

Doxorubicin dose for congestive heart failure modeling and the use of general ultrasound equipment for evaluation in rats. Longitudinal in vivo study.

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Abstract

Introduction: For the evaluation of the congestive heart failure (CHF) in rat models, the use of special equipment for echocardiography for dynamic evaluation is suggested. The optimal doxorubicin dose for CHF induction has still not been established. **Aims:** The aims of our study was to find a reliable doxorubicin CHF rat model using a general ultrasound (US) equipment for in vivo ultrasound examination of the systemic circulation, to establish the optimal doxorubicin dose, and to assess the feasibility of US guided administration of the drug. **Material and methods:** Sixty Wistar rats, weighing 180-200 g were assigned to 3 groups (n=20 in each): group A - 4 time intraperitoneal doxorubicin “Sigma” administration, cumulative dose 2.49 mg/animal or 12.45 mg/kg; group B and 5 time doxorubicin administration, cumulative dose 3.03 mg/animal or 15.15 mg/kg; and group C- controls (injected same volume of saline). Dynamic US using linear 12 MHz transducer was used to establish the CHF modifications. Two rats with CHF were injected under US guidance in the pleural cavity with 0.06 ml cardiotropic drug levosimendan and two rats in the pericardial cavity. **Results:** We established the optimal cumulative doxorubicin dose for CHF induction at 12.45 mg/kg. At a higher dose, more than 40% of animals died. A lower dose did not induce significant clinical and US CHF criteria. Congestion (followed by weight gain) led to lower animal mortality. Preliminary results indicate a similar positive cardioprotective effect of drug injection into pericardial and pleural cavities under US guidance, demonstrating that this technique is useful for drug administration. **Conclusions:** US is an effective modality for in vivo monitoring of the rat organs for the study of cardiovascular function or for drug administration under US guidance. Suggested model (optimal dose of doxorubicin for simulation of CHF of 2.5 mg/animal, a cumulative dose of 12.45 mg/kg in 4 injections every 3 days) can be used for research purposes.

Keywords: heart failure; ultrasound; doxorubicin; rat models.

Introduction

Mouse and rat models are widely used for research purposes, but the inability of non-invasively collecting the anatomical and physiological data during studies still

limit their utility [1]. In the majority of experimental studies the animals have to be killed and dissected. Therefore intravital evaluation of changes in animals' tissues is a relevant task. Recent technical developments allow the use of high frequency transducers for this objective.

Animal models have permitted the study of the early stages of cardiovascular disease. Doggrell et al [2] suggested that an ideal animal model for the human cardiovascular diseases investigation should meet five characteristics: “(i) mimic the human disease, (ii) allow studies in chronic, stable disease, (iii) produce symptoms which are predictable and controllable, (iv) satisfy economical,

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technical and animal welfare considerations, and (v) allow measurement of relevant cardiac, biochemical and haemodynamic parameters”.

Echocardiography, using special equipment, was largely used for *in vivo* examination of cardiac function [3-8] but systemic circulation of the animals was not sufficiently evaluated. For congestive heart failure (CHF) treatment studies, the selection of animals with similar degree of cardiac dysfunction, the longitudinal follow-up, and the reversibility in drug testing is essential. The CHF diagnosis is difficult to be established in animal models without using invasive procedures. Martinez et al [9] detected the CHF in long-term follow-up of postinfarcted rats using only echocardiographic criteria through a J-tree cluster analysis and Fisher’s linear discriminant function. Echocardiographic analysis was shown to be useful to accurately predict the CHF with 100% specificity and 80% sensitivity.

Doxorubicin, one of the most effective anticancer drugs, is characterized by severe cardiotoxic effects (cardiac remodeling and CHF). The drug was injected intraperitoneally [10,11] and a cumulative dose of 15 mg/kg was suggested for doxorubicin-induced CHF onset [11]. Until now the US guidance of doxorubicin injection for CHF modelling in rats has not been used.

The aims of our study were to find a reliable doxorubicin CHF rat model using a general ultrasound (US) equipment for *in vivo* ultrasound examination of the systemic circulation, to establish the optimal doxorubicin dose, and to assess the feasibility of US guided administration of the drug.

Material and methods

For the study white 2 month old Wistar rats of both sexes, selected on the basis of analogies, weighing 180-200 g, were used. All animals were kept in a vivarium in plastic cages in separate rooms at a constant temperature (20-25°C), and inversed cycle of light-dark (12-12 hours), with free access to food and water. Their condition was observed daily for 21 days. The animals were fed by standard granulated food, according to guidelines “Pets in the vivarium” (Kyiv, 1976), approved by the Ministry of Health of Ukraine. The medical Ethics Committees of the Zabolotny Institute of Microbiology and Virology of National Academy of Sciences of Ukraine and the Clinical hospital “Pheophania” of State Affairs Department approved the study.

The 60 specimens of Wistar rats were assigned in three equal groups (n=20). Group A and B received intraperitoneal injections every 3 days of doxorubicin “Sigma”: 4 time administration, cumulative dose 2.49

mg/animal or 12.45 mg/kg for group A and 5 time administration, cumulative dose 3.03 mg/animal or 15.15 mg/kg for group B. Rats of group C (n=20), the controls (placebo) group, were injected 5 times with the same volume of pure saline.

For US scanning Sonosite Turbo and Hitachi HV 900 machines with 12 MHz linear transducer were used. US examinations were performed by two independent radiologists. The rats were examined while sedated, in a supine position (fig 1), with the chest closed, and the transducer placed gently in the left parasternal position for echocardiography. For abdominal and pleural cavities examination multi-planar approaches were utilized. Soft fixation of animals was provided. General anesthesia was carried out using approved methods. Echocardiography was performed in longitudinal and transverse axis, using M-mode (fig 2) and color/pulse Doppler at the level of the left ventricular outflow tract (LVOT). The heart function was evaluated from the left parasternal long axis with the beams directed 25° cranially. The focus area was



Fig 1. Modality of ultrasonographic examination of the fixed rat.

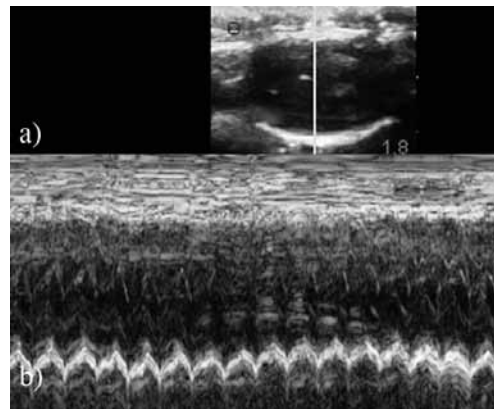


Fig 2. Echocardiography of the fixed rat: a) B-Mode; b) M-mode

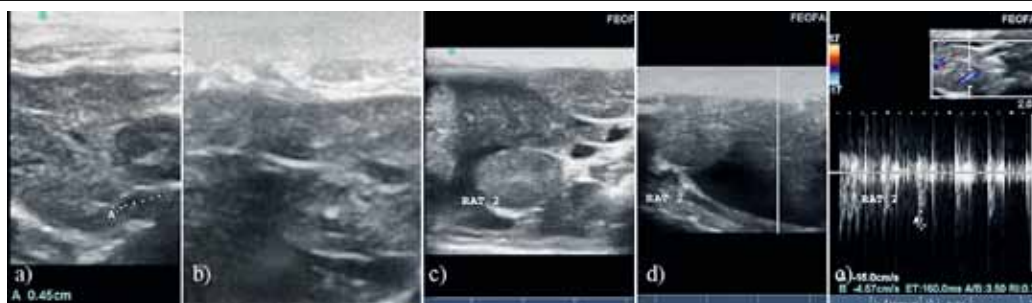


Fig 3. Abdominal US in congestive heart failure rats: a) expanded inferior vena cava to 4.5 mm; b) dilated hepatic veins; c) severe ascites; d) severe hydrothorax; e) resistive index in renal segmental artery 0.71, sign of nephropathy

Table I. Ultrasonography measurements in the study groups

Parameter	Model of heart failure groups (groups A+B)	Control group (group C)	P
Longitudinal liver size, mm	18.0 ± 1.7	15.0 ± 1.5	< 0.05
Longitudinal kidney size, mm	17.0 ± 1.4	16.0 ± 1.3	> 0.05
longitudinal spleen size, mm	17.0 ± 1.8	14.0 ± 1.7	< 0.05
Inferior vena cava, diameter, mm	3.7 ± 2.6	3.0 ± 2.3	> 0.05
Portal vein, diameter, mm	1.78 ± 0.2	1.5 ± 0.3	< 0.05
Ejection fraction, %	46.0 ± 8.0	78.0 ± 6.0	< 0.01
Maximum systolic velocity aortic root, cm/sec	6.5 ± 1.6	11.0 ± 1.3	< 0.01
Resistive Index in the renal segmental arteries	0.7 ± 0.04	0.68 ± 0.04	> 0.05

set at 10–15 mm. Two-dimensional (2D) images of the liver, kidneys, chest, and abdominal cavities were obtained. Doppler techniques were applied for blood flow analysis in the portal vein, inferior vena cava, and renal vessels with 60° angle of insonation beam.

The rat organs were characterized by US using similar criteria and patterns as in humans. We considered US criteria of CHF as following: liver enlargement, expanding of inferior vena cava, decrease of ejection fraction, and presence of ascites and hydrothorax.

Intervention for drug testing. Four supplementary rats that received 15.15 mg/kg doxorubicin in the same modality as group B, were injected on the 15th day with 0.06 ml Simdax (levosimendan), a inotropic and vasodilator agent, under US guidance: two into the pleural cavity and two into the pericardial cavity. Fine needles, 29-31 G, were used. US was additionally performed three times: on the same day, on the 1st day, and on the 2nd day after the levosimendan administration, in order to verify the CHF regression.

Statistical method: The student t-test was applied to perform comparison between groups.

Results

In all 60 rats the target organs were visualized in good conditions at US examinations. The US parameters determined in the study groups are summarized in Table I (table I, fig 2, fig 3).

Establishing dose, weight changes. The dose of doxorubicin lower than 8.59 mg/kg did not lead to modifications in the general condition of animals, mass gain, or US changes in heart and systemic blood circulation.

After the 3rd injection subjective clinical signs of heart failure - cyanosis of tails and paws, animals passivity, and appetite decreased were observed. After the 4th and 5th injections the rats' weight increased significantly comparing to controls (medium for 212.5 ± 8.75 g group A+B and 202 ± 3.2 g in controls, p < 0.05). No significant differences (p > 0.05) were observed when comparing the weights of males or females between group A and B (males 208.75 ± 26.4 g group A and 216.6 ± 14.14 g group B; females 196.6 ± 19.03 g group A and 200 ± 4.5 g group B).

The death of 4 (20%) animals of group A was reported after 3-4 days following the cumulative doses

of 12.25 mg/kg. In group B the death of 8 rats (40% of animals) was observed after 3-4 days after 15.15 mg/kg doxorubicin administration. The difference between the groups was significant ($p < 0.01$). In all these cases the dead animals had the most important weight loss compared with living rats (the medium weight of dead rats was 188.46 ± 2.43 g compared to 201.54 ± 3.2 g of live rats, $p < 0.01$; the medium weight loss of dead rats was 27 ± 2.37 g and of the live rats 13 ± 2.77 g, $p < 0.01$).

Interventions for drug testing. A reduction of symptoms of CHF in all rats injected with cardiotropic drug into the pleural and the pericardial cavity under US guidance was observed (table II).

Discussions

The research studies using mouse and rat models are limited by the inability to obtain noninvasively anatomical and physiological informations, especially in longitudinal studies.

Magnetic resonance imaging (MRI) is considered to be an attractive noninvasive imaging modality without ionizing radiation, offering a sufficient spatial resolution [1]. However, the MRI scanners with a very high magnetic field - up to 14T should be used to study mice and rats and such equipment is available only in few research laboratories.

The US used for research purposes as ultrasound biomicroscopy (UBM) may overcome this obstacle as it confers near-microscopic resolution through the use of high-frequency ultrasound waves [12]. Mircea et al [13] demonstrated the reliability of UBM for the study of ovarian follicles and corpora lutea in mice. This kind of preclinical imaging techniques used for small animals, are available only in the few research centers around the world [14].

In cardiovascular research, animal models have allowed the study of cardiovascular disease in the early

stages, as well as the investigation of the mechanisms of the pathogenesis of cardiovascular disease and the effects of drug intervention. The application of ultrasonic methods using special equipment for examination of cardiac function in vivo was described in rats [3-8].

Bjornerheim et al evaluated the echocardiographic data and found Doppler techniques to be useful than M-mode [3]. The regional heart function was reported to be exactly evaluated using tissue Doppler and 2D strain echocardiography [4]. Color Doppler-guided evaluation of aortic flow and aortic root measurement was reported for the assessment of stroke volume and cardiac output in mice [5]. Recently, reports regarding the use of intravascular probes adapted for the transoesophageal study of rat heart have been published [6].

Few research studies have focussed on the study of echocardiography changes in post infarction CHF and spontaneously hypertensive rats [7-8].

In studies of CHF treatment, it is essential to select animals with a similar degree of cardiac dysfunction. However, this is difficult to establish without hemodynamic evaluation. In all cited papers special US equipment was used. We demonstrated that the US equipment of general use can be effectively applied for in vivo mice examination for preclinical and basic science experiments [15,16].

Summarizing the results of our study, we concluded that 12 mg/kg of doxorubicin can be considered to be the optimal dose for CHF (only 5 rats died after 3-4 days). The higher dose - 15mg/kg - caused deaths of 40% animals. The doses higher than 23.1 mg/kg are inappropriate for CHF model as it led to the death of all animals (unpublished data), while the doses lower than 8.59 mg/kg did not induce significant clinical symptoms and US criteria for CHF.

Doxorubicin is largely used to induce cardiac remodeling and CHF. The drug was administrated as an

Table II. US findings in rats with heart failure rats after levosimendan administration under US guidance

Animal	1 st examination	2 nd examination	3 rd examination
Rat 1*	IVC expanded, liver enlargement	Mild ascite, mild hydrothorax, IVC expanded, liver enlargement	Reduction of pericardial and pericardial effusions
Rat 2*	Mild ascite, IVC expanded, liver enlargement	Mild ascite, hydrothorax, pericardial effusion	Reduction of pericardial and pericardial effusions
Rat 3**	Mild ascites IVC expanded, liver enlargement	Mild ascite, IVC expanded, liver enlargement	Reduction of pericardial and pericardial effusions
Rat 4**	Mild ascite, IVC expanded, liver enlargement	Ascite, hydrothorax, pericardial effusion	Reduction of pericardial and pericardial effusions

*received intrapleural injection; **received intrapericardial injection; IVC- inferior vena cava

intraperitoneal (10 injection of 1 mg/kg [10] or six equal intraperitoneal injections in a 2-week period until a cumulative dose of 15 mg/kg was achieved [11]). Recently 2 mg/kg/week doxorubicin was administered by endovenous infusion [17]. Studies for dose estimation in modeling heart failure in longitudinal rat observations were not conducted. In our study the optimal dose of doxorubicin for simulation of CHF was established to be 2.5 mg/animal, a cumulative dose of 12.45 mg / kg in 4 injections every 3 days.

We observed that CHF with an accumulation of fluid, followed by weight gain, did not lead to animal deaths since animals with less weight died faster. If there is an accumulation of fluid, with high weight, the prognosis seems to be more favorable than the persistent weight loss.

Interventional experiment data confirm that: a) US guided injections were correctly performed because in all 4 rats there were signs of improvement in the cardiac function. This proves that the technique is a feasible technique for an expanding experiment; b) injection into the pericardial cavity and the pleural rat cavities showed a non specified difference between the two approaches. It was supposed that the pericardial pores may function in an allied self-defense mechanism between the pleural and pericardial cavities in mice [18]. Numerous circular fenestrations or pores indirectly connecting the right and left pleural cavities are present in the pericardium of the golden hamster and rat [19]. This phenomenon could be relevant for the choice of the place of injection – the effect should be expected to be similar in both cases.

Although this research was carefully prepared with sufficient number of observations, we are still aware of its limitations. First of all, the research was conducted as an initial part for a large study, concerning the creation of a rat model for testing new medications based on nanoparticles and drug delivery with assistance of US. That is why some parameters were not presented in this paper. Second, our study was limited methodologically by use of general, not special US equipment for precise assessment of the heart tissue. The lack of gold standard to compare in vivo the functional parameters does not allow us to estimate the validity of the method. As CHF occurs in elderly humans, age-related comparative studies on rat models could be relevant. In addition, due to a certain degree of subjectivity in performing the assessment of heart function, the design of study should be double blinded and randomized.

Conclusions

US is an effective modality for in vivo monitoring the rat organs targeted to experiment for the study of cardiovascular function. The interventional ultrasonography is

effective for the expanding of the utility of modeling and drug testing. However, the method is operator related, requires specialized training in US, particularly in small animals.

Suggested model (optimal dose of doxorubicin for simulation of CHF of 2.5 mg/animal, a cumulative dose of 12.45 mg / kg in 4 injections every 3 days) can be used for research purposes, for basic or pre-clinical studies of new drugs, and can be recommended for implementation in research institutes.

Conflict of interest: none

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