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Strain Analysis in the Assessment of a Mouse Model of Cardiotoxicity due to Chemotherapy: Sample for Preclinical Research

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Abstract. *Background: In recent years, the development of more effective anticancer drugs has provided great benefits in patients' quality of life by improving both prognosis and disease-free survival. Nevertheless, the frequency and severity of side-effects, with particular reference to cardiac toxicity, have gained particular attention. The purpose of this study was to create a precise and sensitive preclinical model, able to identify early contractile dysfunction in mice treated with chemotherapy, through use of speckle-tracking echocardiography. Materials and Methods: We generated a mouse model of cardiotoxicity induced by doxorubicin. C57BL 6 mice were divided into two groups, treated for 7 days by intraperitoneal injections of placebo (vehicle) or doxorubicin (2.17 mg/kg), in order to characterize the cardiac phenotype in vivo. Results: We demonstrated that doxorubicin caused ealy remodeling of the left ventricle: after two days of therapy, the radial, circumferential and strain rates were reduced respectively by 35%, 34%, and 39% (p-value ≤0.001). Moreover, histological analysis revealed that doxorubicin treatment increased fibrosis, cardiomyocyte diameter and apoptosis. Conclusion: In a murine model of doxorubicin-induced cardiac injury, we detected left ventricular dysfunction followed by alterations in conventional echocardiographic indices. Our study suggests that a change in strain could be an effective early marker of myocardial dysfunction for new anticancer treatments and, in preclinical studies, it might also be a valuable indicator for the assessment of activity of cardioprotective agents.*

In recent years, the development of more effective anticancer drugs has led to a great increase in overall survival rates with great benefit on patient prognosis, and a substantial decrease in mortality rate. Cardiac toxicity from cancer therapy represents a leading cause of morbidity and mortality in survivors. Patients who develop heart failure from cancer therapy have a mortality rate of 60% at 2 years (1). Consequently, cardiac toxicity from cancer therapeutics has become a fascinating area of interest (2). The term cardiotoxicity includes many possible pathological manifestations such as: arrhythmias, changes in blood pressure, myocardial ischemia or necrosis, pericarditis and venous thrombosis; however, the most important manifestation is a reduction in cardiac function, resulting in heart failure. The development of cardiac dysfunction may occur immediately after drug administration, or up to several years later (3, 4).

Therefore, appropriate management of patients with cancer should include careful consideration of potential cardiotoxicity during therapy, with a focus on early detection and intervention. In this context, a prominent role is, therefore, played by the analysis of changes in myocardial markers, and by the development of preventive strategies involving the use of established cardioprotective medications (1). Among the currently used chemotherapeutic agents usually associated with cardiovascular side-effects, are anthracyclines and monoclonal

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antibody against human epidermal growth factor receptor type 2 (HER2; trastuzumab). The use of anthracycline (both alone and in combination with other drugs) is strongly limited by cardiotoxic effects, which include the induction of cardiomyopathy associated with congestive heart failure (5).

Doxorubicin is an anti-neoplastic agent used to treat various solid and hematological tumors. It inhibits nucleic acid synthesis through intercalation into DNA/RNA strands; in addition, doxorubicin triggers the production of iron-mediated free oxygen radicals that damage DNA and cell membranes (3, 5). Emerging evidence from animal and human studies indicates that doxorubicin-induced cardiomyopathy is mainly due to increased production of reactive oxygen species (ROS), resulting in inflammation, apoptotic cell death and vacuolization of myocardial cells, which are typical changes found in heart failure (5). Such evidence is found both in single treatments, and in combined therapies with molecular-targeted agents.

In order to assess and constrain cardiotoxicity induced by antineoplastic drugs, it is essential to develop new screening protocols aimed at properly studying the toxic effects in animal models. Recently, a novel imaging technique has been developed, speckle-tracking (ST) echocardiography, a non-Doppler-based system, more advanced in both sensitivity and precision than tissue Doppler imaging (6). ST echocardiography is used to detect myocardial wall motion, analyzed by velocity and displacement, and myocardial deformation, analyzed by strain and strain rate (SR). Displacement is a parameter that defines the distance that speckle or cardiac structures move between two consecutive imaging frames; velocity is referred to as the displacement per unit time (7, 8). Strain represents the fractional change in length of a myocardial segment and is one of the most robust of such techniques (9), since it offers enhanced quantification of global and regional function, which are both important in the assessment of left ventricular (LV) remodeling (10, 11).

Echocardiographic image and data acquisition are relatively standardized in humans; however, murine myocardial characteristics require high spatial and temporal resolution (12). Recently, a small-animal ultrasound system was developed, equipped with a high-frequency transducer based on ST analysis (13). The limitations, represented by small animal size (*e.g.* mouse, ~18 g), heart orientation and high heart rates (HRs) of 500-650 beats/min, have been overcome by using high-frequency transducers (up to 70 MHz) (9, 10). While on the one hand, conscious human transthoracic echocardiography is standard practice, on the other, conscious mice cannot be employed for cardiac applications because of the difficulty in positioning of the transducer; therefore, alterations in sympathetic and parasympathetic tone will inevitably result. Anesthetics have significant effects on cardiovascular functions, such as reduction of HR, hypothermia and ventricular repolarization. For this reason, image acquisition under mild anesthesia is strongly recommended (9). The most common anesthetic procedures require the use of ketamine and xylazine, tribromoethanol (Avertin), chloral hydrate, barbiturates (pentobarbital, thiobutabarbital), or the inhalation of volatile anesthetics, such as isoflurane, and halothane; these agents have diverse pharmacological profiles and produce multiple and variable cardiovascular effects in animals. The ideal anesthesia for mouse echocardiography, in addition to being mild (13), should also be easy to administer, reproducible, rapid in onset and recovery, and should cause minimal variations in HR, minimal cardiovascular depression, and low toxicity (14).

In this study, we evaluated the utility of strain analysis in mice during treatment with doxorubicin in order to identify early impairment of left ventricular function, and to propose a preclinical model of cardiotoxicity useful for identifying early contractile dysfunction in mice treated with chemotherapy.

Materials and Methods

Drugs. Doxorubicin (ADRIBLASTINA*IV 2 mg/ml; Pfizer) was administered to mice *via* intraperitoneal injection at the dose of 2.17 mg/kg for 7 days. This schedule of treatment was calculated considering the combination of several factors, such as basal metabolism of mice and metronomic chemotherapy (15, 16). The anesthetic agent used was Zoletil 100 (Virbac), a combination at 50% of tiletamine and zolazepam; it was used at a dose of 50 mg/kg, by adding atropine sulfate at 0.04 mg/kg, and administered *via* intraperitoneal injection.

Animal models. The C57Bl/6 is the most widely used inbred mouse strain, and is commonly employed in cardiovascular research (6). A total of 20 C57BL/6 JOlaHsd female mice (Harlan Italy, San Piero al Natisone, UD, Italy), 8 weeks old and weighing 19.00-19.50 g, were divided into two groups on the basis of different treatments (n=10 per group): a placebo group, receiving only a saline solution, and a doxorubicin group, treated with doxorubicin (2.17 mg/kg). All mice were treated by intraperitoneal injection for 7 days. The mice were monitored for body weight and heart function before the start of the experiment and 2 days and 7 days later. They were housed in standard Plexiglas cages and maintained on a 12 h light/12 h dark cycle in a temperature-controlled room (22±2˚C), with food and water *ad libitum*. At the end of the experiments, after 7days, animals were sacrificed, by cervical dislocation. All the animal experiments were performed according to European Directive 63/2010/UE and Italian Law (DL 26/2014, authorized by the Minister of Health, Italy).

Echocardiography. *In vivo* cardiac function was assessed by transthoracic echocardiography in sedated mice by using a Vevo 2100 (Visualsonics, Toronto, Canada) high-resolution imaging system, with 22-55 hMHz transducer. Mice were sedated and placed in supine position on a temperature-controlled surgical table to maintain rectal temperature at 37°C. Continuous echocardiographic monitoring was obtained *via* limb electrodes (17); limbs were kept in position using a small amount of silk plaster.

Table I. *Heart and basic parameters measured in the studied mice. Data were measured at baseline, and after 2 and 7 days of treatment with placebo, and with doxorubicin.*

	Basal		2 Days		7 Days	
	Placebo	Doxorubicin	Placebo	Doxorubicin	Placebo	Doxorubicin
n	10	10	10	10	10	10
BW (g)	19.43±0.63	19.56±0.34	19.67±0.64	19.32±0.41	20.15±0.59	19.08±0.29†
HR (bpm)	555.8±34.8	557.3±26.3	551±34.9	556.5±57.8	552±31.5	558.1±40.3
HW (g)				0.09±0.01	0.12±0.01†	
LV Mass (mg)	50.9±7.0	51.1±8.3	52.4±10.2	54.8±9.5	50.4±10.2	55.1±11†
LV Vol (µl)	3.2±0.3	3.0±0.4	3.1±0.4	6.0±2.0 *	2.7±0.8	7.3± 3.7†
LVIDd (mm)	3.04±0.1	3.04±0.1	3.08±0.12	3.36±0.3	2.96±0.26	3.0±0.2
LVIDs (mm)	1.16±0.05	1.18±0.04	1.18±0.06	1.40±0.2	1.19±0.17	1.60±0.3†
FS (%)	61.9±1.2	61.9±1.2	62.2±1.03	53.7±6.2	62±0.82	48±6.9†‡
EF%	91.2±0.5	90.9±0.6	91.6±0.7	85.2±5.2	91±0.5	78.9±6.3†‡
RS (%)	39.3±4.5	39.5± 4.5	41.1±5.03	26.2±7.6*	37.8±3.7	18.5±5.3†‡
CS (%)	–40.9±6.2	–40.9±6.2	–36±5.3	–27.2±5.3*	–36.2±4.6	–19.2±6.6†‡
SR (1/s)	13.4±0.5	13.1±0.4	12.8±1	8.2±2.3*	12.4±1.2	5.4±3†‡

Significantly different at $p<0.001$ for placebo vs. 2 days of doxorubicin, †placebo vs. 7 days of doxorubicin, ‡2 days vs. 7 days of doxorubicin. Data are presented as the mean±SD. BW: Body weight; HR: heart rate; HW: heart weight; LV Mass: left ventricular mass; LV Vol: left ventricular volume; LVIDd: diastolic left ventricular internal dimensions; LVIDs: systolic left ventricular internal dimensions; FS: fractional shortening; EF: ejection fraction; RS: radial strain; CS: circumferential strain; SR: strain rate.

Cardiac function was evaluated by echocardiography under basal conditions and after intraperitoneal treatment of doxorubicin and placebo at 2 and 7 days. LV echocardiography was assessed in both parasternal long-axis and short-axis views at a frame rate of 233 Hz. End-systole and end-diastole dimensions were defined as the phases corresponding to the ECG T-wave, and to the R-wave, respectively. Measurements of diastolic LV internal dimensions (LVIDd) and systolic LV internal dimensions (LVIDs) were averaged from three to five beats. LVIDd and LVIDs were measured from the LV M-mode at the mid papillary muscle level. Fractional shortening (FS) percentage was calculated as [(LVIDd−LVIDs)/LVIDd]×100, and ejection fraction (EF) percentage was calculated as [(EDvol−ESvol)/EDvol]×100 (18), where EDvol and ESvol stand for end-diastolic volume and and-systolic volume, respectively. Strain, expressed as a percentage, is the deformation of the myocardial wall compared to its original size.

Acquired B-mode loops were imported into VevoStrain software (VisualSonics, Toronto, ON, Canada). Three consecutive cardiac cycles were selected and, upon adequate tracing of the endocardium, an epicardial tracing was performed. ST-based strain recording allowed assessment of strains specific to six myocardial segments per LV view. Internally, eight points were measured for each of the six segments, resulting in 48 data points in total (9). Strain measurement is useful in the detection of regional myocardial function. Strain was evaluated in three axes: radial, circumferential and longitudinal. Radial strain (RS), defined as the percentage change in myocardial wall thickness, can be measured in both the short- and long-axis views and is expressed in 1/s (12); it describes a positive curve reflecting increasing myocardial thickness during systole and diminishing wall thickness during diastole, and represents myocardial deformation toward the center of the LV cavity. Circumferential strain (CS) represents the percentage change in LV myocardial fiber shortening along the circular perimeter of the heart and is measured from a short-axis view. Longitudinal strain (LS) detects the percentage change in length of the ventricle, typically measured from the endocardial wall in the long-axis view (9, 12). We also calculated the SR, which represents the myocardial deformation rate and which is expressed in 1/s.

Morphological examination. Hearts from the studied mice were fixed with 10% formalin and embedded in paraffin. Sections were stained with hematoxylin and eosin (H&E) for morphological tissue analysis.

Cardiac fibrosis analysis. Interstitial fibrosis was evaluated by staining 5-µm-thick tissue sections with 1% Sirius red in picric acid (Carlo Erba Laboratories, Milan, Italy), as previously described (19). The positively stained (red) fibrotic area was measured using a computer-assisted image analysis system (Nikon NIS ELEMENTS BRV, Melville, NY, USA) and expressed as a percentage of total area. The percentage of red staining was calculated from all samples, with two sections for each sample and five images for each section.

Cardiac apoptosis. Cardiac sections were also examined for the presence of apoptotic cardiomyocytes (performed on 6-µm sections) by TdT-mediated dUTP nick-end labeling (TUNEL) assay using a Promega DeadEnd™ colorimetric TUNEL system (Promega, Madison, WI, USA) with a streptavidin-peroxidase system. Controls were obtained by omitting TdT enzyme from the reaction mixture. The percentage of TUNEL-positive myocytes was determined by counting 10 random fields per section under a microscope (Nikon NIS ELEMENTS BRV, Melville, NY, USA). Using this procedure, apoptotic nuclei were stained dark brown. Labeled nuclei were counted and expressed as the percentage of positively stained cells (20).

Statistical methods. The number of animals required per group (n=10 mice) was calculated using the software G * Power (Erdfelder, Faul, & Buchner, Dusseldorf, Germany), in particular the F tests (ANOVA:

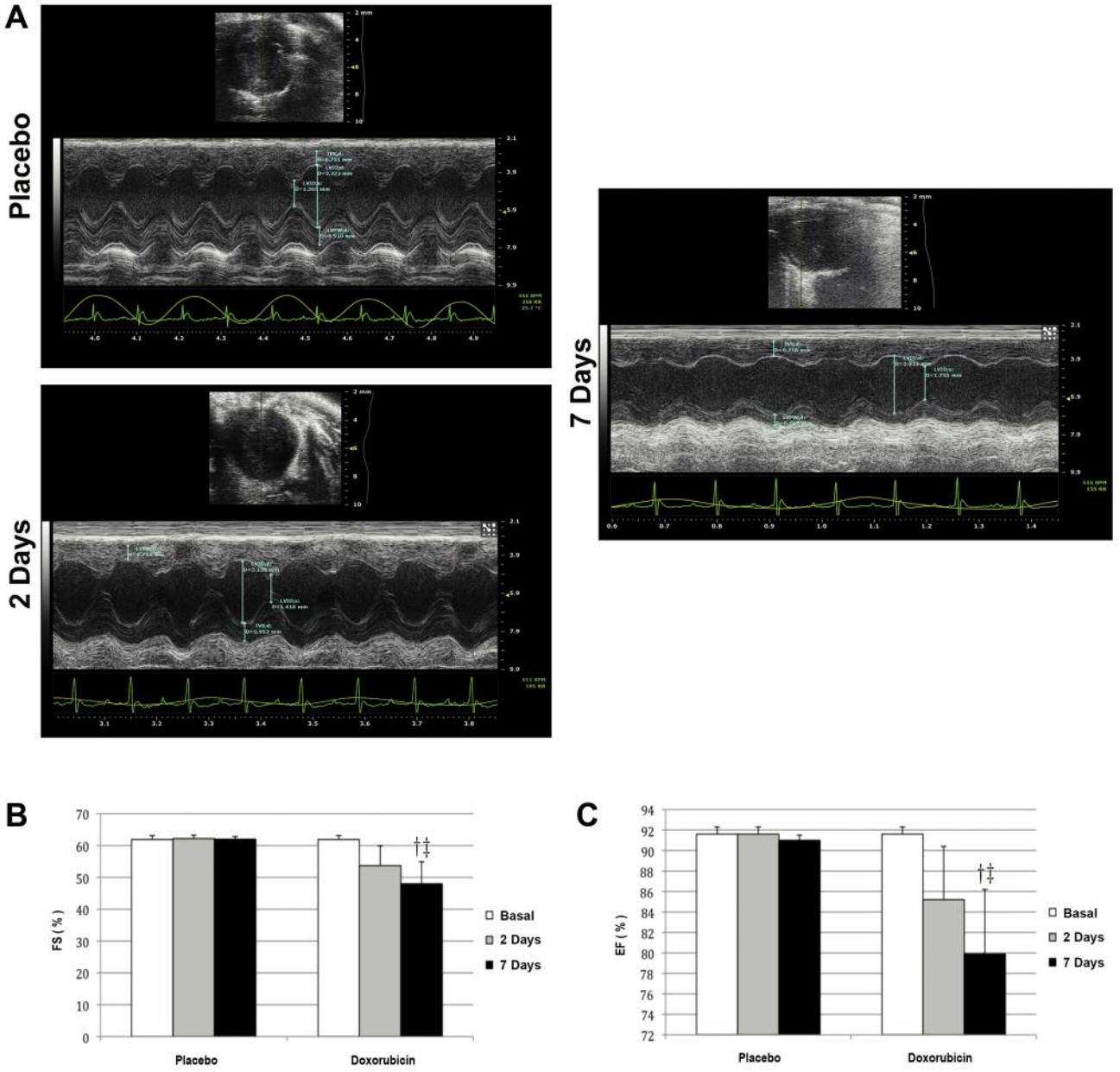

Figure 1. *Cardiac function evaluated by echocardiography. A: M-Mode imaging with the measurements of diastolic left ventricular internal dimensions (LVIDd), systolic left ventricular internal dimensions (LVIDs), interventricular septal end diastole (IVSd) and left ventricular posterior wall end diastole (LVPWd) in mice treated with placebo at 2 and 7 days of treatment with doxorubicin. B: Fractional shortening (FS). C: Ejection fraction (EF). Significantly different at p<0.001 for †placebo vs. 7 days of doxorubicin, ‡2 days vs. 7 days of doxorubicin. Data are presented as the mean±SD of n=10 mice.*

fixed effects, omnibus, one-way). This number of subjects has a power of 95%, with an α (error probability) of 0.05 and an effect size f equal to 1. Analysis of echocardiography and histological data was performed by two researchers blinded to experimental conditions. The statistical analysis was performed using Ri386 3.1.3. Data are presented as the mean±SD. Between-group differences were assessed by Student's *t*-test or ANOVA as appropriate. Statistical significance was defined as $p \leq 0.001$ (significance level of 0.1%).

Results

Doxorubicin treatment and mouse survival. C57BL/6 mice, under basal conditions, exhibited the structural and functional features of normal heart, in accordance with the literature. Data on the heart characterization are reported in Table I.

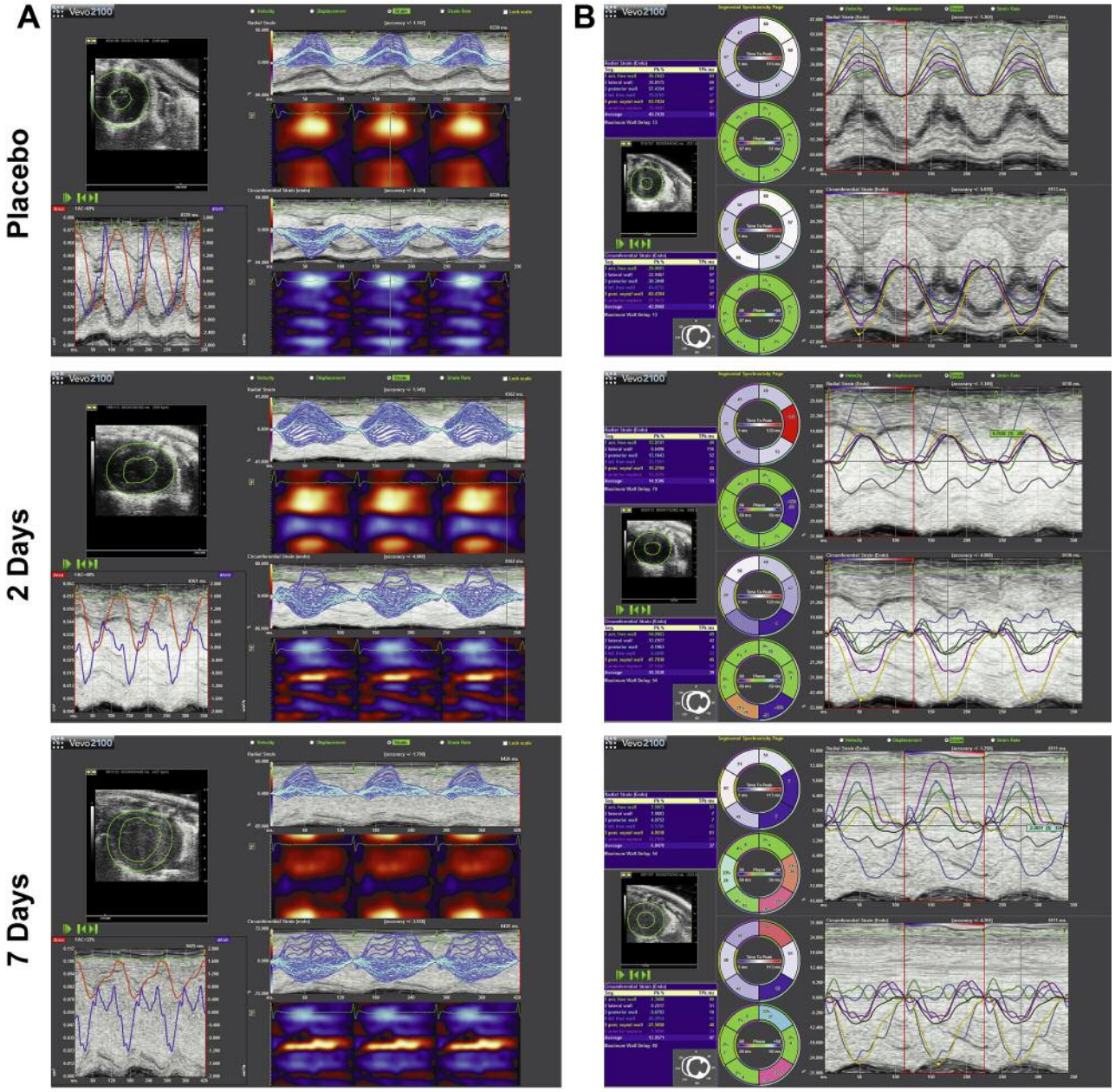

Figure 2. *Strain analysis. A: Parametric imaging of myocardial deformation in two dimensions and velocity data: regional and global wall motion tracking, offering quantification of wall velocity, as anatomical M-Mode heart tracings and displacement. B: Time-to-peak analysis and radial strain (RS) and circumferential strain (CS) curves showing the amount of time following the electric stimulus responsible for heart contraction, where the strain reaches its maximum. Separate strain curves were generated for each of the six standard myocardial regions, with a seventh line (black) denoting the average strain at each time point. These parameters were evaluated at basal, and at 2 and 7 days of treatment with doxorubicin.*

Doxorubicin was administered daily to mice of the doxorubicin group at 2.17 mg/kg until the end of the experiment; this dosage is 1/10 of the toxic concentration reported in the literature (5, 21) (20 mg/kg for intraperitoneal injection). All mice survived until the end of the study. In the doxorubicin group, on the seventh day of treatment, body weight was less than that of the placebo group ($p \leq 0.001$); however, it did not reach the toxic cut-off weight, set at a weight reduction of more than 20% as compared to the initial weight (Table I), thus confirming the safety of the experimental procedure.

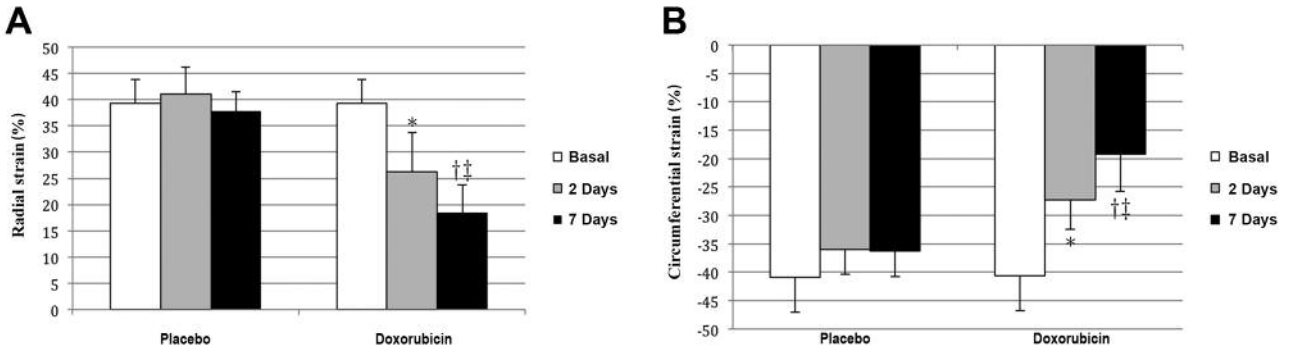

Figure 3. *Strain measurements of radial strain (RS) (A) and circumferential strain (CS) (B). Significantly different at p<0.001 for *placebo vs. 2 days of doxorubicin, †placebo vs. 7 days of doxorubicin, ‡2 days vs. 7 days of doxorubicin. Data are presented as the mean±SD of n=10 mice.*

Optimal anesthesia for echocardiographic examination. Measurements were performed at baseline and after 2 and 7 days of treatment. Acquisition of B-mode images in the parasternal short-axis view, followed by acquisition of M-mode, involved 2-5 min of image acquisition time per view. The image acquisition was performed under mild anesthesia with a mixture of Zoletil (see Materials and Methods) and atropine (0.006 ml/10 g of body weight). Upon this dosage, the average HR was 557±54 bpm, and the average FS and EF were 61% and 90%, respectively; these results were collected through all time points of ultrasound analysis *i.e.* at baseline, 2 days and 7 days (Table I).

LV function and doxorubicin-induced cardiomyopathy. Two days after treatment, the doxorubicin group displayed impaired systolic functions reflected in reduced LVIDs, FS and EF (all at $p<0.05$; Table I). After 7 days of treatment, cardiac function of the doxorubicin group was extremely impaired with respect to the placebo group. Values for LVIDs, FS and EF were all statistically significantly lower as compared to those of the placebo group at 7 days ($p<0.001$); remarkable differences were also observed in comparison with values after 2 days of treatment (Figure 1 and Table I). In the doxorubicin group, there was a significant ($p<0.001$) increase of both LV volume and LV mass: LV mass was increased at 7 days, and LV volume was higher after 2 and 7 days of treatment compared with placebo values at the same times (Table I). Therefore, at 7 days, the doxorubicin-treatment induced significant pathological remodeling of global LV function.

Strain analysis is a powerful technique for measuring early disturbances of heart function. Strain on short-axis images and ventricular function were studied by myocardial deformation along the radial, and circumferential axes. The strain analysis showed statistically significant alterations, as early as 2 days after treatment with doxorubicin, reducing RS and CS ($p<0.001$) compared to placebo; myocardial deformation was highly compromised after 7 days of treatment with doxorubicin. These data are statistically significant compared to baseline values and values at 2 days of doxorubicin treatment (Figures 2 and 3, and Table I). With regard to the LS, our results show a decrease of values at both 2 and 7 days, as demonstrated for RS and CS (data not shown). Interestingly, SR values in the doxorubicin group were reduced after two days of treatment ($p<0.001$), and were further reduced after 7 days ($p<0.001$) (Figure 4). The analysis of time-to-peak of SR indicates evident dys-synchrony of segment shortening as early as 2 days of doxorubicin treatment, becoming overt at 7 days (Figure 4). Myocardial dys-synchrony of segments was observed in all imaging: after 2 and 7 days of doxorubicin treatment, as compared with images from the placebo group (Figures 2-4). The displacement and velocity of the heart, represented as vectors, can be used for characterization of global and regional myocardial mechanics; treatment with doxorubicin resulted in reduced strain compared with the basal and placebo groups, as demonstrated by the smaller size of the vectors. The length of arrows in the figure was, in fact, substantially reduced after 2 and 7 days of doxorubicin treatment, with respect to the placebo group (Figure 5).

Increase in collagen deposition, apoptosis, and cytoplasmic vacuolization in hearts with impaired function. Mice of the placebo group (n=10) exhibited normal cardiac histology, whereas the hearts of mice treated with doxorubicin had impaired morphology, as revealed by the presence of cardiac hypertrophy and cytoplasmatic vacuolization. After 7 days of treatment, the diameter of cardiomyocytes in the doxorubicin group (n=10) was significantly greater than that of the placebo group ($p<0.001$) (Figure 6). Cardiac hypertrophy developed in mice after doxorubicin treatment was significantly increased with respect to mice of the placebo

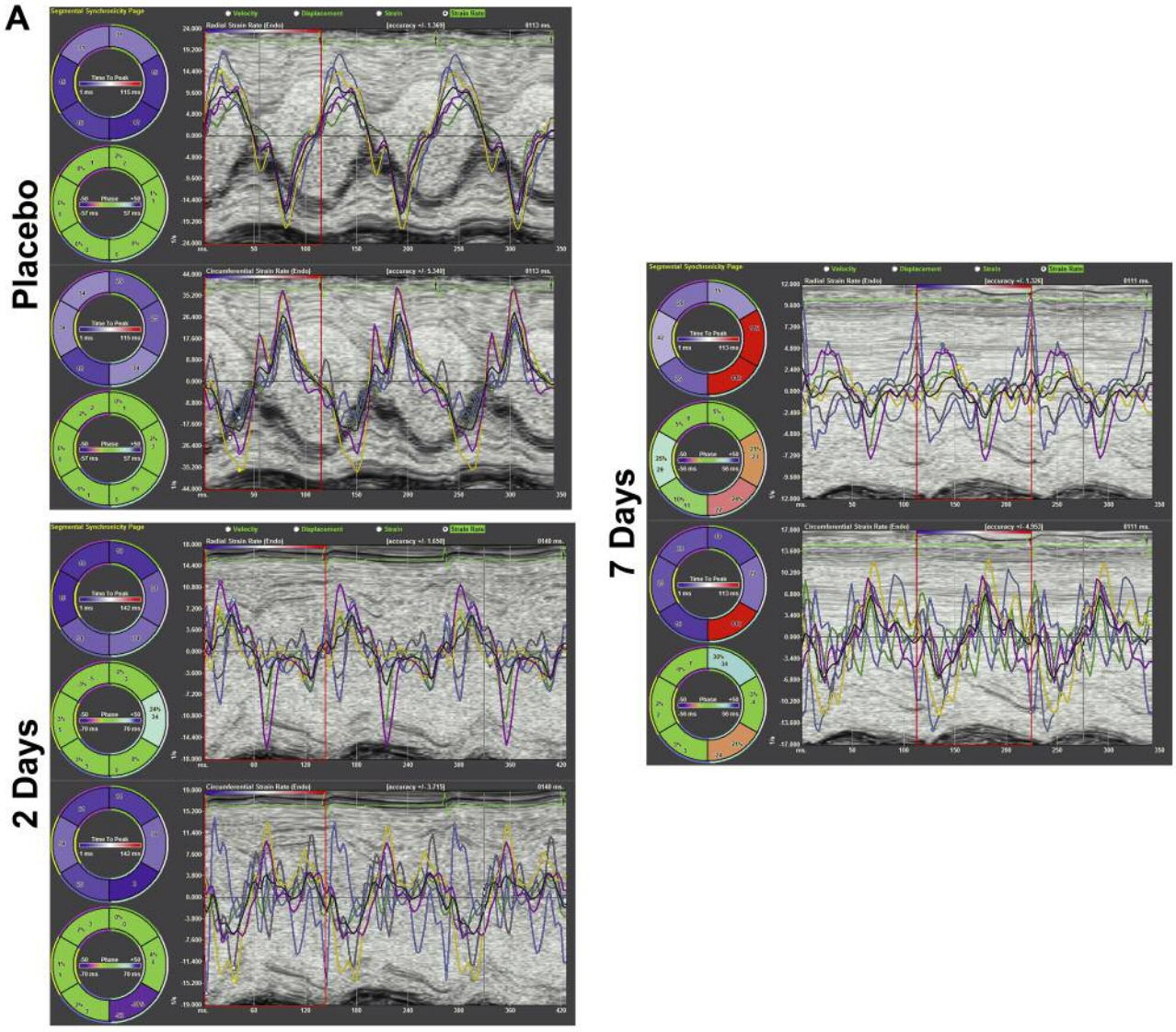

Figure 4. *Strain rate (SR). A: Time-to-peak analysis of SR, showing synchronous (placebo) and dys-synchronous (2 days and 7 days of doxorubicin) velocity components. B: SR graphic. Significantly different at p<0.001 for *placebo vs. 2 days of doxorubicin, †placebo vs. 7 days of doxorubicin, ‡2 days vs. 7 days of doxorubicin. Data are presented as the mean±SD of n=10 mice.*

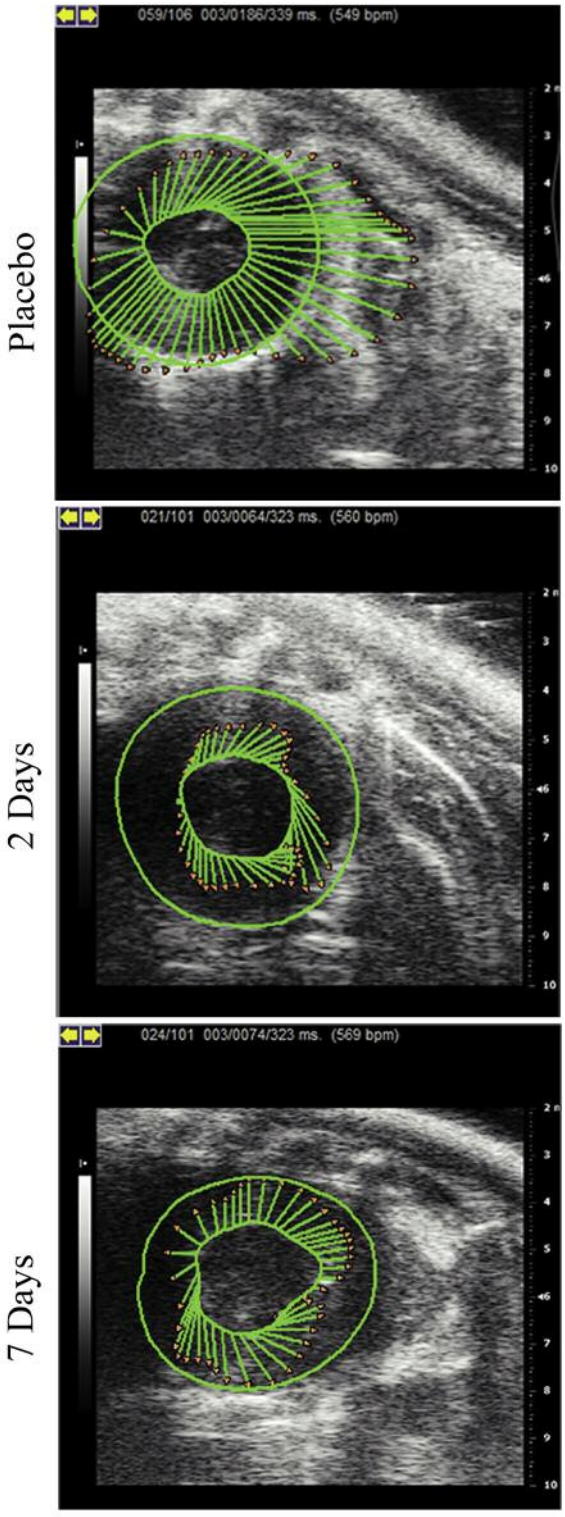

Figure 5. *Strain-based vector mapping of cardiac function. Displacement encoding with echocardiographic vector map imaging for regional strain in basal, two days and seven days of treatment. The displacement (length in cm) of vectors was measured as the distance traveled by the kernels from peak diastole to full systole and serial vectors (arrows), reflecting tracking of segmental myocardial deformation during contraction.*

group. Interstitial fibrosis was expressed as a relative percentage by color intensity; the percentage collagen values were greater than those observed for the placebo group ($p<0.001$) (Figure 7). We also observed an increased heart weight in doxorubicin-treated mice, as compared with those of the placebo group ($p<0.001$) (Table I).

The histopathological investigation revealed statistically significant greater apoptosis in cardiac tissue of the doxorubicin group *vs.* the placebo group (Figure 7).

Discussion

This study provides a complete structural and functional echocardiographic characterization of LV remodeling in a mouse model of cardiotoxicity. Our mouse model offers a number of advantages: low costs, little variability and easy availability of inbred models (22). The evaluation of cardiac function in mice to date has been hampered by technical issues, such as fast mouse HRs, difficulty in obtaining clear echocardiographic imaging, and translational motion during image acquisition.

Some studies demonstrate that the type of anesthesia and the timing of echocardiographic measurements after anesthesia may have significant effects on echocardiographic parameters in mice (13, 14). The intraperitoneal agents tribromoethanol and ketamine and xylazine, as well as the inhalation of volatile anesthetic isoflurane, led to early depression of cardiac function, with an increase in FS over 20-min study, and were associated with increased HR after 15 min (14, 23). The effects of these anesthetics are all time-dependent, in fact the FS, EF and HR parameters stabilize around the optimal values for a good examination at the time points between 15 and 20 min (24). A fair compromise for these problems has been found using a mixture of tiletamine/zolazepam with 0.01% atropine. Atropine was used for two reasons: the first was to maintain a high HR and the second to reduce sialorrhea induced by the vagus nerve. Used at this dose, atropine has a minimal impact on cardiac function. Our study demonstrates that this type of anesthesia is mild and echocardiographic parameters are optimal as soon as a few minutes from sedation; moreover, repeated ultrasound analysis in a relatively short time does not produce adverse effects in animals.

Doxorubicin, commonly used for the treatment of different cancer types, is characterized by induction of cardiotoxic effects even after the first treatment, hence its use is strongly limited (25); for this reason, this anticancer agent was utilized as a positive control in our model of cardiac damage. The doxorubicin dosage used here for the determination of LV remodeling and congestive heart failure, was 10-times lower than that most frequently used in experiments with mice (20 mg/kg) (3, 21); this latter dosage is very similar to the reported intravenous for mice (21.1 mg/kg) (26). Similarly to our previous studies (27), mice were injected

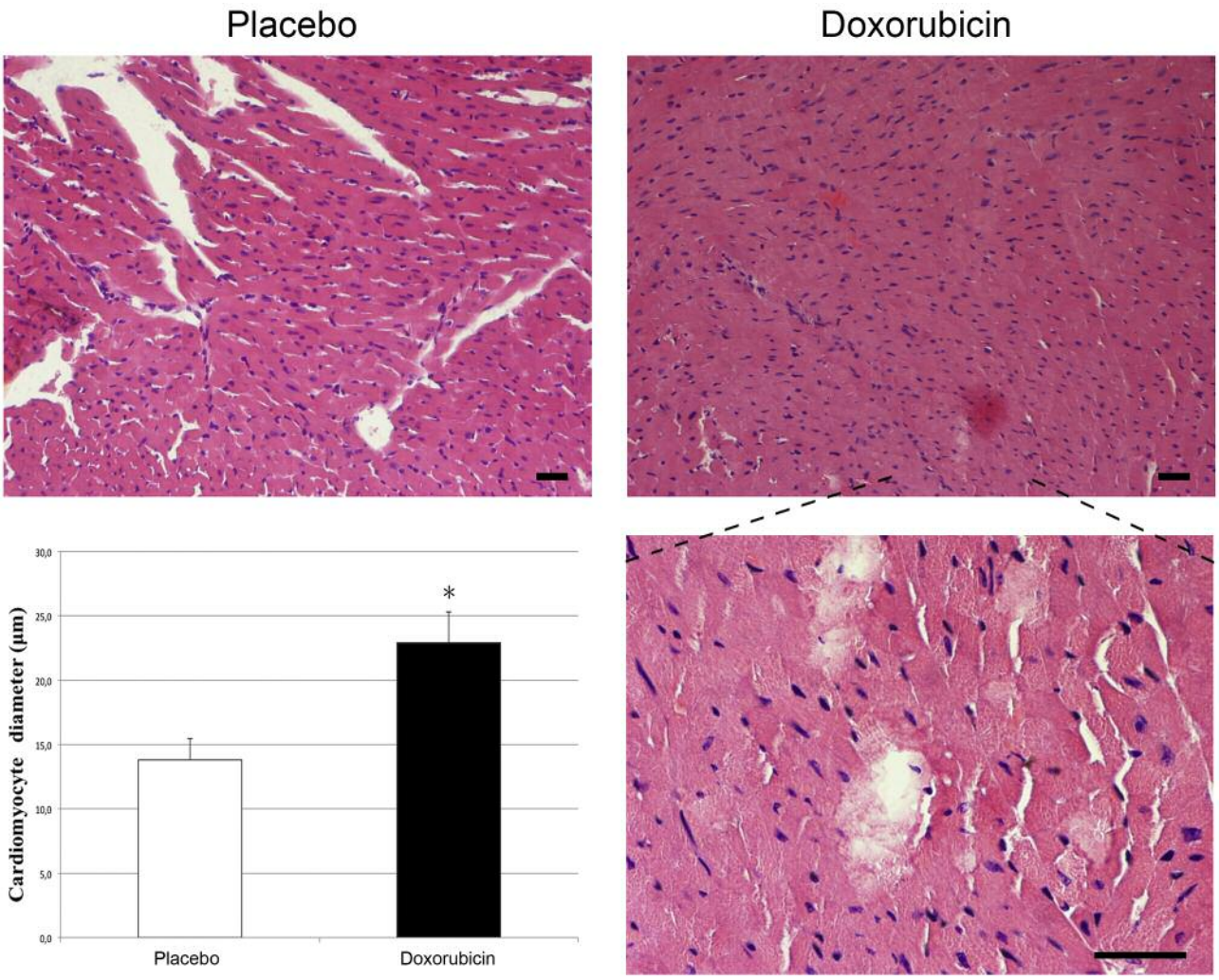

Figure 6. *Cell injury due to doxorubicin therapy. Morphology of cardiomyocytes was examined on histological sections stained with hematoxylin and eosin. In mice treated with doxorubicin (n=10), the diameter of cardiomyocytes increased, and there was hypertrophy (right) and cytoplasmic vacuolization when compared to mice of placebo group (left). The graph shows the diameter of cardiomyocytes in the studied mice; the diameter was increased in those treated with doxorubicin as compared to those of the placebo group. *Significantly different at p<0.001 for placebo vs. 7 days of doxorubicin. Scale bar=20 µm. Data are presented as the mean±SD of n=10 mice.*

with a cumulative dose of 15 mg/kg doxorubicin for 7 consecutive days by intraperitoneal injection. Dose was calculated taking into account the body surface area and the basal metabolism, and was in agreement with the schedule of treatment using metronomic chemotherapy (15, 16). This method is the most appropriate and is far superior to the simple conversion based on body weight alone (15).

In agreement with previous reports (28, 29), myocardial strain analysis in our doxorubicin-induced cardiomyopathy model indicated that LV dysfunction and hypertrophy caused a decrease in CS and RS, as compared with baseline, as early as after 2 days of doxorubicin treatment. The quantitative data (parametric 2-D imaging) allowed a quick visual assessment of LV function, thus enabling the identification of global cardiac alterations following or concurrently with anticancer therapies (9, 30). Safety of the experimental procedure was demonstrated by the lack of any adverse reactions or deaths of the animals over the duration of the study. This model was confirmed by histological analysis performed on hearts of mice treated with doxorubicin.

The main mechanisms by which doxorubicin exerts its cardiotoxicity is through the development of oxidative stress, which in turn is involved in sarcopenia, as well as altered calcium metabolism and mitochondrial activity, which may cause ultrastructure alterations, consisting of the loss of myofibrils and the dilatation of the sarcoplasmic reticulum and

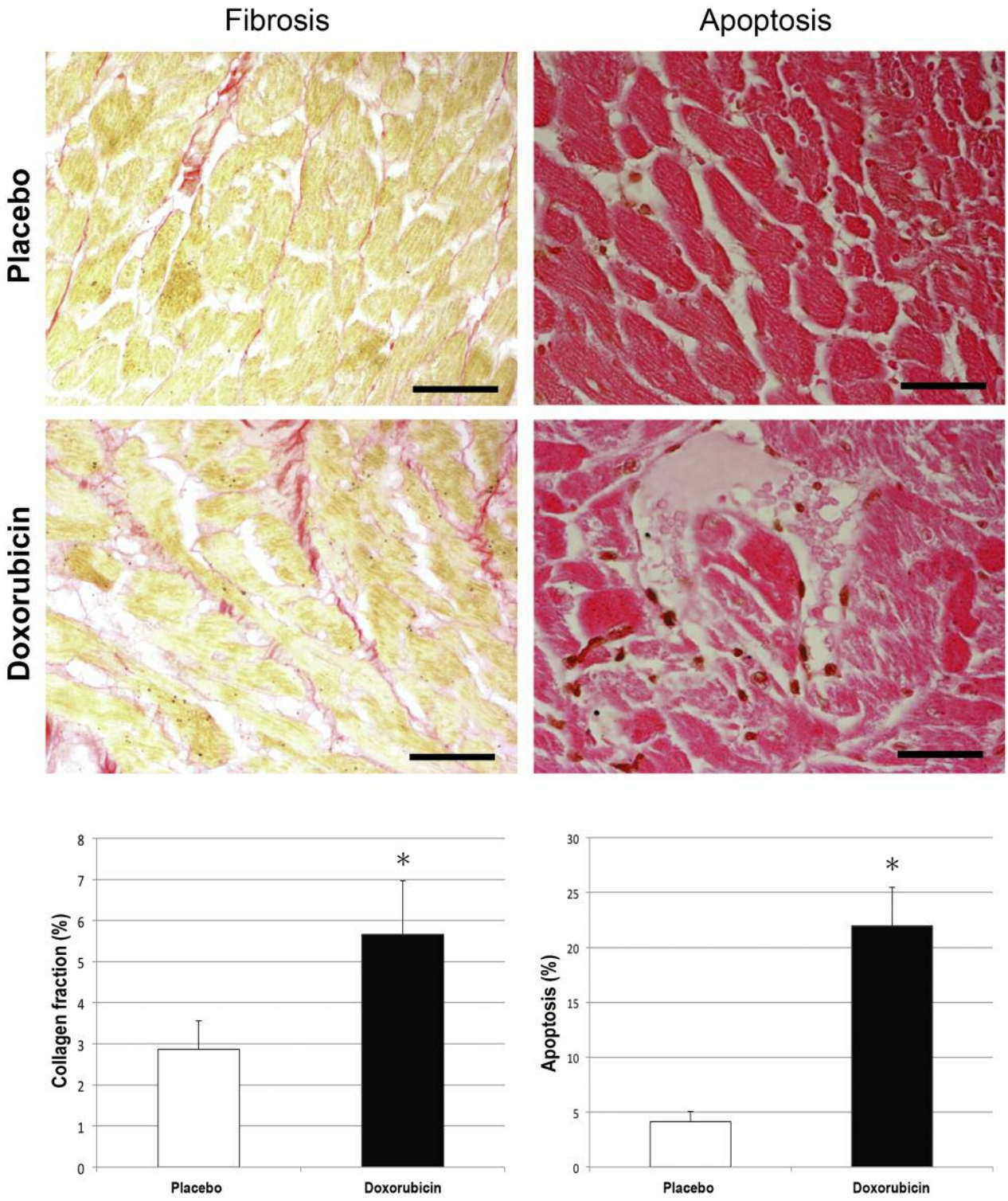

Figure 7. *Quantification of fibrosis and apoptosis. The left column shows the analysis of collagen, indicative of fibrosis, and the right column shows the analysis of apoptosis. The images display the histological examination in placebo and doxorubicin groups at 7 days. *Significantly different at p<0.001 for placebo vs. 7 days of doxorubicin. Scale bar=20 μm. Data are presented as the mean±SD of n=10 mice.*

cytoplasmic vacuoles (26). Doxorubicin also causes cardiomyocyte death, leading to necrosis and apoptosis. Furthermore, it alters the cellular interstitium by inactivating the inhibitors of metalloproteases, resulting in the production of pathological collagen. These alterations lead to two main consequences: formation of cardiac fibrosis, with subsequent alteration of diastolic function, and misalignment of cardiomyocytes, resulting in impairment of systolic function (5).

The principal findings of this study suggest that myocardial strain analysis may be more sensitive and specific than FS and EF in detecting changes in LV systolic function following chemotherapy (4, 28). Today, the identification of cardiac abnormalities after the first few days of treatment is considered of great importance and RS could become a reliable marker for the rapid detection of myocardial changes, allowing to the early prediction of cardiac dysfunction, for example after trastuzumab treatment (29). Several anti-neoplastic drugs have been shown to induce cardiac toxic effects during or after chemotherapeutic treatment; this concern has assumed notable relevance to the radical change in life expectancy in several malignancies, and to the increasing number of long-term survivors (31). For this reasons, the present *in vivo* studies indicate that RS and CS, measured by ST echocardiography, could become reliable markers (4). We have used this model for performing studies of cardiotoxicity, and cardioprotection (27, 29), with the aim of evaluating premature ventricular remodeling and myocardial recovery in response to possible cardioprotective therapy administered before, after and concomitantly with chemotherapy for cancer (27).

Conclusion

Considering that cardiac toxicity remains an important side-effect of anticancer therapy, early detection of cardiac injury is crucial, since it may facilitate therapeutic measures (3, 4). Our study suggests that strain analysis may be a reliable tool for detecting early or subtle myocardial impairment during chemotherapy, with highly sensitive and non-invasive procedures. This model represents a novel approach that may be used for rapid cardiovascular assessing during treatment with antineoplastic drugs. These advances may have powerful diagnostic and therapeutic benefits in humans, and may provide an important thrust for promoting further research on initial cardiac remodeling in patients undergoing chemotherapy, thus permitting early detection of abnormalities that can represent a helpful guide for 'tailoring' dosage regimens of antineoplastic drugs and for testing cardioprotective agents designed to attenuate cardiac injuries induced by chemotherapy (4).

Competing Interest

The Authors confirm that this article content has no potential conflict of interest.

Founding sources

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References

1 Thavendiranathan P, Paulin F, Lim K Plana JC, Woo A and Marwick TH: Use of myocardial strain imaging by echocardiography for the early detection of cardiotoxicity in patients during and after chemotherapy: a systematic review. J Am Coll Cardiol *63*: 2751-2768, 2014.

2 Minotti G, Menna P, Salvatorelli E, Cairo G and Gianni L: Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. Pharmacol Rev *56*: 185-229, 2004.

3 Perrino C, Schiattarella GG, Magliulo F, Ilardi F, Carotenuto G, Gargiulo G, Serino F, Ferrone M, Scudiero F, Carbone A, Trimarco B and Esposito G: Cardiac side-effects of chemotherapy: state of art and strategies for a correct management. Curr Vasc Pharmacol *12*: 106-116, 2014.

4 Raschi E, Vasina V, Ursino MG, Boriani G, Martoni A and De Ponti F: Anticancer drugs and cardiotoxicity: Insights and perspectives in the era of targeted therapy. Pharmacol Ther *125*: 196-218, 2010.

5 Zhang Y, Kang YM, Tian C, Zeng Y, Jia LX, Ma X, Du J and Li HH: Overexpression of Nrdp1 in the heart exacerbates doxorubicin-induced cardiac dysfunction in mice. PLoS One *6*: e21104, 2011.

6 Neilan TG, Jassal DS, Perez-Sanz TM, Raher MJ, Pradhan AD, Buys ES, Ichinose F, Bayne DB, Halpern EF, Weyman AE, Derumeaux G, Bloch KD, Picard MH and Scherrer-Crosbie M: Tissue Doppler imaging predicts left ventricular dysfunction and mortality in a murine model of cardiac injury. Eur Heart J *15*: 1868-1875, 2006.

7 Bauer M, Cheng S, Jain M, Ngoy S, Theodoropoulos C, Trujillo A, Lin FC and Liao R: Echocardiographic speckle-tracking based strain imaging for rapid cardiovascular phenotyping in mice. Circ Res *108*: 908-916, 2011.

8 Geyer H, Caracciolo G, Abe H, Wilansky S, Carerj S, Gentile F, Nesser HJ, Khandheria B, Narula J and Sengupta PP: Assessment of myocardial mechanics using speckle tracking echocardiography: fundamentals and clinical applications. J Am Soc Echocardiogr *23*: 351-369, 2010.

9 Ram R, Mickelsen DM, Theodoropoulous C and Blaxall BC: New approaches in small animal echocardiography: imaging the sound of silence. Am j Physiol Heart Circ Physiol *301*: 1765-1780, 2011.

10 Bham A, Sirke A, Zhang J, Protti A, Catibog N, Driver W, Botnar R, Monaghan MJ, Shah AM: High-frequency speckle tracking echocardiography in the assessment of left ventricular function and remodeling after murine myocardial infarction. Am J Physiol Heart Circ Physiol *306*: 1371-1383, 2014.

11 Perk G, Tunick PA and Kronzon I: Non-Doppler two-dimensional strain imaging by echocardiography from technical considerations to clinical applications. J Am Soc Echocardiogr *20*: 234-243, 2007.
12 Peng Y, Popovic ZB, Sopko N, Drinko J, Zhang Z, Thomas JD and Penn MS: Speckle-tracking echocardiography in the assessment of mouse models of cardiac dysfunction. Am J Physiol Heart Circ Physiol *297*: 811-820, 2009.
13 Tremoleda JL, Kerton A and Gsell W: Anaesthesia and physiological monitoring during *in vivo* imaging of laboratory rodents: considerations on experimental outcomes and animal welfare. EJNMMI Res *2*: 44, 2012.
14 Roth DM, Swaney JS, Dalton ND, Gilpin EA and Ross J Jr.: Impact of anesthesia on cardiac function during echocardiography in mice. Am J Physiol Heart Circ Physiol *282*: 2134-2140, 2002.
15 Reagan-Shaw S, Nihal M and Ahmad N: Dose translation from animal to human studies revisited. FASEB J 22: 659-661, 2008.
16 Scharovsky OG, Mainetti LE and Rozados VR: Metronomic chemotherapy changing the paradigm that more is better. Curr Oncol *16*: 7-15, 2009.
17 Barbieri A, Barretta ML, Rea D, Picone C, Fabozzi O, Palma G, Antonio L, Federico A, Bimonte S, Setola F, De Lutio Di Castelguidone E, Castellano M, D'Errico AG, Caraglia M and Arra C: Intraluminal gel ultrasound and eco-color doppler: new tools for the study of colorectal cancer in mice. In Vivo *27*: 443-450, 2013.
18 Moens AL, Takimoto E, Tocchetti CG, Chakir K, Bedja D, Cormaci G, Ketner EA, Majmudar M, Gabrielson K, Halushka MK, Mitchell JB, Biswal S, Channon KM, Wolin MS, Alp NJ, Paolocci N, Champion HC and Kass DA: Reversal of cardiac hypertrophy and fibrosis from pressure overload by tetrahydrobiopterin: efficacy of recoupling nitric oxide synthase as a therapeutic strategy. Circulation *117*: 2626-236, 2008.
19 Cittadini A, Isgaard j, Monti MG, Casaburi C, Di Gianni A, Serpico R, Iaccarino G and Saccà L: Growth hormone prolongs survival in experimental postinfarction heart failure. J Am Coll Cardiol *41*: 2154-2163, 2003.
20 Cittadini A, Monti MG, Castiello MC, D'Arco E, Galasso G, Sorriento D, Saldamarco L, De Paulis A, Napoli R, Iaccarino G and Saccà L: Insulin-like growth factor-1 protects from vascular stenosis and accelerates re-endothelialization in a rat model of carotid artery injury. J Thromb Haemost *7*: 1920-1928, 2009.
21 Riad A, Bien S, Westermann D, Becher PM, Loya K, Landmesser U, Kroemer HK, Schultheiss HP and Tschöpe C: Pretreatment with statin attenuates the cardiotoxicity of doxorubicin in mice. Cancer Res *69*: 695-699, 2009.
22 Benavid es-Vallve C, Corbacho D, Iglesias-Garcia O, Pelacho B, Albiasu E, Castaño S, Muñoz-Barrutia A, Prosper F and Ortiz-de-Solorzano C: New strategies for echocardiographic evaluation of left ventricular function in a mouse model of long-term myocardial infarction. PLoS One *7*: e41691, 2012.
23 Täng MS, Redfors B, Shao Y and Omerovic E: Velocity vector imaging fails to quantify regional myocardial dysfunction in a mouse model of isoprenaline-induced cardiotoxicity. Echocardiography *29*: 818-826, 2012.
24 Uechi M, Asai K, Osaka M, Smith A, Sato N, Wagner TE, Ishikawa Y, Hayakawa H, Vatner DE, Shannon RP, Homcy CJ and Vatner SF: Depressed heart rate variability and arterial baroreflex in conscious transgenic mice with over expression of cardiac Gsa. Circ Res *82*: 416-423, 1998.
25 Strigun A, Wahrheit J, Niklas J, Heinzle E and Noor F: Doxorubicin increases oxidative metabolism in HL-1 cardiomyocytes as shown by ^{13}C metabolic flux analysis. Toxicol Sci *25*: 595-606, 2012.
26 Bertazzoli C, Chieli T, Grandi M and Ricevuti G: Adriamycin: toxicity data. Experientia *26*: 389-390, 1970.
27 Tocchetti CG, Carpi A, Coppola C, Quintavalle C, Rea D, Campesan M, Arcari A, Piscopo G, Cipresso C, Monti MG, De Lorenzo C, Arra C, Condorelli G and Di Lisa F: Ranolazine protects from doxorubicin-induced oxidative stress and cardiac dysfunction. Eur J Heart Fail *16*: 358-366, 2014.
28 Stoodley PW, Richards DA, Hui R, Boyd A, Harnett PR, Meikle SR, Clarke J and Thomas L: Two-dimensional myocardial strain imaging detects changes in left ventricular systolic function immediately after anthracycline chemotherapy. Eur J Echocardiogr *12*: 945-952, 2011.
29 Fedele C, Riccio G, Coppola C, Barbieri A, Monti MG, Arra C, Tocchetti CG, D'Alessio G, Maurea N and De Lorenzo C. Comparison of preclinical cardiotoxic effects of different ERBB2 inhibitors. Breast Cancer Res Treat *133*: 511-521, 2012.
30 Bauer M, Cheng S, Unno K, Lin FC and Liao R: Regional cardiac dysfunction and dys-synchrony in a murine model of afterload stress. PLoS One *8*: e59915, 2013.
31 Tocchetti CG, Gallucci G, Coppola C, Piscopo G, Cipresso C, Maurea C, Giudice A, Iaffaioli RV, Arra C and Maurea N: The emerging issue of cardiac dysfunction induced by antineoplastic angiogenesis inhibitors. Eur J Heart Fail *15*: 482-489, 2013.

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