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A short-time model to study relevant indices of cardiotoxicity of doxorubicin in the rat

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Running title: A short-time model of cardiotoxicity.

Abstract

Aim: Short-time models (STM) to study the cardiotoxicity (acute or chronic) of doxorubicin in rats are of interest to assess protective interventions and pathways. STM promotes more ethical animal treatment with less stress, and at a lower cost compared to established long-time models (LTM). We wanted to investigate if a STM of 9 days yields the same information regarding cardiotoxicity as a LTM of 9 weeks.

Methods: Male Wistar rats received identical drug administration protocols in STM and LTM. The two intervention groups (n = 6) received intraperitoneal (i.p.) injections of 2mg/kg doxorubicin every day on 5 consecutive days, with a total cumulative dose of 10mg/kg. The two control groups (n = 6), received an equivalent volume of saline injected every day on 5 consecutive days. Hearts from STM and LTM were excised and Langendorff-perfused after 9 days or 9 weeks, respectively, after the first drug injection. Cardiotoxicity was assessed in paced Langendorff hearts by release of hydrogenperoxide (H₂O₂) and troponin T (TnT) in effluent, by myocardial accumulation of doxorubicin and its metabolite doxorubicinol, and by physiological parameters recorded during pressure, or volume regulated perfusion.

Results: In STM, hearts exposed to doxorubicin demonstrated a 15% reduction in left ventricular developed pressure (LVDP) irrespective of flow mode, and a 13% increase in aortic pressure (AoP), during volume regulated perfusion, an index of coronary resistance, compared to controls. Left ventricular end-diastolic pressure (LVEDP) was increased 72% during pressure regulated perfusion and 100% during volume regulated perfusion in STM. In LTM, hearts exposed to doxorubicin demonstrated a 40% reduction in LVDP during pressure regulated perfusion and a 20% reduction during volume regulated perfusion. LVEDP was 70% higher in doxorubicin treated hearts during pressure regulated perfusion and 80% higher during volume regulated perfusion. In addition, aortic pressure was increased 30% during volume regulated perfusion. In both STM and LTM, hearts exposed to doxorubicin

demonstrated a higher H₂O₂ and TnT release, compared to respective controls. The difference was most pronounced in STM. Myocardial content of doxorubicin was detectable in both STM and LTM. However, doxorubicinol was only detectable in STM.

Conclusion: STM is comparable to LTM to study relevant indices of cardiotoxicity of doxorubicin in rat hearts. Biochemical differences are more pronounced in STM, while contractile differences are more pronounced in LTM. STM could be a preferred model for preliminary studies of protective interventions.

Key words: Doxorubicin, troponin, hydrogenperoxide, reactive oxygen species, heart, rat, doxorubicinol, cardiotoxicity

Introduction

The anthracycline doxorubicin is one of the most frequently prescribed anticancer drugs because of its activity in solid tumours as well as haematological malignancies. However, its pronounced cardiotoxicity limits long term use and prevents effective anticancer therapy.(1) A common approach to evaluating cardiac function is monitoring left ventricular ejection fraction. A weakness in this method is that cardiac damage is usually detected only when a functional impairment has already occurred, which leaves little room for early, preventive strategies.(2) Therefore, measurement of cardiospecific biomarkers can be a valid diagnostic tool for early identification, assessment, and monitoring of cardiotoxicity.(2) Cardiac troponins have been suggested as valuable biomarkers of anthracycline cardiotoxicity, both in animal and clinical studies.(3, 4) Furthermore, accumulation of reactive oxygen species (ROS), like hydrogenperoxide (H_2O_2) is associated with oxidative stress during myocardial injury. Thus, release of biomarkers like TnT and H_2O_2 are of relevance to study reduced cardiac function associated with doxorubicin, and should be supplemented by measurement of myocardial accumulation of the anthracycline and its metabolite doxorubicinol in experimental studies.

Previous cardiotoxicity models include long term exposure to doxorubicin for 5-12 weeks.(5-7) LTMs are time consuming, and represent long-time stress for the animals, and high mortality rates have been reported.(8, 9) Therefore, it is of interest to develop and test short-time models (STM) when studying cardiotoxicity of anthracyclines. A combination of *in vivo* and *ex vivo* animal models has the advantage that it includes relevant pharmacokinetic phases after administration of drugs *in vivo*. In addition, such a model allows controlled evaluation of relevant end points (cardiotoxicity) *ex vivo*, without interference of systemic effects. In this methodological study in rats we wanted to investigate if a STM model of 9 days is comparable to a LTM of 9 weeks to study relevant indices of cardiotoxicity of

doxorubicin in hearts. Both models involved repetitive intraperitoneal injections of doxorubicin or saline *in vivo*, prior to evaluation of cardiotoxicity in *ex vivo* isolated Langendorff hearts. Previously described STMs have not studied the generation of free radicals and release of TnT, however, they have addressed physiological parameters describing reduction of cardiac function, and accumulation of doxorubicin in the myocardium.(10, 11) Our STM model includes these important biomarkers, supplemented by measurement of doxorubicinol, and as such, is of interest to study protective interventions. Furthermore, the model allows switching between pressure and volume regulated flow, and the latter adds measurement of the resistance in the coronary vasculature induced by drug treatment.

Methods

Materials

Doxorubicin was purchased from Meda AS (Slemmestad, Norway), pentobarbital from Haukeland Hospital Pharmacy (Bergen, Norway), heparin from Leo Pharma A/S (Oslo, Norway), and ingredients for the Krebs-Henseleit bicarbonate buffer from Merck KGaA (Darmstadt, Germany). This study conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the Animal Care and User Committee in Norway.

Animals

Male Wistar rats (200 ± 2 g) were purchased from Taconic (Ejby, Denmark). The animals were housed in grid-bottom metal wire cages in a room maintained at a 12 hour light/dark cycle at a temperature of 20-22°C. They were acclimatised for 2 weeks, housed 3 per cage and allowed free access to food pellets (Pellets rodent, Special Diets Service, UK) and tap water until injection of doxorubicin or saline. The animals were separated in individual cages based on their respective treatments protocols.

Langendorff perfusion model

The perfusion medium was a modified, oxygenated (95% O₂ and 5% CO₂) Henseleit bicarbonate buffer (KHBB) (pH 7.4) containing in mM: 118.5 NaCl, 25.0 NaHCO₃, 1.2 MgS₄, 4.7 KCl, 1.2 KH₂PO₄, 1.2 D-glucose and 1.25 CaCl₂. Hearts were excised after anaesthesia of the rats with an i.p. injection of pentobarbital 50mg/kg (0.1ml/100g bodyweight) and heparinised i.p. (0.1ml 500IU/100g bodyweight). Anaesthesia was evaluated by the pedal-withdrawal reflex. The heart was rapidly excised and immediately placed in cold (4°C) KHBB to temporarily stop its beating and preserve it from ischemic injury prior to

perfusion. The heart was mounted on a steel cannula placed in the aorta and perfused retrogradely in a Langendorff system with the use of thermostated (37°C) Lauda reservoirs (Lauda-Königshofen, Germany), perfusion lines, and heart chamber. Pressure regulated flow was performed at 100cmH₂O (73mmHg), while volume regulated flow (12.5ml/min) was performed by use of an Alitea peristaltic pump (Stockholm, Sweden). A water-filled latex balloon was placed in the left ventricle and connected to a SensoNor 840 pressure transducer (Memscap AS, Skoppum, Norway) for the recording of left ventricular developed pressure (LVDP) and secondarily derived contractility indices. Left ventricular end-diastolic pressure (LVEDP) was adjusted between 4 and 8mmHg. A second pressure transducer was connected to a side arm on the aortic cannula for the recording of aortic pressure (AoP), an index of coronary vascular resistance during volume-regulated perfusion. Pressure signals were amplified (Quadbridge, AD Instruments, London, UK) and recorded using a PowerLab data acquisition system (AD Instruments, East Sussex, England). AoP, LVDP, LVEDP and LVDP first derivatives maximum (dp/dt_{max}) and minimum (dp/dt_{min}) were continuously displayed and recorded. Pacing (300 beats per minute by electric stimulation of 5V amplitude of 3ms duration) was obtained by placing one electrode on the right auricle and one on the steel cannula. Coronary flow rate was measured by timed collection of the coronary perfusate that dripped from the heart. At the end of the perfusion protocol hearts were removed from the Langendorff system and myocardial tissue from the left ventricle was dissected free and immediately frozen in liquid helium and stored at -80°C until analysis of doxorubicin and doxorubicinol, within 14 days of termination of the Langendorff protocol. Effluent samples of 1mL were collected in 1.5mL polypropylene Eppendorf micro test tubes (Eppendorf Vertrieb, Wesseling-Berzdorf, Germany) from each heart, at the end of the perfusion protocol, and stored at 0°C, until analysis for TnT within 4 days of termination of the Langendorff protocol. Effluent samples of 1mL were collected in Eppendorf tubes from each heart, at the end of the

perfusion protocol, and immediately analysed for H₂O₂, the samples were placed in a thermostated (37°C) Eppendorf rack heated by a Lauda reservoir (Lauda-Königshofen, Germany). All experiments and analysis were carried out between 7am and 7pm.

Experimental groups, drug treatments and Langendorff protocols (Figure I)

Two protocols were tested: STM and LTM. Each protocol had an intervention group demarcated dox (doxorubicin) and a control group demarcated sal (saline).

The intervention groups STMdox (n = 6) and LTMdox (n = 6) received an i.p. injection of 2mg/kg doxorubicin every day on 5 consecutive days up to a total cumulative dose of 10mg/kg doxorubicin.

The control groups STMsal (n = 6) and LTMsal (n = 6): received an i.p. injection of equivalent volume 0.9% saline every day on 5 consecutive days.

STM: On day 10, hearts from STMdox and STMsal were excised for the *ex vivo* experiments and Langendorff-perfused.

LTM: After 9 weeks, hearts from LTMdox and LTMsal were excised for the *ex vivo* experiments and Langendorff-perfused.

All hearts were subjected to the following perfusion protocol: A 15 minute stabilization period with pressure regulated flow, followed by 5 minutes with pressure regulated flow, and 5 minutes with volume regulated flow. During the latter 10 minutes physiological data were recorded, and cardiac effluent samples collected for evaluation of biochemical and pharmacological parameters. The perfusion protocol is illustrated in Figure I.

Determination of doxorubicin and doxorubicinol

Doxorubicin and doxorubicinol were quantified by high performance liquid chromatography (HPLC-MS/MS) (1200 series RRLC, Agilent Technologies, USA) coupled to an Agilent

6410 triple quadrupole mass spectrometer using positive electrospray ionisation (Agilent Technologies, USA). Frozen left ventricular tissue was minced and weighted out in a glass tube with a screw cap and homogenized in physiological saline (2ml/100mg tissue) with a tissue homogenizer (Ultra Turrax, Sigma Aldrich, Germany). 1000µl of sample was added 100µl of daunorubicin as internal standard (IS), and 200µl of buffer (1M TRIZMA, pH 11.1) and mixed well before extraction with 4ml ethylacetate/heptane (80/20 vol/vol). The samples were mixed using a rotary blender for 15 minutes and then centrifuged at 3500rpm for 10 minutes at 10°C. The organic phase was evaporated to dryness at 50°C under nitrogen then dissolved in 100µl of methanol followed by 100µl of distilled water. The extract was mixed thoroughly and transferred to silanized vials before analysis. 25µl of extract was injected and separated on a Zorbax SB-Aq (2.1 x 50mm, 1.8µm particles, Agilent Technologies, USA) column using gradient elution with acetonitrile and 0.1% formic acid in water. Quantification were performed using multiple reaction monitoring (MRM) mode at m/z 546.1 → 363.1 for doxorubicinol, m/z 544.1 → 361.1 for doxorubicin and m/z 528.1 → 321.1 for daunorubicin (IS).

Effluent content of TnT

TnT in cardiac effluent was measured using an Elecsys 2010 immunoassay analyzer (Roche Diagnostics Norway AS, Oslo, Norway), based on the sandwich principle. Total duration of assay: 9 minutes. 1st incubation: 50µL of sample, a biotinylated monoclonal cardiac TnT-specific antibody, and a monoclonal cardiac TnT-specific antibody labeled with a ruthenium complex (Tris(2,2-bipyridyl)ruthenium(II)-complex (Ru(bpy))) reacted to form a sandwich complex. 2nd incubation: After addition of streptavidin-coated microparticles, the complex became bound to the solid phase via interaction of biotin and streptavidin. The reaction mixture was aspirated into the measuring cell where the microparticles were magnetically

captured onto the surface of the electrode. Unbound substances were then removed with ProCell. Application of a voltage to the electrode then induced chemiluminescent emission which was measured by a photomultiplier. Results were determined via a calibration curve which was instrument-specifically generated by 2-point calibration and a master curve (5-point calibration) provided via the reagent barcode. Detection limit was 5.0ng/L

Effluent content of H₂O₂

H₂O₂ in cardiac effluent was measured using an Apollo 4000 electrochemical detection system (World Precision Instruments, Sarasota, Florida, USA). The electrode was calibrated using 9 serial dilutions of H₂O₂ in phosphate buffered saline with added aniline. The current recorded from the effluent was then calculated as $\mu\text{M H}_2\text{O}_2$. Samples were kept at 37°C during measurement. The electrode was allowed 3 minutes of stabilisation and 1 minute of recording.

Statistics

All results are reported as mean values \pm standard deviation (SD) in tables. Groups were compared with regards to parameters with a student t-test. Only groups within similar protocols (STMdox with STMsal, and LTMdox with LTMsal) were compared. Differences between models were only described. SPSS for Windows version 17.0 was used, and $p < 0.05$ was considered statistically significant.

Results

H₂O₂ release in STMdox ($77.4 \pm 2.8\mu\text{M}$) was significantly higher compared to H₂O₂ release in STMsal ($12.8 \pm 1.7\mu\text{M}$). Similarly, H₂O₂ release in LTMdox ($22.8 \pm 5.3\mu\text{M}$) was significantly higher compared to H₂O₂ release in LTMsal ($9.9 \pm 2.7\mu\text{M}$). Myocardial content of doxorubicin and doxorubicinol in STMdox was $1.2 \pm 0.18\text{nmol/g}$ and $0.45 \pm 0.14\text{nmol/g}$ respectively. In LTMdox we found a doxorubicin content of $0.19 \pm 0.02\text{nmol/g}$.

Doxorubicinol was undetectable after 9 weeks in LTMdox. TnT release was significantly higher in STMdox ($345.3 \pm 37.3\text{ng/L}$) compared to STMsal ($63.5 \pm 9.7\text{ng/L}$). Similarly, TnT release was significantly higher in LTMdox ($152.2 \pm 22.1\text{ng/L}$) compared to LTMsal ($48.0 \pm 14.8\text{ng/L}$). In STM, hearts exposed to doxorubicin demonstrated a 15% reduction in left ventricular developed pressure (LVDP) irrespective of flow mode, and a 13% increase in aortic pressure (AoP), during volume regulated perfusion, an index of coronary resistance, compared to controls. Left ventricular end-diastolic pressure (LVEDP) was increased 72% during pressure regulated perfusion and 100% during volume regulated perfusion in STM. In LTM, hearts exposed to doxorubicin demonstrated a 40% reduction in LVDP during pressure regulated perfusion, and a 20% reduction during volume regulated perfusion. LVEDP was 70% higher in doxorubicin treated hearts during pressure regulated perfusion and 80% higher during volume regulated perfusion. In addition, aortic pressure was increased 30% during volume regulated perfusion. In both STM and LTM, hearts exposed to doxorubicin demonstrated a higher H₂O₂ and TnT release, compared to respective controls. dp/dt_{max} was lower and dp/dt_{min} higher in STMdox and LTMdox compared to their respective controls, irrespective of perfusion mode. Coronary flow was decreased during pressure regulated perfusion, in the doxorubicin treated groups in both models, compared to controls. All physiological results are presented in Table I and II and biochemical and pharmacological results in Table III and IV.

Discussion

Our study shows that a 9 day STM is sufficient time to demonstrate relevant indices of cardiotoxicity of doxorubicin in isolated Langendorff-perfused rat hearts, irrespective of flow mode. Our model allows parallel assessment of ROS and TnT release, myocardial content of anthracyclines and measurement of cardiac function. Biochemical differences are more pronounced in STM, while contractile differences are more pronounced in LTM. The latter reflects the accumulated myocardial contractile damage associated with doxorubicin in the tissue. However, STM represents a preferred model for preliminary preclinical studies of protective interventions with less stress for the animals, and at a lower cost.

HPLC-MS/MS measurements of doxorubicin and its metabolite doxorubicinol showed significant accumulation of anthracyclines in the myocardial tissue. The results from the LTM, demonstrate the slow elimination of doxorubicin from myocardial tissue. Disposition of doxorubicinol in patients is formation rate limited, with the terminal half-life of the metabolite being similar to doxorubicin. The relative exposure of doxorubicinol, i.e., the ratio between area under the curve (AUC) for doxorubicinol compared to AUC for doxorubicin ranges between 0.3 and 0.6.(12, 13) Thus, doxorubicinol is present in a much lower concentration than doxorubicin, though it is proposed to be more toxic compared with doxorubicin, and it has been established that doxorubicinol is nearly 30 times more potent than doxorubicin at inhibiting calcium-handling proteins and at depressing contractility and systolic myocardial function.(14, 15)

We used a 3rd generation troponin T test, Elecsys 2010 immunoassay analyser. This test uses the same monoclonal antibodies (M11.7 and M7) as the 2nd generation ES 300 test, but is standardized with human recombinant cardiac TnT instead of bovine cardiac TnT, which has been used previously. However, previous studies(16) have concluded that there

were no differences in coronary TnT concentrations in isolated rat heart effluent between 2nd and 3rd generation cardiac TnT assays, ES 300 and Elecsys 2010, respectively.

In our STM, a significant reduction in contractile parameters due to anthracyclines could be studied in Langendorff hearts irrespective of flow mode. Reduction of cardiac function can be directly related to measurements of myocardial release of relevant biomarkers and content of doxorubicin and doxorubicinol. With volume regulated flow, the effects on coronary vascular resistance of the anthracycline can be studied in parallel with effects on myocardial contractility. Thus, reduced contractility as a result of reduced coronary flow during pressure regulated perfusion could be assessed. Our results demonstrate that flow mode is of minor importance when studying myocardial effects of doxorubicin in this model.

In our STM, LVDP in doxorubicin treated rats were significantly reduced compared to controls both during pressure regulated and volume regulated perfusion. A similar short term model found comparable results (17), they studied cardiac performances of *ex vivo* perfused hearts from rats that had been treated with various anthracyclines within 12 days, administrated by repetitive injections. One group received 3mg/kg doxorubicin every other day for 12 days, up to a total cumulative dose of 18mg/kg. Even though they used a higher total cumulative dose compared to our study, they found that their model was able to predict correctly what was already known concerning the cardiotoxicity of anthracyclines. These findings support our STM.

Different laboratories use different doses of doxorubicin to induce cardiotoxicity. In general, i.p. injections of 2.0-4.0mg/kg are used, up to total cumulative doses of 10-20mg/kg, but higher doses have been reported. In our study, we used 2mg/kg doxorubicin, up to a total cumulative dose of 10mg/kg, to induce cardiotoxicity. Selection of dose and concentration of doxorubicin was done to give the rats a total cumulative dose high enough to induce myocardial damage, but at the same time low enough to keep them alive. Choice of dose is

particularly important to reduce the possibility of masking beneficial effects of protective interventions. In our results, a reduction of 15% of LVDP compared to controls in STM provides frame for evaluating such effects, and an increase in dose is possible. The dosage and concentration frame of doxorubicin used in this study has been applied in previous studies, and has yielded comparable results.(11, 17, 18)

Reduction of the cardiotoxicity of doxorubicin and its main metabolite doxorubicinol, while retaining their anticancer effect, is desirable. Thus, interventions that reduce accumulation of anthracyclines and improve the drug's effect on cardiac function can be measured directly in our STM. Interestingly, diastolic dysfunction, reflecting poor ventricular compliance, is reported to be an early sign of anthracycline cardiotoxicity in the clinic.(19, 20) Diastolic dysfunction and increased coronary vascular resistance was observed both in STM and LTM in the present results, and flow mode did not influence the results. Interestingly, an increase of LVEDP between 70-100% was apparent already in our 9 day model and was still present after 9 weeks. However, the increase in coronary vascular resistance was doubled in the same period from 13-30%. Thus, long time effects of doxorubicin in the heart, affect both cardiomyocytes and the coronary arteries.

Finally, use of pacing gave us the opportunity to study contractile indices without interference of arrhythmias associated with anthracyclines.(21) Thus, our STM seems robust and reproducible, and it should be possible to add new indices of cardiotoxicity into this model in future studies.

Limitations

The present STM is suitable for preclinical evaluation of new protective interventions to reduce anthracycline cardiotoxicity. However, protection in compressed doxorubicin regimens is not necessarily equiprotective when tested in a more clinically relevant chronic

regimens. Such studies should be reserved for interventions proven effective and safe in STM. Furthermore, the model allows the possibility to elucidate important pathways associated with cardioprotective principles.

Repeated i.p. injections could give development of scar tissue and thickened skin with reduced and unpredictable absorption of drugs. Furthermore, doxorubicin could give local tissue damage and inflammation. We were careful to alternate injection sites, using correct injection techniques including a suitable needle to reduce this problem. The compartmental distribution of doxorubicin in the myocardium or within cardiomyocytes is not known due to the fact that heart tissue was minced and homogenized. Precise cellular and subcellular distribution of the anthracycline is of relevance to understand mechanisms of cardiotoxicity. Assessment of effect on cardiomyopathy is important in studies of anthracyclines. In our study, the condition of the heart at the two time points would be of interest. However, our model did not allow for cardiac pathology due to the tissue being used to detect doxorubicin and doxorubicinol.

We used cardiac pacing, which excludes the possibility of evaluating heart rate and electrocardiograms. This would have been valuable and interesting information considering the well-known association between arrhythmias and anthracyclines. However, assessment of cardiac function (recording of left ventricular developed pressure (LVDP) and secondarily derived contractility indices) was of particular importance in this study, and pacing was used to obtain optimal recordings not influenced by arrhythmias.

An experimental STM with a combination of in vivo doxorubicin administration and subsequent ex vivo evaluation of cardiotoxicity in the rat must make some compromises in comparison to the clinical administration of the drug. The development of cardiotoxicity in animal models with weekly injections is previously described in the literature.⁽¹⁷⁾ Repetitive low doses are preferred compared to a single high dose in the rat, and every-other-day

administration has been proposed.(22) In our STM we used daily i.p. injections to reduce duration of the experiments.

Non-invasive transthoracic echocardiography is suitable for studying development and course of anthracyclines cardiomyopathy, and development of heart failure in animal models.(23) Our global isovolumetric STM cannot reflect in vivo heart function, and reduction of contractile indices represents only indirect measures of heart failure. However, our STM is ideal to study contractile function in parallel with release of relevant biochemical parameters in real time specific to the heart. Release of biomarkers in blood associated with in vivo models could be subject to several systemic effects and be less specific to the organ of interest.

Our STM does not allow measurement of intracellular production and compartmental distribution and release of H_2O_2 . Thus, release in effluate represents a sum of venous drain (majority) and exudate from the surface of the heart (minority) from the organ. After nine weeks, pathological changes in the heart could allow for a different distribution and release of H_2O_2 . However, release is specific to the organ compared to in vivo models where development of heart failure would affect potential release from other organs.

Conclusion

STM is comparable to LTM to study relevant indices of cardiotoxicity of doxorubicin in rat hearts. Thus, STM could be a preferred model for further studies of protective interventions. STM is a better alternative than previously described LTMs, because it submits the animals to less stress and discomfort. In addition a STM is less time consuming, more cost effective and more in accordance with the idea of refinement for use of laboratory animals. This study shows that our STM is a promising alternative for future preclinical studies of cardiotoxic mechanisms of doxorubicin and doxorubicinol, and for studies of protective interventions.

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Conflict of interest: None declared.

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Table I**Physiological results from hearts in the short-time model during pressure and volume regulated perfusion**

STM	Pressure regulated perfusion		Volume regulated perfusion	
	STMdox n = 6 2mg/kg doxorubicin	STMsal n = 6 Saline injection	STMdox n = 6 2mg/kg doxorubicin	STMsal n = 6 Saline injection
LVDP (mmHg)	112.1 ± 7.6 *	130.9 ± 4.4	130.3 ± 6.9 *	150.4 ± 2.4
LVEDP (mmHg)	14.8 ± 3.1 *	8.6 ± 0.7	21.9 ± 2.3 *	10.7 ± 2.9
AoP (mmHg)	73.0 ± 0	73.0 ± 0	144.8 ± 0.5 *	127.5 ± 10.5
Heart rate (beats per minute)	300 ± 0	300 ± 0	300 ± 0	300 ± 0
dp/dt max (mmHg/s)	3490.2 ± 448.9 *	4064.8 ± 201.3	2955.9 ± 362.2 *	4184.6 ± 202.8
dp/dt min (mmHg/s)	-1870.8 ± 153.8 *	-2362.5 ± 190.8	-1266.1 ± 503.2 *	-2514.9 ± 65.5
Coronary flow (ml/min)	8.1 ± 0.5 *	11.1 ± 0.6	12.5 ± 0	12.5 ± 0

Table I Values presented as mean ± standard deviation (SD). * = significantly different from control hearts, p<0.05. Short time model doxorubicin treated hearts (STMdox). Short time model saline treated hearts (STMsal). Left ventricular developed pressure (LVDP). Left ventricular end diastolic pressure (LVEDP). Aortic pressure (AoP).

Table II**Physiological results from hearts in the long-time model during pressure and volume regulated perfusion**

LTM	Pressure regulated perfusion		Volume regulated perfusion	
	LTMdox n = 6 2mg/kg doxorubicin	LTMsal n = 6 Saline injection	LTMdox n = 6 2mg/kg doxorubicin	LTMsal n = 6 Saline injection
LVDP (mmHg)	64.1 ± 3.9 *	106.9 ± 7.5	95.8 ± 17.3 *	119.2 ± 8.1
LVEDP (mmHg)	11.4 ± 1.7 *	6.7 ± 1.5	12.9 ± 0.8 *	7.1 ± 0.8
AoP (mmHg)	73.0 ± 0	73.0 ± 0	130.6 ± 4.7 *	97.2 ± 8.8
Heart rate (beats per minute)	300 ± 0	300 ± 0	300 ± 0	300 ± 0
dp/dt max (mmHg/s)	1667.2 ± 299.6 *	3099.2 ± 202.6	2707.6 ± 298.7 *	4413.9 ± 488.2
dp/dt min (mmHg/s)	-834.2 ± 98.9 *	-2137 ± 285.7	-1235.1 ± 320.1 *	-2568.0 ± 186.2
Coronary flow (ml/min)	6.3 ± 0.9 *	11.9 ± 0.6	12.5 ± 0	12.5 ± 0

Table II Values presented as mean ± standard deviation (SD). * = significantly different from control hearts, p<0.05. Long time model doxorubicin treated hearts (LTMdox). Long time model saline treated hearts (LTMsal). Left ventricular developed pressure (LVDP). Left ventricular end diastolic pressure (LVEDP). Aortic pressure (AoP).

Table III**Biochemical and pharmacological results from hearts in the short-time model**

STM	STMdox 2mg/kg doxorubicin	STMsal Saline injection
Doxorubicin tissue concentration (nmol/g)	1.2 ± 0.18	0 ± 0
Doxorubicinol tissue concentration (nmol/g)	0.45 ± 0.14	0 ± 0
Troponin -T effluate concentration (ng/L)	345.3 ± 37.3 *	63.5 ± 9.7
H ₂ O ₂ effluate concentration (μM)	77.4 ± 2.8 *	12.8 ± 1.7

Table III Values presented as mean ± standard deviation (SD). * = significantly different from controls, p<0.05. Short time model doxorubicin treated hearts (STMdox). Short time model saline treated hearts (STMsal).

Table IV**Biochemical and pharmacological results from hearts in the long-time model**

LTM	LTMdox 2mg/kg doxorubicin	LTMsal Saline injection
Doxorubicin tissue concentration (nmol/g)	0.19 ± 0.02	0 ± 0
Doxorubicinol tissue concentration (nmol/g)	0 ± 0	0 ± 0
Troponin -T effluate concentration (ng/L)	152.2 ± 22.1 *	48.0 ± 14.8
H ₂ O ₂ effluate concentration (μM)	22.8 ± 5.3 *	9.9 ± 2.7

Table IV Values presented as mean ± standard deviation (SD). * = significantly different from controls, p<0.05. Long time model doxorubicin treated hearts (LTMdox). Long time model saline treated hearts (LTMsal).

Figure I Perfusion protocols

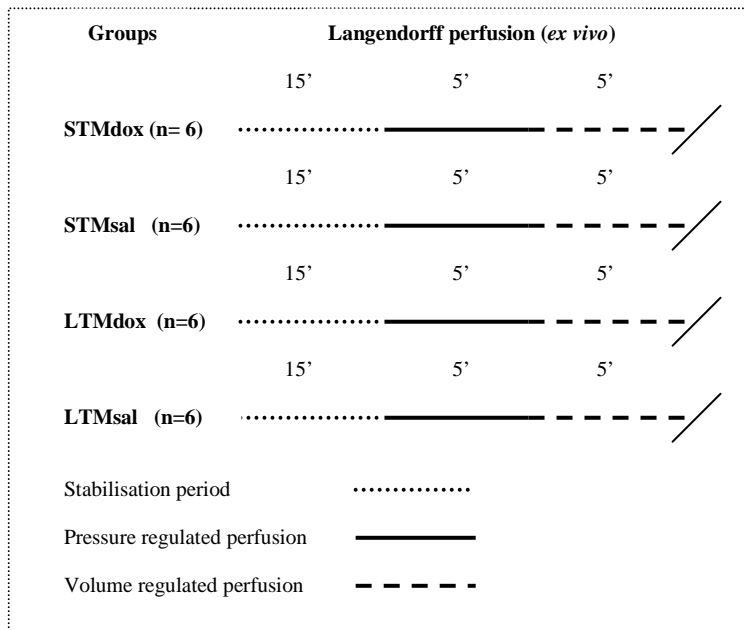


Figure 1. Short time model doxorubicin treated hearts (STMdox). Short time model saline treated hearts (STMsal). Long time model doxorubicin treated hearts (LTMdox). Long time model saline treated hearts (LTMsal).