (RWT), increased significantly in the AAB group, however at 24 weeks a decrease of RWT was observed due to increasing left ventricular diastolic dimensions (Figure 2). Left ventricular mass (LV mass) was similar in the two groups at baseline and increased gradually over time. LV mass-index, calculated as LV mass per body weight, was similar at baseline and significantly higher in the AAB group at 8, 18 and 24 weeks compared to sham rats (Table I.). We observed a constant increase in LV mass-index in the AAB group, whereas in the sham group we observed a constant decrease in LV mass-index (Figure 2).

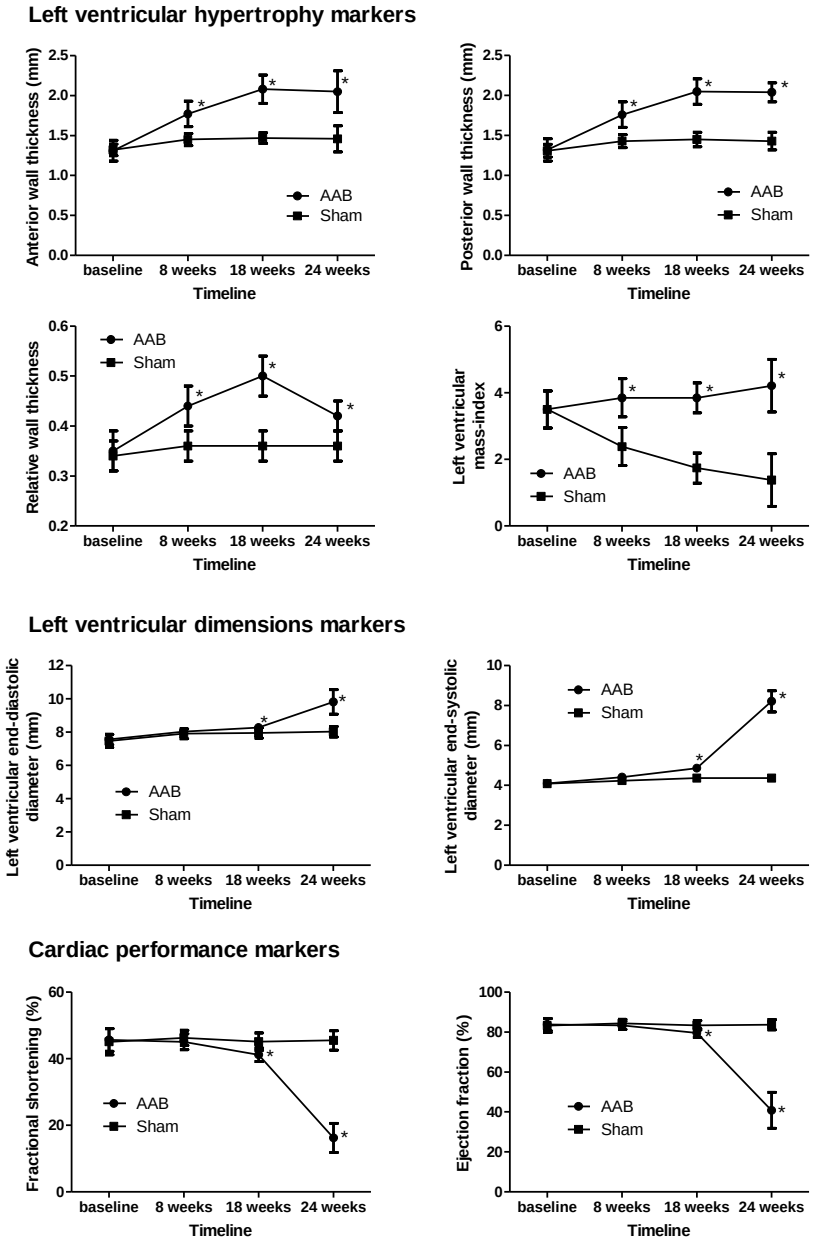

Figure 2.

Evolution of echocardiography parameters of left ventricular hypertrophy, dimension and cardiac performance from baseline to 24 weeks

*p < 0.05 (AAB *vs.* sham)

Left ventricular dimensions

Left ventricular diameters in diastole and in systole were similar at baseline (Figure 5).

At 8 weeks there was no significant difference between the two groups for the LVDd, only at 18 weeks a significant difference could be observed. Both LVDd and LVDs increased gradually in the AAB group over time, with an increase at 24 weeks suggesting significant dilation of the left ventricle.

Parameters of cardiac contractile performance

Fractional shortening (FS%) was not significantly different at baseline and at 8 weeks between the two groups (Figure 2). However, at 18 weeks FS% started showing a significant decrease in the AAB group, resulting at 24 weeks in a dramatic decrease suggesting left ventricular impairment and dilation in this group (Figure 2). Ejection fraction (EF%) was not significantly different at baseline and at 8 weeks between the two groups (Figure 2). However, at 24 weeks EF% decreased significantly in the AAB group.

Histology analysis findings

At 18 weeks, the rat heart showed concentric hypertrophy while at 24 weeks post ligature the rat heart showed eccentric hypertrophy and dilatation. Histological examination of the AAB rat hearts (at 24 weeks) showed hypertrophy of the cardiomyocytes and myofibrillar disarray on haematoxylin-eosin staining (Figure 3). However, by trichrome staining, fibrosis in AAB rats was not extensive, with an in homogeneous distribution, mainly perivascular, and seemed to be concentrated in clusters.

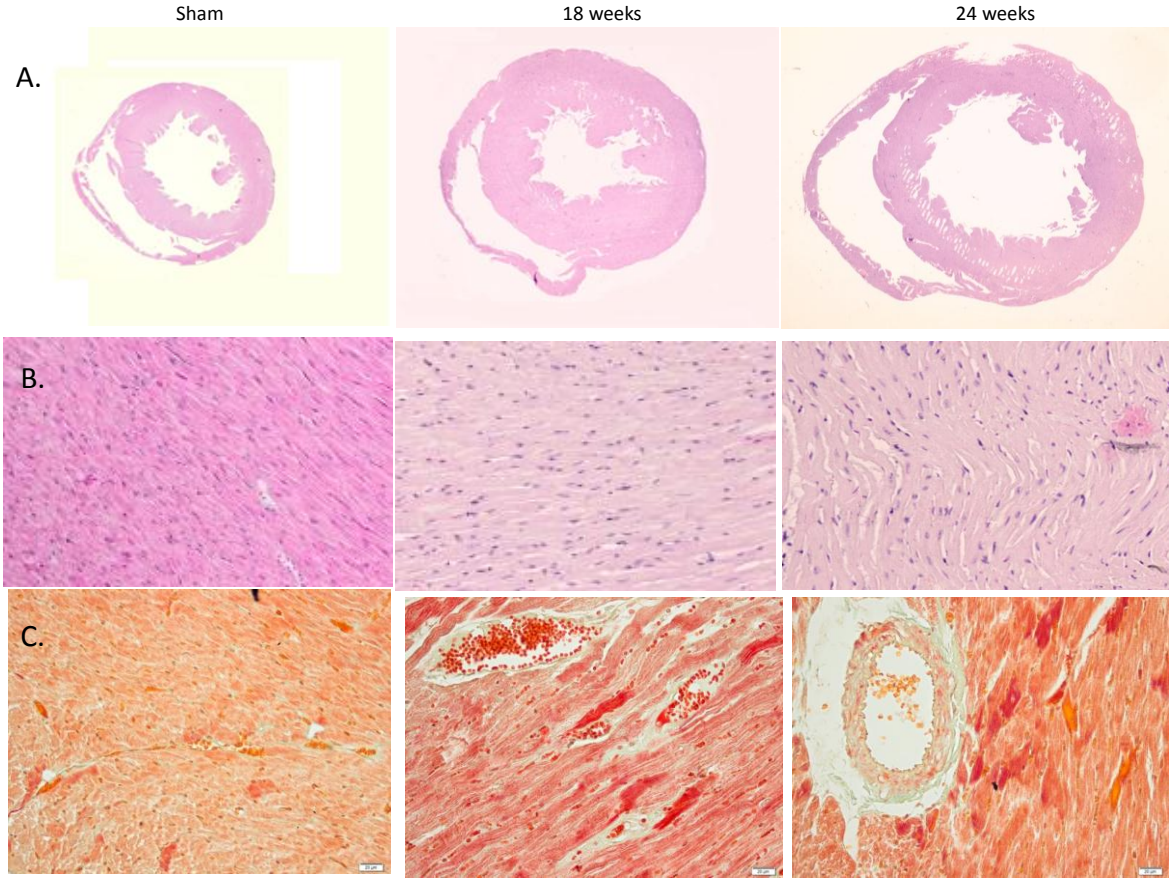

Figure 3.

Representative images of cardiac histology. **A.** Representative images of rat whole-heart transversal cross section obtained by microscopic analysis (haematoxylin-eosin stain). **B.** Magnification x10 of haematoxylin-eosin stained rat heart sections, showing at 18 and 24 weeks post ligature hypertrophy of the cardiomyocytes and disarray of myofiber. **C.** Magnification x40 of trichrome stain showing perivascular fibrosis at 24 weeks post-operation.

Evaluation of oxidative stress

To determine the effects of aortic banding on plasma reactive oxygen species (ROS), plasma levels of malondialdehyde (MDA) and superoxide dismutase (SOD) were determined, as markers of myocardial oxidative stress. In the AAB rats, plasma levels of MDA at 18 weeks were significantly higher than those of sham rats, as well as at 24 weeks (Figure 4 and Table I). However, no significant difference in MDA plasma levels could be found between AAB rats at 18 and 24 weeks. At 18 weeks, SOD plasma levels were not significantly different between AAB and sham rats. However, SOD plasma levels were significantly lower in AAB rats at 24 weeks,

compared to SOD levels in sham rats. MDA and SOD levels at 24 weeks post-operation correlated with delta EF% (EF% at 24 weeks – EF% at 18 weeks), p = 0.01, Pearson r = -0.83 and 0.87 (Figure 5).

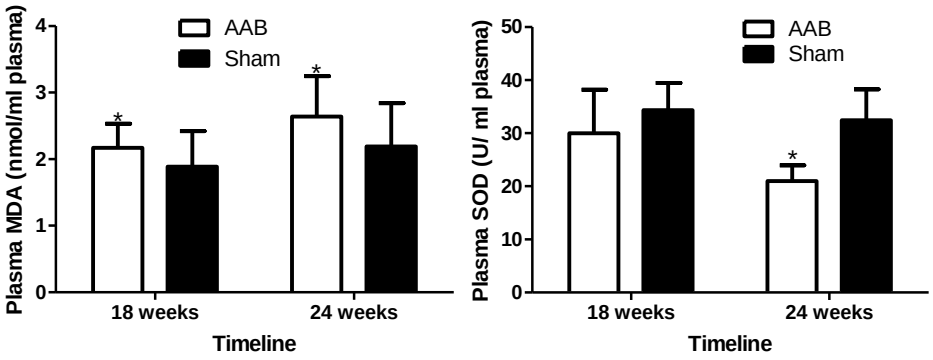

Figure 4.

Plasma malondialdehyde (MDA) and superoxide dismutase (SOD) levels at 18 and 24 weeks for AAB and sham rats
*p < 0.05 (AAB *vs*. sham)

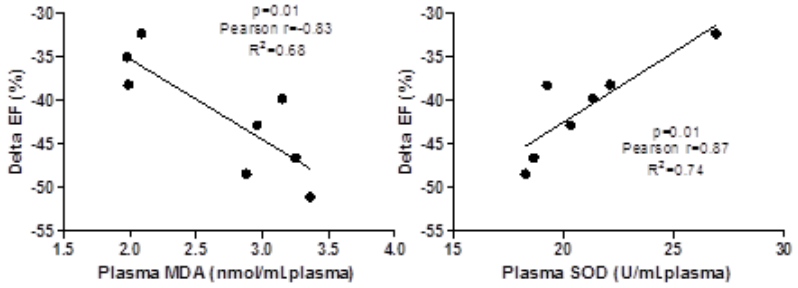

Figure 5.

Correlation of plasma malondialdehyde and superoxyde dismutase with delta EF% at 24 weeks post-operation

Abdominal aortic banding has been used before to induce ventricular hypertrophy in rats. However, in most studies, the constriction of the aorta was made using 21 gauge needles (0.8 mm diameter), resulting in a 30% reduction of blood flow at the point of stenosis. Such a reduction of blood flow usually produces LVH but not always HF [14, 16, 27]. The presented modification to the standard method of inducing ventricular hypertrophy in rats was the use of a 23 gauge (0.6 mm) needle which helped produce a higher reduction of the blood flow at the site of the stenosis.

Evolution of body and heart weight

The slow rate of increase in body weight in the AAB group is consistent with other models of cardiac dysfunction such as ascending aortic banding, Dahl salt-sensitive rats or spontaneously hypertensive rats [7, 9, 27]. This phenomenon may be caused either by anorexia or by an increase in caloric expenditure, or a combination of both. Anorexia and malnutrition have been found in clinical studies to be consequences of chronic heart disease, named by some authors "cardiac cachexia" [17, 22]. On the other hand, several clinical trials have reported an increase in energy expenditure in HF patients, related to a chronic imbalance in the activation of anabolic or catabolic pathways caused by the activation of compensatory mechanisms such as the sympathetic nervous system, immunological or metabolic processes [17]. Further studies are needed to determine underlying causes of weight discrepancies between sham and AAB rats in this particular model and to identify the implicated signalling pathways.

Pressure overload models are characterized by a rapid development of cardiac hypertrophy produced by neuro-hormonal responses, including the activation of sympathetic nervous system and renin-angiotensin-aldosterone system [8] HW and HW/BW ratio are informative markers for the evaluation of cardiac hypertrophy [26]. Thus, our results confirm that the cardiac hypertrophy was present at 18 and 24 weeks post-ligature placement.

Evolution of echocardiographic parameters

The transthoracic echocardiography is a useful clinical diagnostic tool, which has clear advantages as a non-invasive method for the assessment of structural and functional cardiac parameters in rats. Several studies have confirmed the capacity of transthoracic echocardiography to detect cardiac dysfunction in small laboratory animals and especially in rats [4, 15, 18, 24]. Recent studies

have used echocardiography to assess left ventricular remodelling and systolic contractile function for the characterization of different animal models of HF [3, 15], the effect of pharmacological interventions [14, 15] or innovative approaches in the treatment of HF [7, 21]. In our study, we used transthoracic echocardiography to monitor the evolution of the heart structure and function in AAB rats in comparison to sham rats, and to determine the time-points when the animals developed hypertrophy and HF.

The use of xylazine and ketamine during echo-cardiography is well validated both in rats and rabbits, with minimal influence on heart rate and satisfactory results [25].

Parameters of left ventricular hypertrophy and dimension

Evaluation of heart structure and size by transthoracic echocardiography was performed by measurement of specific parameters such as AWT, PWT, RWT, LVDs, LVDd, LV mass and LV mass-index [24].

RWT is a commonly used parameter to describe the degree of left ventricular hypertrophy. RWT can be calculated in different ways: 2PWT / LVDd, 2AWT / LVDd, or a combination of both (AWT + PWT) / LVDd. The combined formula is largely used for the evaluation of left ventricular hypertrophy and is considered to be more accurate [24]. RWT can also distinguish between compensated and decompensated hypertrophy, or between concentric and eccentric hypertrophy as it influences LVDd [1, 24]. In our model, RWT at 24 weeks post ligature placement was significantly decreased compared to RWT at 18 weeks in AAB rats, suggesting increasing left ventricular diastolic dimensions, with preservation of AWT and PWT.

LV mass is a calculated parameter used for the characterization of LVH. Reffelman and Kloner compared the results of the calculated LV mass with results from the autopsy and proved that the formula is accurate for revealing the real LV mass [24]. LV mass has been found to be increased in other studies involving pressure overload models of LVH [20, 21, 27]. However, more accurate in the evaluation of LVH is the LV mass-index, LV mass normalized per body weight [24]. In the present study, LV mass-index has distinguished even better between AAB and sham rats, as over time, LV mass-index increased in the AAB group, while in the sham group it decreased. The same pattern can be observed in other pressure overload models, suggesting that in control rats, up to a certain age, LV mass-index is decreasing and in AAB rats the proportion between LV mass and body weight is inversed [20, 21, 24].

Parameters of cardiac contractile performance

Fractional shortening (FS) and ejection fraction (EF) are the two parameters most frequently used in studies, in order to evaluate cardiac contractile performance. They reveal the capacity of the left ventricle to properly contract and provide oxygenated blood to the body and are able to detect HF [21]. FS and EF can also distinguish compensated from decompensated HF. In the investigated AAB group, up to 18 weeks post-ligature, FS and EF were similar to the sham group, suggesting normal contractile performance in spite of high afterload, due to compensatory hypertrophy. At 24 weeks, both FS and EF decreased significantly, suggesting a decompensated state with HF. Other pressure overload models identified decompensated states with the help of either FS or EF, suggesting the utility of the two parameters in the identification of the exact moment when rats enter the decompensated state [3].

Effects of pressure overload on oxidative stress biomarkers

Oxidative stress is defined as an imbalance between reactive oxygen species and antioxidants production. Increased oxidative stress may cause contractile dysfunction and structural damage in the myocardium, leading to apoptosis of cardiomyocytes [6, 21]. The amount of oxidative stress was assessed by measuring MDA plasma levels and the plasma levels of endogenous antioxidant enzyme SOD. MDA levels were higher in AAB rats compared to sham rats at both time points (18 and 24 weeks), while SOD levels were lower at 24 weeks in AAB rats compared to sham rats. These findings suggest that oxidative stress could be implicated in left ventricular systolic dysfunction in the pressure overload heart failure rat model and that MDA and SOD could represent markers of myocardial dys-function. MDA levels in AAB rats were not significantly different at 18 and 24 weeks post-ligature, suggesting MDA may indicate cardiac dysfunction but do not differentiate between compensated and decompensated HF. However, SOD may be used as a marker of decompensated HF. The use of MDA and SOD as markers of myocardial dysfunction has been also reported by other researchers [12, 21]. Pang *et al.* have reported increased MDA and decreased SOD levels as a consequence of pressure overload and myocardial hypertrophy produced by the placement of a silver clip around the left renal artery of rats [21]. In a recent study, Koyama *et al.* produced a SOD knockout mouse by specifically deleting SOD in the heart and skeletal muscle. SOD-knockout mice exhibited dilated cardiomyopathy and died by six months of age, suggesting SOD is an important antioxidant molecule for the integrity of cardio-myocytes [12].

Additionally, SOD was found to be decreased in a doxorubicine-induced heart failure model and beneficial effects on antioxidant defence were registered after treatment with aliskiren [23].

Conclusions

This particular pressure overload model of HF is potentially clinically relevant to HF progression in humans as the stimulus for HF (high afterload) developed gradually, making the pathological process evolve from compensated hypertrophy to decompensated HF, as is the case in humans. Thus, this particular animal model can be further used for the evaluation of drugs that may prevent or treat LVH and HF.

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