A Rat Model to Assess Interventions to Reduce Cardiotoxicity of Anthracyclines

Does pre-treatment with morphine reduce the cardiotoxicity of doxorubicin?

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ABSTRACT

A rat model to assess interventions to reduce cardiotoxicity of anthracyclines is of interest. The model should be able to induce significant myocardial damage rapidly but at the same time allow for measurement of protective interventions. A proposed model includes an in vivo model where rats are treated with repeated intraperitoneal injections of an anthracycline for a relatively short period of time. Effects on physiological variables of cardiotoxicity are subsequently assessed in an ex vivo isolated rat heart model (Langendorff), and effects on biochemical variables by measurement of heat shock proteins (HSP) in myocardial tissue.

A model with these elements was tested in this thesis. The model was designed to include a pilot study to assess whether pre-treatment with morphine, an opioid, could reduce cardiotoxicity of the anthracycline doxorubicin. The use of morphine as an analgesic is established in human cancer treatment, and opioids have been reported to induce protection against ischemia and oxidative stress in animal models. Proposed mechanisms of cardiotoxicity of anthracycklines include elements of oxidative stress and impaired calcium homeostasis. Thus, morphine could represent an intervention to reduce cardiotoxicity of anthracyclines.

Doxorubicin (DOX), an anthracycline, was administered to female Wistar rats (n = 16) every second day in a dose of 3 mg/kg for 11 days intraperitonealt. A subgroup of rats (n = 8) received pre-treatment with morphine (MO) 3 mg/kg 60 minutes prior to DOX (MO-DOX), while the other eight rats received saline (SAL) of identical volume (SAL-DOX). In the MO–DOX group, 6 out of 8 rats died prior to the end of the protocol, while the remaining two animals were moribund and therefore put to death before end of the protocol to collect tissue for analysis of HSP. The remaining animals (SAL-DOX) were tested in an isolated rat heart model (Langendorff) to measure physiological parameters. Due to loss of the intervention group, a group of animals (n = 5) with no treatment with MO, DOX or SAL were used as control (control hearts) for reduction in physiological parameters. The SAL-DOX group showed a significant reduction (40-60%) in physiologic parameters compared to control hearts irrespective of flow mode (pressure regulated or volume regulated perfusion) in the isolated rat heart model. Furthermore, this group showed a trend for higher (40%) coronary perfusion pressure, an index of coronary resistance during volume regulated perfusion. Analysis of HSP demonstrated no significant differences in the HSP expression except from HSP94 which only gave positive results in the SAL-DOX treated group. We also analyzed content of DOX (2.7 $\pm$ 0.8 nmol/g wet weight) and the major metabolite doxorubicinol (0.14 $\pm$ 0.1 nmol/g wet weight) in hearts from SAL-DOX group, respectively.

The results demonstrate that the present model can be used for rapid induction of cardiotoxicity in rats, and this has been reported by others. The cardiotoxicity can be measured irrespective of flow mode in the isolated rat heart model, and treatment results in significant accumulation of doxorubicine and its major metabolite in heart tissue. Treatment with doxorubicine in this protocol upregulates expression of HSP 94, but not other HSPs. Pre-treatment with morphine increased mortality when combined with doxorubicine. The results of the pilot study are in conflict with recent studies which have demonstrated a protective effect of morphine on the cardiotoxicity of doxorubicine. However, the experimental methods are not identical in the studies. Furthermore, different doses and periods of administration of the drugs were used.

In conclusion the present rat model is suitable to assess interventions to reduce cardiotoxicity of anthracyclines. Furthermore, pre-treatment with morphine increases mortality of doxorubicin in the in vivo model.

SAMMENDRAG

Det er av interesse å studere intervensjoner og rottemodeller for å redusere hjertetoksisitet indusert av antracykliner. Modellen skal kunne indusere signifikant og rask hjerteskade, men den skal samtidig kunne brukes til å måle beskyttende intervensjoner. En foreslått modell inkluderer en in vivo modell hvor rotter behandles med gjentatte intraperitonale injeksjoner med et antracyklin over en relativt kort periode. En ex vivo isolert rottehjertemodell brukes for å måle effekten av fysiologiske parametre som følge av hjertetoksisitet, og effekten på biokjemiske parametre måles ved graden av uttrykte heat shock proteiner (HSP) i myokardialt vev.

En modell med disse elementene ble prøvd ut i dette prosjektet. Modellen var konstruert til å inkludere et pilot studium for å bestemme hvorvidt forbehandling med morfin (MO), et opioid, kan redusere hjertetoksisiteten av antracyklinet doxorubicin. Bruken av morfin som smertestillende middel er vel etablert i human kreftbehandling, og opioider har vist seg å indusere beskyttelse mot ischemi og oksidativt stress i flere dyremodeller. Foreslått mekanismer for hjertetoksisitet av antracykliner inkluderer bl.a. oksydativt stress og endret kalsium homeostase. På denne måten kan MO representere en klinisk aksepterbar intervensjon for å redusere hjertetoksisitet som følge av antrasyklinbehandling.

Doxorubicin (DOX) ble administrert til Wistar hunnrotter (n = 16) intraperitonalt annenhver dag i en dose på 3 mg/kg over 11 dager. En undergruppe av disse rottene (n = 8) ble forbehandlet med MO 3mg/kg 60 minutter før de ble injisert med DOX, (MO-DOX). De resterende åtte rottene fikk likt volum med saltvann (SAL), (SAL-DOX). I MO-DOX gruppen døde 6 av 8 dyr før forsøksperioden var over. De resterende to dyrene var døende, og ble derfor avlivet før forsøksperioden var over for å kunne utføre analyse av HSP. Fysiologiske variabler i dyrene i SAL-DOX gruppen ble studert i en isolert rottehjerte modell (Langendorff). Som følge av stort frafall i intervensjonsgruppen, ble en ubehandlet gruppe dyr (n = 5) benyttet som kontroll (kontrollhjerter) for reduksjon i fysiologiske variabler. SAL-DOX gruppen viste en signifikant reduksjon (40-60%) i fysiologiske variabler sammenlignet med kontroll dyrene uavhengig av type perfusjon (trykkregulert eller volumregulert) i modellen. Denne gruppen viste en trend mot høyere (40 %) koronart perfusjonstrykk, et indicator på koronar motstand ved volumregulert perfusjon. HSP analyser viste ingen signifikante forskjeller mellom de to gruppene, men HSP94 var bare positiv i SAL-DOX gruppen. Det ble også målt konsentrasjon av DOX (2,7 $\pm$ 0,8 nmol/g wet weight) og hovedmetabolitt doxorubicinol (0,14 $\pm$ 0,1 nmol/g wet weight) i hjertevev fra SAL-DOX gruppen.

Resultatene demonstrerer at nåværende modell kan benyttes for rask induksjon av hjertetoksisitet i rotter, et resultat som også er blitt rapportert av andre. Hjertetoksisitet kan måles uavhengig av type perfusjon i den isolerte rottehjertemodellen, og behandling fører til signifikant akkumulering av doxorubicin og dets hovedmetabolitt i hjertevev. Behandling med doxorubicin etter denne protokollen oppregulerer utrykk av HSP 94, men ikke andre HSP. Resultatene i pilotstudien samsvarer ikke med andre nylige studier som har vist at morfin gir beskyttelse mot hjertetoksisitet som skyldes doxorubicin. Metodene, endepunktene, dosering og administrasjonsperiode varierte imidlertid mellom studiene.

Det konkluderes med at nåværende rottemodell egner seg til å fastsette intervensjoner for å redusere hjertetoksisitet som følge av antracykliner. Forbehandling med morfin øker dødeligheten av doxorubicin i denne in vivo modellen.

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CONTENTS

1. INTRODUCTION

1.1 Aim of the study

The main purpose of this study was to test an animal model to assess interventions to reduce cardiotoxicity of anthracyclines based on physiological and biochemical endpoints The model should be able to induce significant myocardial damage rapidly (in less than 2 weeks), but at the same time allow for measurement of protective interventions. Furthermore, the model was designed to include a pilot study to assess whether pre-treatment with morphine, an opioid, could reduce cardiotoxicity of the anthracycline doxorubicin. Effects on cardiotoxicity was assessed in an *ex vivo* isolated rat heart model (Langendorff 1895), and measurement of heat shock proteins (HSPs). The use of morphine as an analgesic is established in human cancer treatment, and opioids have been reported to induce protection against ischemia and oxidative stress in animal models. Proposed mechanisms of cardiotoxicity of anthracyclines include elements of oxidative stress and impaired calcium homeostasis. Thus, morphine could represent an intervention to reduce cardiotoxicity of anthracyclines acceptable for patients.

1.2 Abbreviations and definitions

Following abbreviations and definitions have been used through the entire thesis.

Afterload	Resistance against the ejection of blood, including the peripheral vascular resistance, aortic stenosis and the blood distending the left ventricle at the end of diastole
AoP	Aortic pressure
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CO	Cardiac output
CVP	Central venous pressure
DOX	Doxorubicin
DOX_{ol}	Doxorubicinol
dP/dt_{min}	Minimum value of 1st derivative of LVDP as a function of time
dP/dt_{max}	Maximum value of 1st derivative of LVDP as a function of time
KHBB	Krebs Hensleits bicarbonate buffer
HR	Heart rate
HPLC	High pressure liquid chromatography
HSP	Heat shock protein
LVDP	Left ventricular developed pressure
LC-MS/MS	Liquid chromatography with mass spectrometry detection
LVDP	Left ventricular diastolic pressure
LVSP	Left ventricular systolic pressure
LVEDP	Left ventricular end-diastolic pressure
MO	Morphine
OR	Opioid receptor
PBS	Phosphate buffered saline
PC	Pharmacological preconditioning
ROS	Reactive oxygen species
SAL	Saline, NaCl (aq)
SVR	Systemic venous resistance

1.3 Background and justifications

Cancer and heart failure are two diseases with a high rate of mortality. Doxorubicin (DOX), an anthracycline, is one of the most used antineoplastic agents. However, DOX, similar to other anthracyclines, is associated with cardiotoxic effects leading to development of heart failure which is one of the main limitations for its use in treatment of cancer.

The mechanisms of DOX induced cardiac toxicity remains unknown, but it has become possible to distinguish between the mechanisms underlying DOX´s antitumour effect and the mechanisms responsible for cardiotoxicity. The fact that these dual effect of anthracyclines seem to differ in mechanisms has raised the possibility of designing treatment protocols that reduce damage to the heart without reducing antitumor effects.

Several protective approaches to avoid cardiotoxicity of anthracyclines have been tried, e.g. liposome-encapsulation of the anthracyclines, co-administration with the iron-chelating agent dexrazoxane and numerous antioxidants. However they have limited ability to protect the heart from anthracyline induced damage, and have failed to be included in a clinical setting. At present there is no specific treatment protocol to avoid DOX-induced cardiac toxicity except dose reduction, and therefore, it is of considerable clinical importance to find methods to avoid or minimize the cardiotoxic effects of anthracyclines in treatment of cancer patients.

1.4 The anthracyclines, doxorubicin and cardiotoxicity

The anthracycline antibiotics are natural products derived from the actinobacteria *Streptomyces peucetius* originally developed as antibiotics. They are cancer remedies and act by binding between adjacent base-pairs in DNA that result in inhibition of nucleic acid synthesis preventing replication and transcription.

Doxorubicin (DOX), an anthracycline, was discovered in the early 1960s and was found to be very effective against both solid and soft tumours, and are now widely used as an antineoplastic agent for a wide range of cancers including leukaemia (Hitchcock-Bryan, Gelber et al. 1986) and solid tumours, i.e. breast cancer (Pai and Nahata 2000; Minotti, Menna et al. 2004).

However, doxorubicin's cumulative myocardial toxicity limits long term use and an optimal dosing with less effective cancer treatment as a result, and represents a clinical problem and a challenge in cancer treatment (Rhoden, Hasleton et al. 1993; Shan, Lincoff et al. 1996). Cardiotoxicity appears in several forms, though cardiomyopathy and congestive heart failure are the most important cumulative dose-limiting adverse effects of anthracyclines (Plosker and Faulds 1993; Shan, Lincoff et al. 1996; Kremer, van Dalen et al. 2001). Cardiotoxicity caused by doxorubicin may be divided into acute, subacute and late forms. The acute form is a myocarditis–pericarditis syndrome with side effects like nausea, vomiting and arrhythmia. The acute cardiotoxicity is rare with current treatments protocols, and is often reversible. The subacute form occurs several weeks or months after end of doxorubicin treatment, and it has about 60% mortality. The late forms may be seen as late as 4-20 years after the end of treatment (Steinherz, Steinherz et al. 1991).

Shan et al. has defined "chronic cardiotoxicity" as cardiotoxcity appearing within a year of treatment and "late-onset cardiotoxicity" as cardiotoxicity occurring more than one year after ended treatment (Shan, Lincoff et al. 1996). Recently several retrospective investigations have shown that late doxorubicin-induced cardiomyopathy occur years after end of treatment (Lipshultz, Colan et al. 1991; Steinherz, Steinherz et al. 1991; Leandro, Dyck et al. 1994).

Dose reduction reduces the acute and early incidences of myocardial failure, but it does not seem to remove the risk of late myocardial failure. The incidence of congestive heart failure is 0.14% at a total dose of less than 400 mg/m^2 body surface area, but increases to 7% at a dose of 550 mg/m^2 body surface area (Von Hoff, Layard et al. 1979). In accordance to the dose dependent clinical toxicity an empirical dose limit at 500 mg/m^2 per surface area was

suggested as a way to reduce the risk of cardiomyopathy (Lefrak, Pitha et al. 1973). A study found that low cumulative doses of doxorubicin, 228mg/m^2 per body surface area, increased afterload or decreased contractility or both in 65% of leukaemia patients up to 15 years after treatment with anthracyclines (Lipshultz, Colan et al. 1991). Additional dose intensity of treatment, female sex, young and old age and combination of anthracyclines with other chemotherapeutic agents are all risk factors for cardiac toxicity (Lipshultz 2006).

Development of DOX-induced cardiotoxicity is probably multifactorial in genesis, and several mechanisms have been suggested. Most studies support the view that an increase of oxidative stress in the mitochondria, as shown by an increase in levels of reactive oxygen species (ROS) and lipid peroxidation, is of primary importance in the pathogenesis of DOX-induced cardiotoxicity. Generation of free radicals damage cellular membranes which result in depletion of ATP generation (Singal, Siveski-Iliskovic et al. 1995). Other mechanisms that might play a role in the pathogenesis of DOX-induced cardiotoxicity are; myocyte damage due to increased Ca^{2+} - levels mediated by DOX and ROS (Kim, Kim et al. 2006), imbalance in myocardial electrolytes, reduced expression of a variety of cardiac muscle-specific proteins including contractile proteins and mitochondrial proteins. The former are directly related to diminished contractility of cardiomyocytes. Further, alteration in adrenergic function, release of vasoactive amine, lysosomal changes, inhibition of nucleic acid and protein synthesis, abnormalities in mitochondia, attenuation in adenylat cyclase, Na^+-K^+-ATPase, and Ca^{2+}-ATPase activities are mechanisms involved (Singal, Iliskovic et al. 1997; Takemura and Fujiwara 2007). Furthermore increased myocardial cell apoptosis in vascular cells and cardiomyocytes has been reported due to DOX (Arola, Saraste et al. 2000).

These mechanisms have similarities to mechanisms involved in heart failure due to myocardial ischemia. The ischemic response in the heart is characterised by reduced oxygen consumption, transition from aerobic metabolism to anaerobic metabolism and depressed contractile activity with electrocardiac changes. Alteration in energy metabolism results in decomposition of the cells ATP storage. ATP demanding membrane pumps will suffer and result in elevated intracellular levels of water, Ca^{2+} and Na^+, while K^+ leak out of the cells.The electrolyte imbalance leads to swelling and myocyte damage. Reduced protein- and lipid synthesis are followed by damaged membrane integrity which may lead to apoptosis. Expression of O_2 – radicals may also occur (Sommerschild 2000).

Doxorubicin follows a linear pharmacokinetic after intravenous administration, and is widely distributed in the plasma and tissues. The plasma protein binding varies from 50-85%. The drug is mainly metabolised in the liver by aldo-keto reductase which converts DOX to a

secondary alcohol metabolite, doxorubicinol, (DOX_{ol}), by reduction of a side carbonyl group, and by the NADPH dependent cytochrome P450 (CYP) reductase which cleaves the glycosidic bond and release aglycone metabolites (Licata, Saponiero et al. 2000). The DOX_{ol} retains antitumour activity, but is together with the aglycone metabolites also implicated in cardiotoxicity which include the inhibition of the Ca^{2+}/Mg^{2+} ATPase of sarcoplasmatic reticulum, the Na^{+} -K^{+} ATPase and the Na^{+}-Ca^{2+} exchanger of sarcolemma (Olson, Mushlin et al. 1988). Elimination occurs via biliary elimination and faecal excretion. Approximately 50% of the biliary eliminated dose is unmetabolised drug, 23% is DOX_{ol}, and the rest is of other metabolites. See figure 1.1 for chemical structure of doxorubicin and its metabolites.

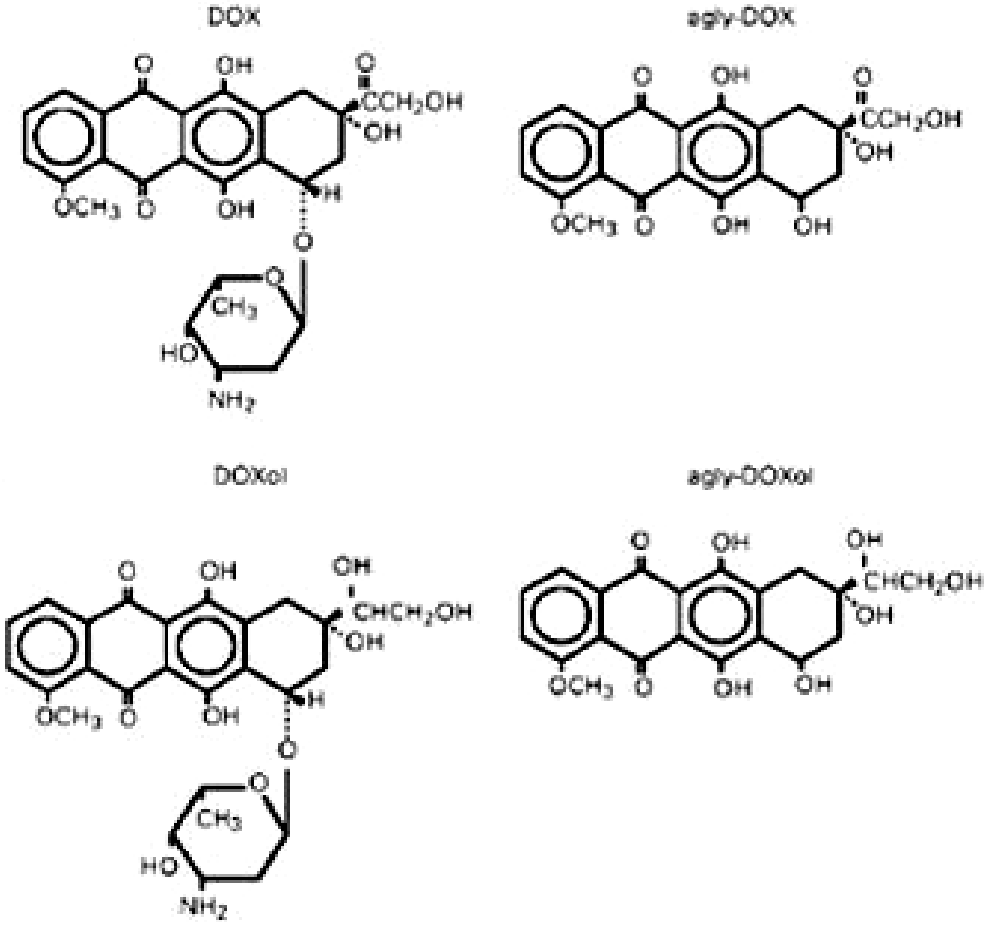

Figure 1.1 Chemical structures of doxorubicin (DOX), doxorubicinol (DOX_{ol}), aglycone-DOX (agly-DOX) and aglycone-DOX_{ol} (agly-DOX_{ol}) (Misiti, Giardina et al. 2003).

1.5 Cardioprotection, opioids and morphine

Ischemic preconditioning (IPC) is a cardioprotective phenomenon in which one or more periods of myocardial ischemia prior to a subsequent prolonged ischemic insult result in an adaption which increases the myocardial tolerance against infarction. Both delayed cell injury and limit myocardial necrosis have been described (Yellon, Baxter et al. 1998). The phenomenon was first described by Murry et al. (1986). They studied the effect of short term coronary occlusions on anaesthetized dogs (Murry, Jennings et al. 1986) and reported that ischemic preconditioning resulted in infarct sizes approximately 25% of those observed in untreated hearts. The phenomenon has an early phase in which cardioprotective effects last for 1-3 hour, and a delayed phase which appears approximately after 24 hours and may last up to 72 hours (Schultz and Gross 2001). Preconditioning is at present known as the best protection form against infarction (Lawson and Downey 1993; Richard, Kaeffer et al. 1996). The cardioprotection achieved by IPC is manifested in a reduction of infarct size, which is used as the most common measure (Dekker 1998).

IPC reduces metabolic activity during the ischemic period which results in better protection of the cells energy storage of ATP (Murry, Richard et al. 1990). Furthermore damaging ionic alterations during ischemia and subsequent reperfusion is reduced. Liu et al. were the first to look into possible activation of receptor pathways by IPC (Liu, Thornton et al. 1991). They demonstrated that pre-treatment with both adenosine and adenosine receptor antagonists induced protection against infarction in rabbit hearts similar to IPC by A_1 receptors. They also showed that an adenosine antagonist administered before IPC abolished the protective effect. Later, another endogenous substance, bradykinin, was demonstrated to induce protection associated with IPC. Importantly, this implies that interventions other than ischemia can induce protection associated with IPC, but with a potential for acceptance in the clinical setting. Pharmacological preconditioning (PC) has been demonstrated with opioids including morphine, and involves activation of delta and kappa receptors (Gross 2003). In a study, Schultz et al. (1995) suggested that opioid receptor activation serves not only as a trigger of the preconditioning (PC) response but also as a mediator of the memory phase of PC in the rat myocardium. Thus, in early preconditioning, a memory phase of up to two hours after trigging in which protection is demonstrated. Interestingly and of clinical relevance, morphine is a first-line analgesic in cancer treatment where anthracyclines are involved.

1.6 Cellular apoptosis, necrosis and intracellular signalling pathways

In addition to their essential role in cell survival, the mitochondria are regulators of cell death via both apoptosis (programmed cell death) and necrosis (accidental death of cells). Through feedback mechanisms between the mitochondria and the cytoplasm, cell survival, energy metabolism and homeostasis are maintained (Javadov and Karmazyn 2007). However, stress e.g. myocardial ischemia increases intracellular Ca^{2+} concentration and oxidative stress generation within the cytoplasm and the mitochondria. The cellular Ca^{2+} overload, oxidative stress and ATP reduction result in a permeability transition associated with the formation of a non-specific permeability transition pore (mPTP) in the inner mitochondrial membrane with subsequent loss of ionic homeostasis. During a mild insult of stress, transient opening of the mPTP and/or irreversible pore opening can result in matrix swelling and outer membrane rupture, leading to the release of apoptotic proteins and induction of apoptosis (Halestrap, Clarke et al. 2007). Apoptosis is only possible if a majority of the mitochondria are still able to synthesize ATP. Under the condition of a severe insult of stress massive swelling and mitochondrial membrane depolarization result in enhanced production of reactive oxygen species (ROS) and ATP hydrolysis. Even though apoptotic proteins are released, cell death will occur via necrosis due to ATP depletion (Javadov and Karmazyn 2007). Most likely there is a link between apoptosis and IPC. Ischemic preconditioning prevents formation of mPTP, and prevents therefore also major influx of electrolytes and water into the mithocondrial matrix, resulting in a decrease of apoptotic myocytes (Tissier, Berdeaux et al. 2008).

Opioids, adenosine and bradykinin are assumed to give cardioprotection via stimulation of G-protein coupled receptors. As a result protein kinase C (PKC) enhances ATP dependent potassium channel (K_{ATP}) activity. It has been demonstrated that in isolated rabbit cardiomyocytes, bradykinin triggered ROS generation, and this effect was abolished by inhibitors of both mitoK_{ATP} and protein kinase G (PKG) (Oldenburg, Qin et al. 2004). Opioid receptor stimulation has also been shown to increase intracellular ROS which also activate PKC. Opening of mitochondrial K_{ATP} channels contributes to an increased level of ROS, which further amplifies activation of K_{ATP} channels (McPherson and Yao 2001). Opening of the K_{ATP} channels are thought to result in a cascade of signals which in turn will lead to inhibition of mPTP formation in mitochondria (Tissier, Berdeaux et al. 2008). See figure 3.1.

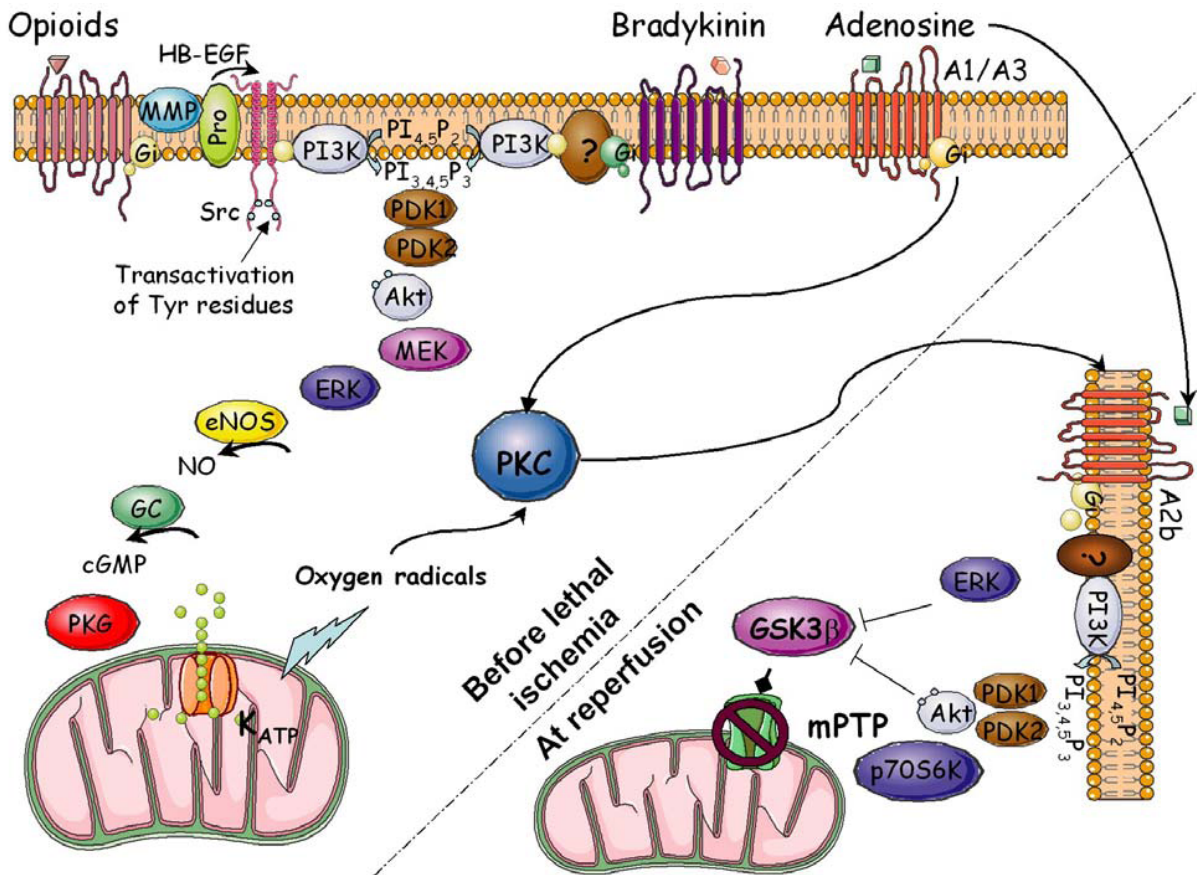

Figure 3.1

The figure illustrates a simplified signaling pathways of myocardial preconditioning (Tissier, Berdeaux et al. 2008)

MMP; matrix metalloproteinases; HB-EGF, heparinbindingepidermal growth factor-like growth factor; Pro, pro-HB-EGF; PI3K, phosphatidylinositol 3-kinase; PI4,5P2, phosphatidylinositol bisphosphate; PI3,4,5P3, phosphatidylinositol trisphosphate; MEK; mitogen activated protein kinase kinase; ERK, extracellular-signal regulated kinase; NO, nitric oxide; NOS, NOS synthase; eNOS, endothelial NOS; GC, guanylyl cyclase; PKG, protein kinase G; PKC, protein kinase C; mKATP, mitochondrial ATP-dependent potassium channel; p70S6K, p70S6 kinase; GSK-3b, glycogen synthase kinase-3b; mPTP, mitochondrial permeability transition pore.

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1.7 Opioids, receptors and protection

Opioid receptors are involved in regulation of cardiovascular tissue in both healthy and damaged myocardium. The G-protein coupled receptors are localized in the central nervous system and peripherally to autonomic presynaptic nerve endings and on cardiac myocytes (Ventura, Guarnieri et al. 1994). Myocardial cells are site for opioid peptide synthesis, storage and release, which are elevated during stress (Barron, Jones et al. 1995; Eliasson, Mannheimer et al. 1998). There are three primary subtypes of the opioid receptors (OR); my (μ), delta (δ), and kappa (κ). Several studies have found that activation of specific ORs induce cardioprotection similar to IPC (Schultz, Rose et al. 1995; Schultz, Hsu et al. 1997; Benedict, Benedict et al. 1999), and the δ- ORs seem to be the primary ORs to mediate the cardioprotective effect of IPC. Schultz et al suggested that the δ_1-subtype mediates the IPC using BNTX, a selective δ_1-OR antagonist, (Schultz, Hsu et al. 1998) and a TAN-67, a selective δ_1-OR agonist (Schultz, Hsu et al. 1998).

It has been shown that remifentanil, a syntetic opioid analgesic drug, mediates cardioprotective effects through the κ- and δ-ORs, but not through the μ-ORs (Zhang, Irwin et al. 2005). An interesting finding observed by Guan-Ying Wang and co-workers is that only the κ-receptors are involved in mediating protection on both infarct and arrhythmia mediated by ischemia, while the δ-ORs only take part in the mediating of arrhythmia protection. They concluded that pre-treatment with a κ-OR agonist might give a more beneficial protection compared with pre-treatment with a δ-OR agonist. It has to be taken into account that a higher concentration of the κ-OR agonist is required to generate cardioprotection. (Wang, Wu et al. 2001).

Morphine (MO) is a commonly used analgesic that, besides having μ-OR mediated analgesic effects, also can stimulate the δ_1-OR. Pre-treatment with MO 24 h before 25 minutes of regional ischemia followed by 2 hours of reperfusion reduced infarct size after 24 hour to 20%. The infarct size reduction with MO was abolished by the μ-OR competitive antagonist naloxone (Frassdorf, Weber et al. 2005).

1.8 Models and methods to assess cardiotoxicity of anthracyclines

The following sub chapters describe different methods used to assess cardiotoxicity of anthracyclines.

1.8.1 The *ex vivo* isolated heart model

The Langendorff technique was first used more then a century ago (Langendorff 1895), and today it is a well established technique in pharmacological and physiological research performing isolated perfused heart experiments. The principle of the Langendorff apparatus consists of an excised animal heart with oxygen and nutritients via a perfusion fluid i.e. physiological solution or blood. Using the technique, cardiac contractile strength, heart rate and vascular effects can be measured and examined in an isolated heart in absence of the effects of other organs. The Langendorff studies are conducted in either constant pressure, or constant flow modes. In the Langendorff preparation, the aorta is cannulated and the heart is retrogradely perfused.

The technique allows a highly reproducible preparation which can be examined quickly and in large numbers in a cost effective manner, and is a useful tool to examine different aspects of ischemia- and reperfusion induced injury as well as for controlled dose-response studies of metabolic and pharmacologic interventions (Sutherland and Hearse 2000). The isolated heart preparation is valuable for assessing the direct cardiovascular effects of different drugs in form of contractile function, electrical activity or metabolic activity. However, it has to be taken into account that the heart is separated from other organs, normal blood flow and the central nerve system, and therefore in absence of hormones and both sympathetic and vagal stimulation. Although neurotransmitters, proteins, catecholamines etc. might be added in the perfusate, in vivo reactions due to the model can not occur.

An acute model were the heart is exposed to anthracyclines when mounted on the Langendorff apparatus have been reported (Schjott, Gjerde et al. 2006). The model has the advantage of eliminating systemic cardiovascular effects when studying physiological responses to pharmacological interventions. However, the model bypasses the pharmacokinetic phases (absorption, distribution and elimination) associated with introduction of a drug in the body, and gives only insight into the direct action of an anthracycline on the myocardium. Furthermore, knowledge of clinical relevant tissue concentrations of substances could be lacking. Toxic metabolites in adequate concentrations could also be excluded from the model.

This is of importance when studying anthracyclines like DOX where part of the cardiotoxicity is believed to result from the metabolite doxorubicinol (Minotti, Parlani et al. 2001).

The most frequently examined, and most studied mammalian heart is the rat heart, although mouse and guinea pig represents alternative species.

1.8.2 The *in vivo* rat model

In vivo (Latin: within the living) research refers to experimentation done in the context of intact organisms. Rat is used for research in several scientific areas such as biochemistry, pharmacology, toxicology and oncology. Their broad similarity to man in physiology as well as on the genetic level makes it possible to measure similar disease parameters in both species.

Research animals respond to many factors, or changes in their environment. These responses may affect the experimental results, and thus animals used in research should be kept under conditions that permit as standardized a response to experimental parameters as possible. Numerous factors including ambient temperature, humidity, ventilation, light, sound etc. influence the response of animals to experimental procedures. Normally rats are housed 4-5 together in cages with plastic walls and a stainless mesh lid with excess to tapped water and appropriate food.

Replacement-, reduction -, and refinement alternatives (the Three Rs) should be kept in mind when performing scientific research. The term reduction alternatives describe methods for obtaining comparable levels of information using fewer animals. The animals used should be kept at a minimum that is consistent with the aims of experience. Refinement alternatives shall aim for the most gentile methods that enhance animal well-being. Replacement alternatives encompass compensatory methods for animal research, e.g. use of cells or organs for *in vitro* studies.

When drugs or other agents are to be administered to an animal, several different routes may be selected. The route selected is decided by type of animal and the purpose of administration. The more common routes of administration used for laboratory rats are:

Gastrointestinal tract:

- Oral per os (p.o.) - through the mouth
- Gavage -into the stomach via tube

Parenteral:

- Intravenous (i.v.)- directly into the circulatory system through a vein
- Intraperitoneal (i.p.)- injected into the abdominal cavity

- Subcutaneous (s.c.)- injected beneath the skin
- Intramuscular (i.m.)- injected into a muscle

Compounds absorbed i.p. will pass into the circulation relatively fast due to the very large surface area for absorption and the abundant blood supply to the organs and tissue of the peritoneal cavity. Compounds absorbed will pass through hepatic circulation prior to distribution to other organs. The abdomen is penetrated in either of the lower quadrants because intestinal mass and organs generally lie in other areas. When injecting intraperitoneal, the needle may go into the intestinal tract and irritant materials may cause peritonitis. The rat should be held in a head-down, stretched-out position during the i.p. injection to avoid damage on internal organs. Compounds injected into the muscle are slower absorbed than from i.p. injections. On the other hand the absorbed material will pass directly into the circulation. The i.m. administration is painful and injection site need to be rotated. The subcutan administration is gentle for the animal, but yields a slower absorption than after i.m. administration. Solid drug forms may be effectively administrated by s.c. implantation. When given intravenously, a drug is immediately delivered to the bloodstream and tends to provides effect more quickly than when given by any other route; thus i.v. administration is the most efficient parenteral route, though prolonged i.v. administration may be accomplished by jugular vein catherization, and requires a surgical approach.

Rats are frequently used when investigating pharmacokinetics, how drugs are absorbed, metabolized and excreted from the body when introducing orally, i.v., i.p., i.m. or s.c., and in toxicology tests in order to discover toxic drug metabolites. High performance liquid chromatography (HPLC) in combination with mass spectrometry (MS/MS) can be used to determine concentration of drug and metabolites in blood and homogenized tissue. With the use of imaging (magnetic resonance imaging, MRI, Positron emission tomography, PET-scan etc.) toxicological effects on structure or function of organs can be studied *in vivo* without tissue dissection, sectioning and staining. In oncology research, the use of imaging allow researcher to follow tumour progression without harming the animal which fulfil refinement.

1.8.3 A proposed combined model

In vivo exposure of anthracyclines to rats can be followed by further *ex vivo* or *in vitro* studies to detect drug concentration and physiological variables (Arola, Saraste et al. 2000; Platel, Pouna et al. 2000). Previous cardiotoxicity models include long term exposure of anthracyklines for 5-10 weeks (Bottone, Voest et al. 1998; Kelishomi, Ejtemaeemehr et al.

2008). These models are time consuming, and represent long-time stress for the animals and do not fulfil the idea of refinement. Thus it is of interest to develop short-time models of two weeks duration which imply cost effectiveness and saving of time. Further, compared with the acute model, such models include distribution, metabolism and elimination of the drug and are therefore more relevant to the clinic.

A combination of the two models, *in vivo* and *ex vivo*, could represent an interesting compromise. This model would include the relevant pharmacokinetic phases as well as controlled evaluation of relevant end points (cardiotoxicity) without interference of systemic effects. Furthermore, it gives the opportunity to collect myocardial tissue for pharmacological and biochemical measurement. The model could represent an attractive approach in animal studies and preclinical testing of protective interventions to reduce cardiotoxicity. However, as with most animal models there are limitations, and the relevance for the clinic and present human protocols should be kept in mind.

An optimal model should be able to induce significant myocardial damage rapidly but at the same time allow for measurement of protective interventions. For instance, it has to be taken into account that an equal cumulative dose administered over a short period versus a long period may cause more damage to the animals and the hearts. Thus, there is a risk of inducing so much damage that the effect of any intervention is masked. On the other side, the effect on biochemical parameters, like HSPs, could be neglected due to the short and intensive duration of the protocol. Thus, the individual and cumulative dose, frequency of administration and duration of drug therapy is of major importance in such a model.

1.8.4 The Langendorff heart preparation

In this model, the aorta of the excised heart is mounted onto a cannula which is connected to a chamber containing oxygenated perfusion fluid which is delivered in the retrograde direction. Retrograde perfusion via aorta to the coronary arteries can be either at a constant flow rate (with the use of a calibrated pump) or at a constant hydrostatic pressure (60-100 mmHg). When the perfusion fluid enters aorta, the aortic valves shut and the fluid is directed into the *coronary ostia* from where the fluid spread and supply the entire heart with oxygen and nutritients. After perfusing the entire ventricular mass of the heart, the perfusate exits the coronary venous circulation via the coronary sinus and out of the open right atrium.

During an experiment, a constant flow preparation will feed the heart with a certain amount of fluid per minute. Unlike constant pressure perfusion,

autoregulatory mechanisms are overruled and the flow does not automatically adjust due to changes in variables like heart rate, myocardial work or ischemic tissue. Irrespective of flow mode, the method allows a broad spectrum of physiological variables including contractile function, heart rate and electrical activity of the heart to be measured from a single experiment. Left ventricular developed pressure (LVDP) = left ventricular systolic pressure (LVSP) – left ventricular end-diastolic pressure (LVDEP), and heart (HR) are recorded by a pressure transducer connected to a small balloon placed in the left ventricle. The maximal (LV dt/dt max) and the minimum (LV dp/dt min) first derivatives of LVDP indicate maximum and minimum changes in pressure with time, and reflect the capacity of the myocard to develop a rapid contraction or relaxation. Pressure transducers are connected to an analogue/digital converter which allows the recording and analysis of data on a computer. Perfusion delivery pressure or aortic pressure AoP, which reflects pressure in the aorta, is recorded by a pressure transducer in the inflow line. Per definition mean aortic pressure, $AoP_{mean,}$ is determined by the cardiac output, CO, the systemic vascular resistance and central venous pressure (AoP_{mean} = (CO x SVR) + CVP). Because we are working with an isolated heart AoP_{mean} in this relation reflects the pressure in the coronary circulation (Skrzypiec-Spring, Grotthus et al. 2007). Under constant pressure the AoP will adjust to changes in the HR or contraction, but when perfusing at constant flow rate, autoregulatory mechanisms are overridden and the amount of perfusate delivered is not altered physiologically in response to changes in HR or contraction. Due to this AoP can be elevated during constant flow due to experimental conditions. The extent of elevation reflects the resistance of the coronary vasculature. Electrocardiogram (ECG) can be attached to the exterior of the heart to monitor electrical activity, and pacing electrodes can be used in a similar fashion to keep HR. Thus, the model is suitable for monitoring physiological and electrophysiological parameters, and in particular delivery of drugs and other substances can be closely monitored. Furthermore, the perfusion system allows control with temperature, delivery of gas and perfusion fluid. See fig 3.7.1 and 3.7.2.

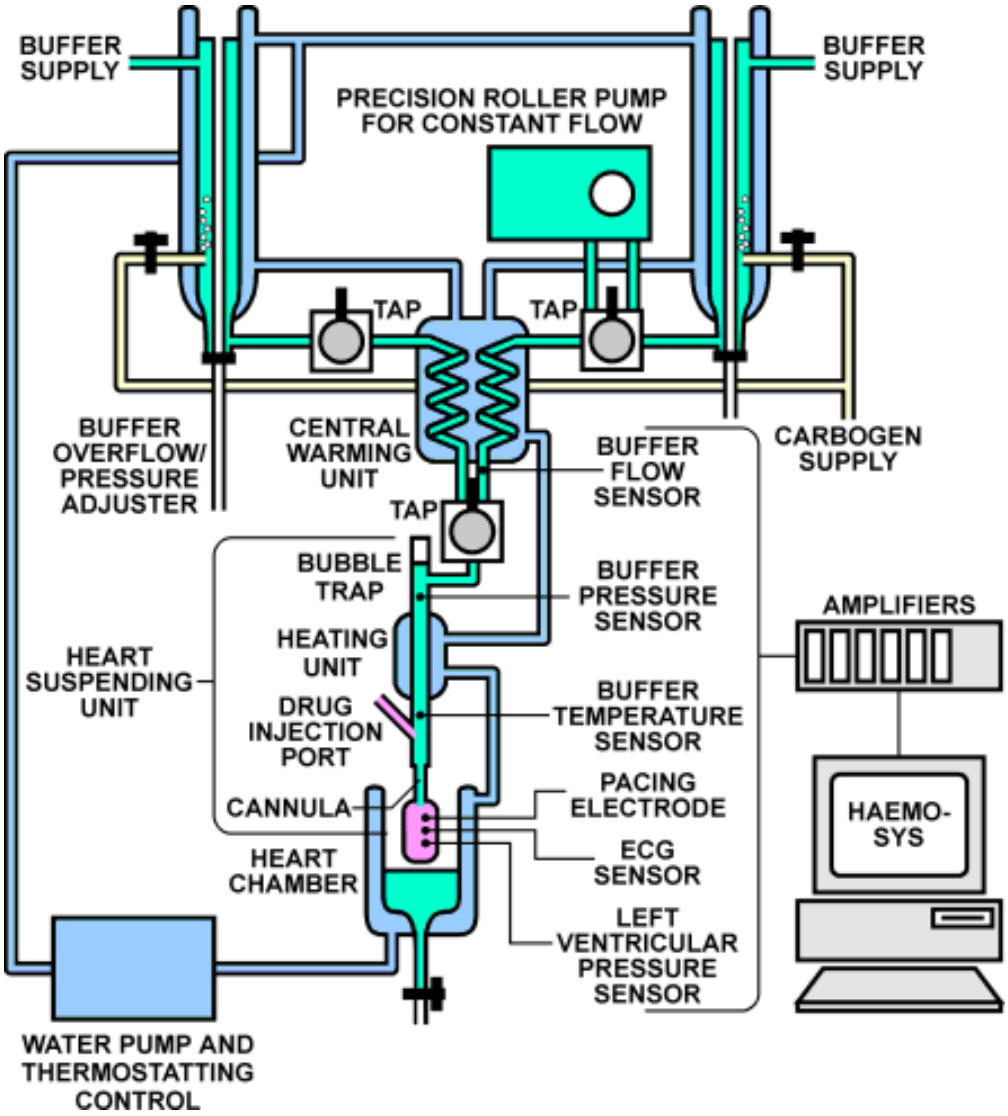

Figure 3.7.1
The figure illustrates a Langendorff isolated heart perfusion system (QuantaMetrix Access year 2008).

The perfusion fluid;

The Krebs-Henseleit bicarbonate buffer (KHBB), first described by Krebs and Henseleit (Krebs and Henseleit 1932), was supposed to replace blood, and imitate the ionic content of blood and have a pH at 7.4 at 37 ºC. KHBB contains the following ingredients (in mM); *NaCl 118.5, $NaHCO_3$ 25.0, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, glucose 11 and $CaCl_2$ 1.2.* Sutherland and Hearse have chosen to correct the calcium content in the buffer due to the fact that much of the calcium in blood is bound to proteins and the realistic plasma ionized calcium concentration is approximately half of the recommended value of 2.5 mM (Sutherland and Hearse 2000).

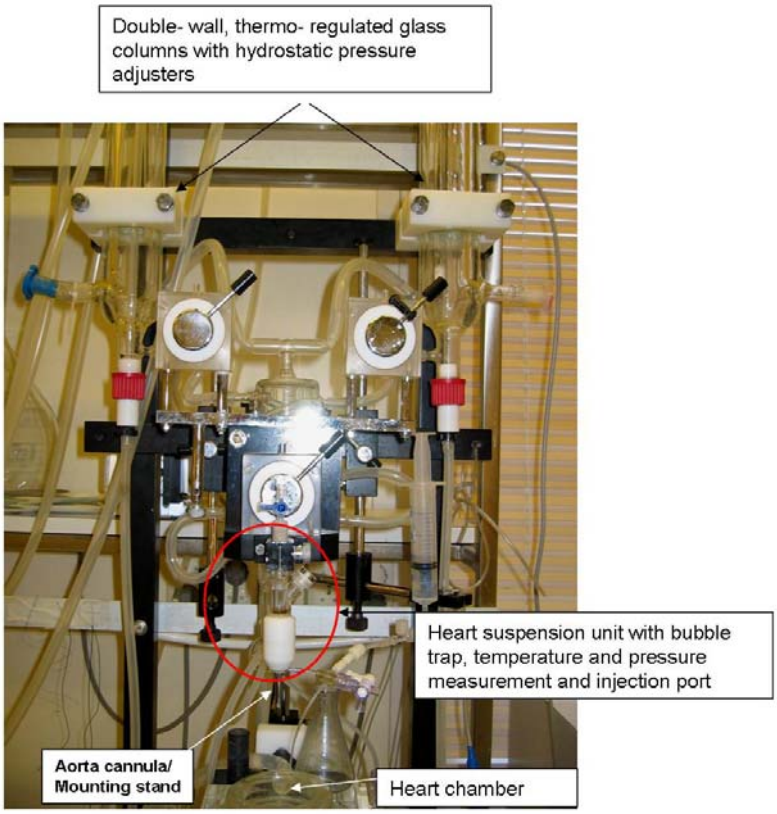

Figure 3.7.2
The photo shows the Langendorff system used at the Vivarium, University of Bergen.
The Langendorff perfusion system shown consists of following components:

- Double-wall, thermo-regulated glass columns with hydrostatic pressure adjusters
- Roller pump and reservoirs
- Heart suspension unit with bubble trap, temperature and pressure measurement and injection port
- Heart chamber/warming jacket
- Mounting stand
- Roller pump for constant flow

1.8.5 Heat shock proteins, antibodies and staining

Heat shock proteins

The cells have their own physiological defence systems to manage stress. Heat shock proteins (HSPs) belong to a family of proteins with chaperone characteristics which provide one kind of defence against stress like ischemia/hypoxia, high temperature, exposure to toxic chemicals and oxidative stress. Molecular chaperones are proteins that facilitate the folding, assembly and disassembly of other proteins, but are not part of the finished product.

Heat shock proteins have been divided into several categories on the basis of their molecular mass. Heat shock protein-27 for instance, has a molecular weight of approximately 27 kDa. Their expression is elevated when the cells are exposed to stress.

It has been demonstrated that HSP90 and HSP70 are elevated in the heart responding to doxorubicin injury (Gabrielson, Bedja et al. 2007). Sprague-Dawley rats were given doxorubicin or saline over a 6-week period and were harvested after 10 weeks. A significant upregulation of the two HSPs was demonstrated in the DOX treated rats, but not in control rats by Western blotting. They also showed that treatment with DOX and the HSP90 inhibitor 17AAG in combination in isolated cardiomyocytes increased the toxicity of DOX compared to DOX treatment alone. HSP27 is also believed to protect against DOX induced injury. It has been demonstrated that transgenic mice with cardiac specific over-expression of HSP27 are more resistant to DOX induced heart failure *in vivo* (Liu, Zhang et al. 2007).

Antibodies

Antibodies belong to a group of proteins called immunoglobulins (Ig) which is part of the immune defence. Immunoglobulins comprise five major classes; IgG, IgA, IgM, IgD and IgE listed in order of decreasing quantity found in plasma. In addition there are subclasses of IgG (IgG_1-IgG_4) and IgA (IgA_1 and IgA_2) depending on their heavy chains, so there are 9 isoforms in total. Each Ig consist of two identical heavy chains (H), (gamma γ, alpha α, mu μ, delta δ, epsilon ε,), and two identical light chains (L), (lambda λ, and kappa κ) linked by disulfide bonds. The general structure of the antibodies does not differ much except from the antigen binding fragment of the protein which provides a great variation (figure 2.7.1). Therefore there exist several millions of different variations of this end structure. IgG (monomer) and IgM (pentamer) are by far the most frequently utilized antibodies in immunochemistry.

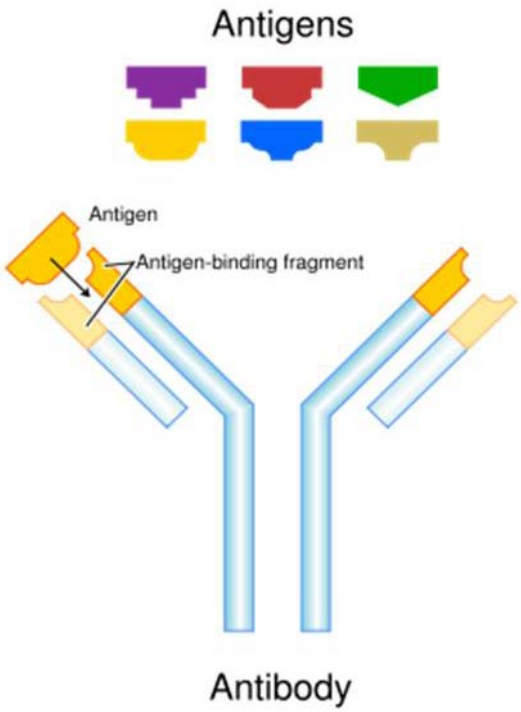

Figure 3.7.1

The figure is an illustration of an antibody. The figure is from Wikipedia (Fvasconellos 2007).

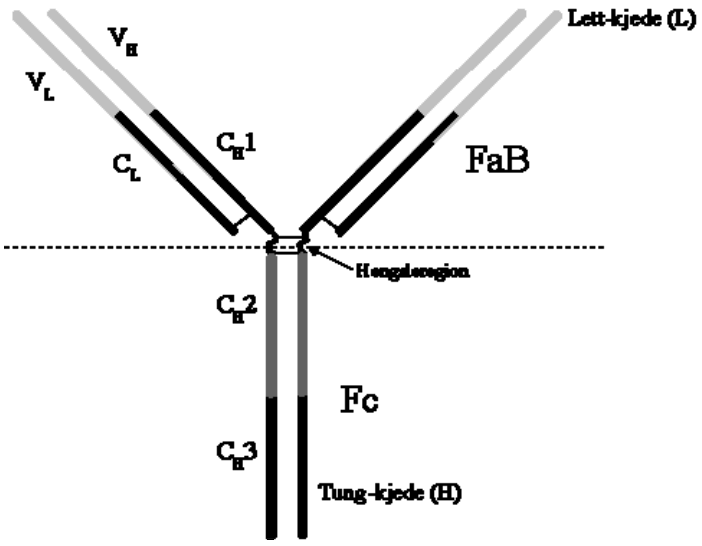

Figure 3.7.2

Illustration of an Y- shaped antibody (folk.UiO 2000) The enzyme papain cleave the disulfide bonding and cleave the monomer in two FaB-fragments (consisting of one light chain and one half heavy chain) and one Fc- fragment (consisting of two heavy chains carboxyterminal residues). The FaB region has antigen bonding function while the Fc region has effector functions.

1.8.6 Staining: an overview

Basic immunochemistry

> *Immunochemistry is the identification of a certain antigen in a histological tissue section or cytological preparation by an antibody specific to that antigen* (Renshaw 2007).

Antibody titer, dilutions, incubation time and temperature are all important co-players to achieve good quality of immunohistochemical staining. The parameters are interdependent of each other.

Antibody titer; "An immunohistochemistry titer is the highest dilution of an antiserum which will result in optimal species staining with the least amount of background"(Boenisch Thomas 2001) .

Antibody dilution; dilutions are usually expressed as the ratio of the more concentrated stock solution (a fairly concentrated solution which may be diluted before use) to the total volume of wanted dilution. A 1:5 dilution is made by dilution 1 part of stock solution with 4 parts of diluent.

Antibody incubation; incubation time and antibody titer is inversely proportional to each other. The higher the antibody titer, the shorter the incubation time can be. Normally antibody titer is set after incubation time is decided. Incubation time vary from a few minutes to several hours with 30 minutes most used. The incubation time of the primary antibody is of crucial importance due to the fact that equilibrium for these reactions rarely is reached within the first 20 minutes.

Incubation temperature; equilibrium in antigen-antibody reactions is reached faster at 37°C compared to room temperature (25°C) and therefore used. Temperature of 4°C is normally used for overnight incubation (Renshaw 2007).

Storage of fluorchrome-conjugates;

Fluorchrome–conjugated reagents are photosensitive and are therefore stored in the dark at 4°C. Tissue slides with fluorchrome-conjugates may also be stored at -20°C.

Indirect immunofluorescence staining method

This method involve an unlabeled primary antibody binding which reacts with the tissue antigen being probed for, and a secondary fluorescent labeled, antibody which reacts specifically to the primary antibody. If the primary antibody reacts specific with tissue an antigen and is cultured in rabbit, the secondary antibody must be against the IgG of the animal species in which the primary antibody was cultured, here rabbit, and raised in a different species, i.e. goat, see figure 3.7.3 below. Localization of the target antigen can be visualized by an UV microscopy.

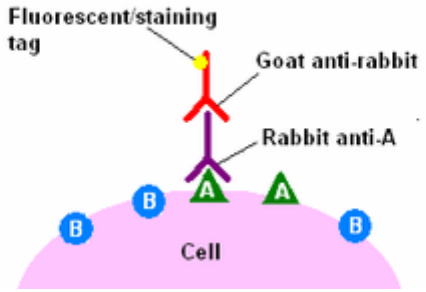

Figure 3.7.3
The figure illustrates how the primary antibody reacts with the antigen being probed for, and the secondary labelled antibody reacts with the primary antibody. Taken from Wikipedia (Male 2005).

Fixation

Fixation is a biochemical process by which biological tissue is preserved from damage or decay. Biochemical tissues are immersed in a fixative fluid which prevents autolysis (self destruction) and bacterial growth which would cause putrefaction.

The formalin based fixatives are the most used. Formaldehyde is a well known cross-linking agent that can inactivate, stabilize, or immobilize proteins. Fomaldehyde (HCHO) dissolves in water to form methylene hydrate ($HO\text{-}CH_2\text{-}OH$) which again forms polymers, termed formalin (37% W/v formaldehyde in water). Formaldehyde reacts with the peptides and proteins which result in formation of stable methylene bridges.

Acetone is often used as a fixative for cell-surface markers. However, acetone does not form covalent bindings between the proteins like formaldehyde, and morphological damage in the tissue like loss of cell membrane may occur.

Background staining

Background staining in immunohistochemistry is a major problem, and caused by hydrophobic and ionic interactions. Tissues which share equal structures to the primary antibody react with the secondary antibody and are then stained.

All proteins are hydrophobic to varying degrees due to the neutral aromatic amino acids phenylalanine, tyrosine and tryptophan linking together to avoid water. Hydrophobic interactions may also occur between different protein molecules. Aldehyde fixation increases hydrophobicity which is a result of cross-linking of reactive epsilon and alpha amino acids between adjacent proteins and within a protein's structure. Fixation time, duration and acidity optimized to avoid cross-reactions. The use of blocking protein is the most used method to avoid cross-reactions by blocking unspecific bindings. The background is then stained to create a clear contrast to what is probed for. Blocking is best performed if the blocking protein is of the same species which produced the secondary antibody (Boenisch Thomas 2001; Renshaw 2007)

Ionic interactions occur when proteins of opposite net charges meet. At any pH proteins have charged groups that may participate in binding them to each other or to other molecules. Most IgG class antibodies have isoelectric points (pH at which net charge is zero) from 5.8-7.3. At buffer pH normally used in immunohistochemistry (pH 7.0-7.8) most proteins will have a net negative charge. Ionic interactions can therefore occur if the tissue proteins have a positive surface charge. Addition of 0.1-0.5 M NaCl to the buffer, or diluent buffers with higher ionic strength are used to avoid ionic interactions (Boenisch Thomas 2001; Renshaw 2007)

Control staining

Due to difficulties determining specificity in immunochemical staining, appropriate controls are imperative. These controls include tissue controls and reagent controls. A positive tissue control includes a slide that contains the antigen being probed for which can give a hint whether the reagents are durable or not and have been applied in correct order. However, this

type of positive control requires the specific antigen being probed for, and is therefore expensive and sometimes difficult to perform. In contrast, a negative tissue control does not contain the antigen being probed for. Visible staining must therefore be from the primary antibody or nonspecific reactions. A negative control, the secondary antibody alone, shows in which extent the antibodies recognize their target antigens.

2. MATERIAL AND METHODS

2.1 Animals

Adult female Wister rats (HanTac WH) initially weighing 200 ± 20 gram were purchased from Taconic, Ebjy, Denmark. The animals were housed in grid-bottom metal wire cages in a room maintained at a 12 hour light/dark cycle and a temperature of 20-22ºC. They were acclimatized for two weeks, housed four per cage and allowed free access to food pellets (Pellets rodent, Special Diets service, UK) and tapped water until induction of anaesthesia. The experiments were performed between 8:00 am and 11 am all days. All procedures were in conformance with the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and User Committee in Norway.

2.2 Drugs

Doxorubicin (DOX) was purchased from Meda AS, Slemmestad, Norway. Penthobarbital was purchased from Haukeland Hospital Pharmacy, Bergen, Norway. Heparin was purchased from Leo pharma A/S, Oslo, Norway while Morphine was delivered by Nycomed Pharma AS, Asker Norway. The ingredients for the KHHB were all purchased from Merck KGaA, Darmstadt, Germany.

2.3 In vivo method and protocol

2.3.1 Pre-treatment

The rats were randomized into 3 groups each comprising 5-8 rats as follows (figure 2.1):
1. Intervention group (n = 8); morphine + doxorubicin (MO-DOX), 2. Control group (n = 8); saline + doxorubicin (SAL-DOX), 3. Control hearts (n = 5) No treatment with drugs (Control hearts).The rats in both intervention and control group were injected intraperitoneally (i.p.) with 3.0 mg/kg of DOX every second day up to a maximal cumulative dose of 12 mg/kg over 11 days. The rats in the intervention group, MO-DOX, were injected with 3.0 mg/kg i.p. of morphine 60 minutes prior to the DOX injection, the control group was injected with equal volume of saline water instead of morphine. Intraperitoneally injections with morphine, saline and DOX were performed with the rat held in a head-down, stretched- out position using a 0.5

x 16 mm needle to avoid damage to internal organs. The rats were weighted at the start of the experiments and at regular intervals during the experiment period. Furthermore, they were observed for nausea, vomiting, diarrhoea and nose bleeding.

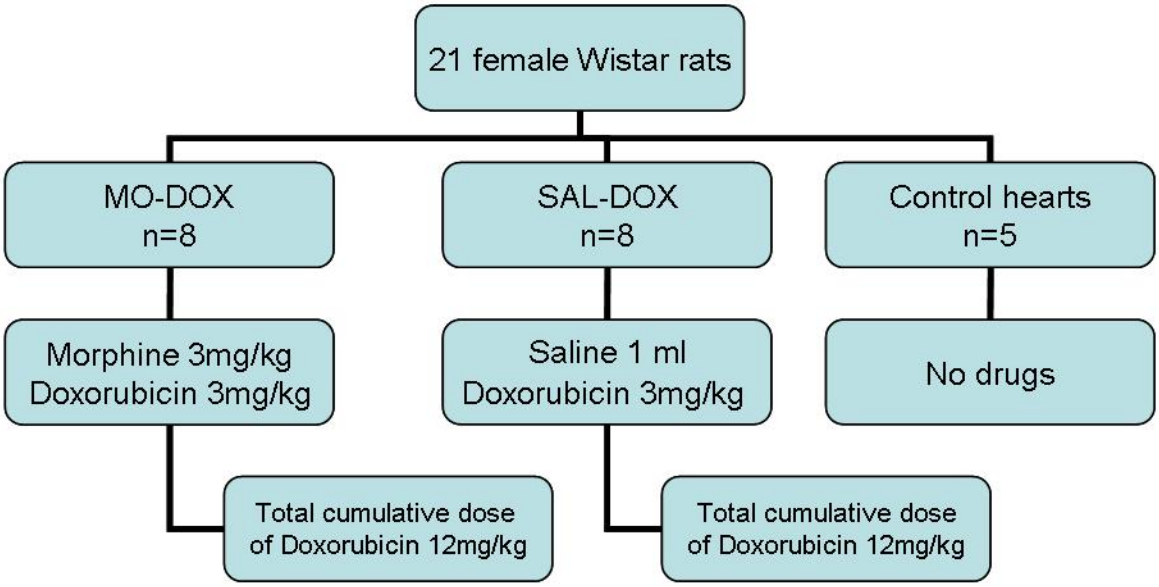

Figure 2.1

Protocol of the *in vivo* treatment. 21 rats were randomly divided into 3 groups where 2 groups were treated with morphine and doxorubicin, and with saline and doxorubicin respectively. Control hearts were not treated with any drugs.

2.4 Ex vivo method and protocol

2.4.1 Buffer, KHBB:

The perfusion medium used was a modified, oxygenated (95% O_2 and 5% CO_2) Krebs-Hensleits bicarbonate buffer, KHBB (pH 7.4) containing (in mM): NaCl 118.5, $NaHCO_3$ 25.0, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.2, D-glucose 11.0 and $CaCl_2$ 1.25. Glucose was added as the sole energy source. The perfusion fluid was gassed with 100% CO_2 to lower pH to avoid precipitation of calcium phosphate particles, and filtrated (0.8µm white AAWP, 47 mm, Millipore Corporation, Bedford, MA 01730). Further, the fluid was equilibrated with 95% O_2

and 5% CO_2 prior to experiments to achieve a pH at 7.35-7.40. Distilled, HPLC grade water was used in the buffer. The perfusion solution was bubbled with gas to secure saturated buffer for 25-30 minutes prior to and throughout the experiments.

2.4.2 Isolated heart

Hearts were excised after anaesthesia of the rats with an injection of pentobarbital 50mg/kg i.p. (0.1 ml/100 gram body weight) and heparinised i.p. (0.1 ml, 500 IU/100 gram body weight). Anaesthesia was evaluated by the pedal withdrawal reflex. Hearts were rapidly excised and immediately placed in cold (4 °C) KHBB to temporarily stop its beating and preserve it from ischemic injury prior to perfusion. Lungs, thymus and fat tissue were excised. Further the aorta was fixed to the aortic cannula with a ligature thread on the Langendorff system within 2-5 minutes, and retrogradely perfused at 100 cm H_2O (73 mmHg) with KHBB. The hearts were allowed to stabilize for 10 minutes with pressure regulated flow. At the start of the stabilization time a latex balloon attached to one end of a catheter was placed in the left ventricle through the mitral valve. The balloon was filled with saline water to obtain a LVEDP of 4-10 mmHg, and the catheter filled with saline water was connected to a pressure transducer (SensoNor 840) to record left ventricular (LV) pressure variables and heart rate (HR). Physiological parameters were monitored by use of a PowerLab data acquisition system (ADI instruments, East Sussex, England) connected to a Dell Latitude D505 computer. Aortic pressure (AoP), LVDP, LVEDP, left ventricular dp/dt_{max} and left ventricular dp/dt_{min}, and heart rate were continously monitored. To avoid hypothermia, the heart was surrounded by a water-jacket organ chamber covered with parafilm.

2.4.3 Experimental protocol

At the end of the stabilization period, hearts from the SAL-DOX group and from control hearts were perfused for 10 minutes in total where physiological variables were monitored in real time; 5 minutes with pressure regulated flow, and 5 minutes with volume regulated flow. At the end of the pressure regulated perfusion period, flow was switched to volume regulated flow (12.5 ml/minute) by use of a peristaltic pump (Alitea, Stockholm, Sweden). Then the hearts were removed from the Langendorff system, sliced and frozen in nitrogen cooled isopentane for myocardial staining. Myocardial tissue residues were put in Eppendorf tubes and frozen (-20°C) for drug concentration (DOX + doxorubicinol) studies. The experimental design of the study is illustrated in figure 2.2

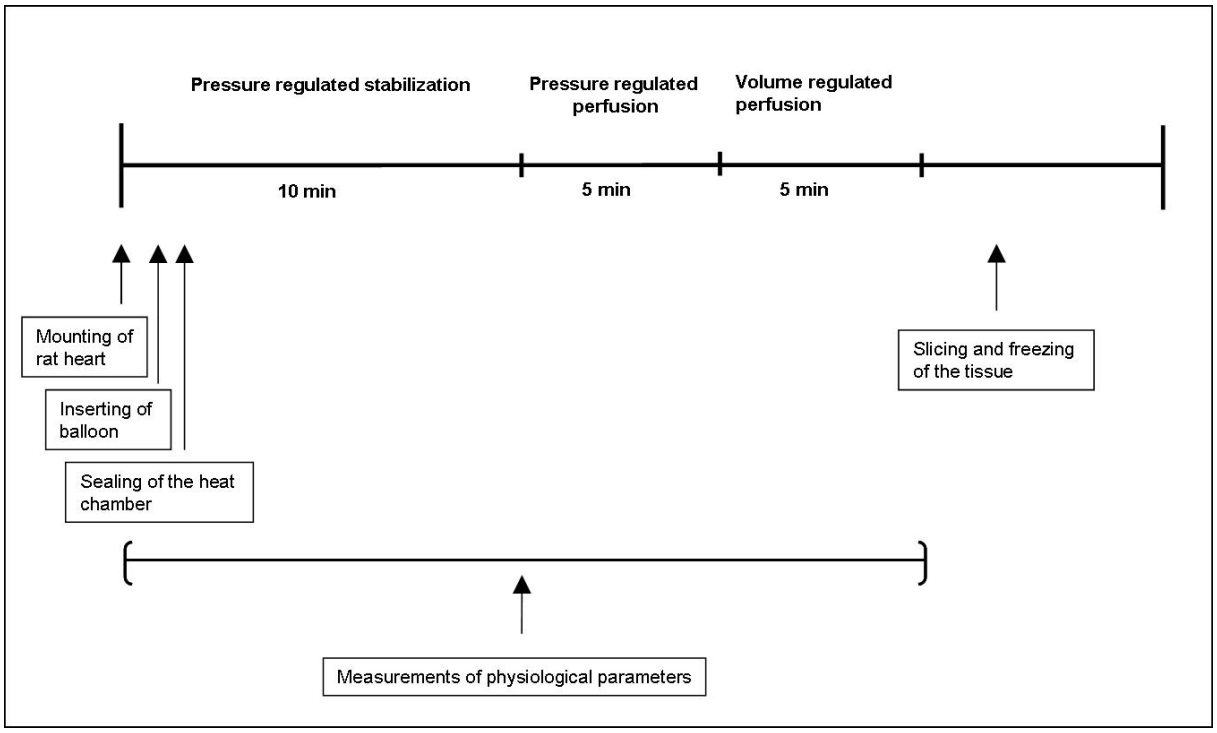

Figure 2.2
Experimental design of the *ex-vivo* study.
Heart was excised, mounted on a cannula and retrogradely perfused with KHBB in a Langendorff system. Pressure regulated perfusion for five minutes was followed by volume regulated perfusion for five minutes. Measurement of physiological parameters was recorded during the entire perfusion time. Then a slice of the perfused heart was frozen in liquid nitrogen for further immunfluorescence studies, and residual tissue were collected for measurement of doxorubicin and doxorubicinol.

2.4.4 Exclusion criteria

Based on the fact that this was a toxicological study it was expected that DOX would significantly impair cardiac function and thereby physiological parameters. Furthermore, the experience with the technique of isolated rat heart perfusion would be of importance. Physiological parameters for some of the hearts in the SAL-DOX group could not be measured satisfactory. The hearts of these cases were excluded. Among the control hearts, those with LVDP below 70 mmHg or HR below 250 were excluded. All hearts was included in studies of HSPs, and all hearts in the SAL-DOX group were included in measurement of myocardial content of DOX and its metabolite doxorubicinol.

2.4.5 Myocardial content of doxorubicin and doxorubicinol

Myocardial content of DOX-concentration and its major metabolite doxorubicinol (DOX_{ol}) was performed by a high performance liquid chromatography (HPLC) (Agilent Technologies 1200 SL) coupled with mass spectrometry detection (MS/MS) using electrospray ionization (Agilent Technologies 6410 LC/MS/MS).

Frozen ventricular tissue was minced and weighted out in a glass tube with a screw cap and homogenized in physiological saline (2ml for 100mg tissue) with a tissue homogenizer (Ultra Turrax, Sigma Aldrich, Germany). 500 µl of the sample was added 50 µl of internal standard (daunorubicin), and 100 µL of buffer (1M TRIZMA, pH = 11.1) and mixed well before extraction with 5 ml ethylacetate/heptane (80/20 vol/vol). The samples were mixed using a rotary blender for 15 minutes and then centrifuged at 3500 rpm for 5 minutes at 10 °C. The organic phase was evaporated to dryness at 50 °C under nitrogen, N_2, before dissolved in 100 µl of methanol and 100 µl of water. The samples were mixed thoroughly and transferred to a microtiterplate before analysis.

The column used for HPLC was a Phenomenex phenylhexyl, 2.0 x 100 mm, 3µm particles. The guard column was a Phenomenex Securityguard Phenyl, 2.1 x 4 mm. Mobile phases used were acetonitrile (A) and 0.01 % formic acid, 5mM ammoniumformiate, pH 3.8 in water (B). The injection volume was 25µl and the flow was 300 µl/minutes.

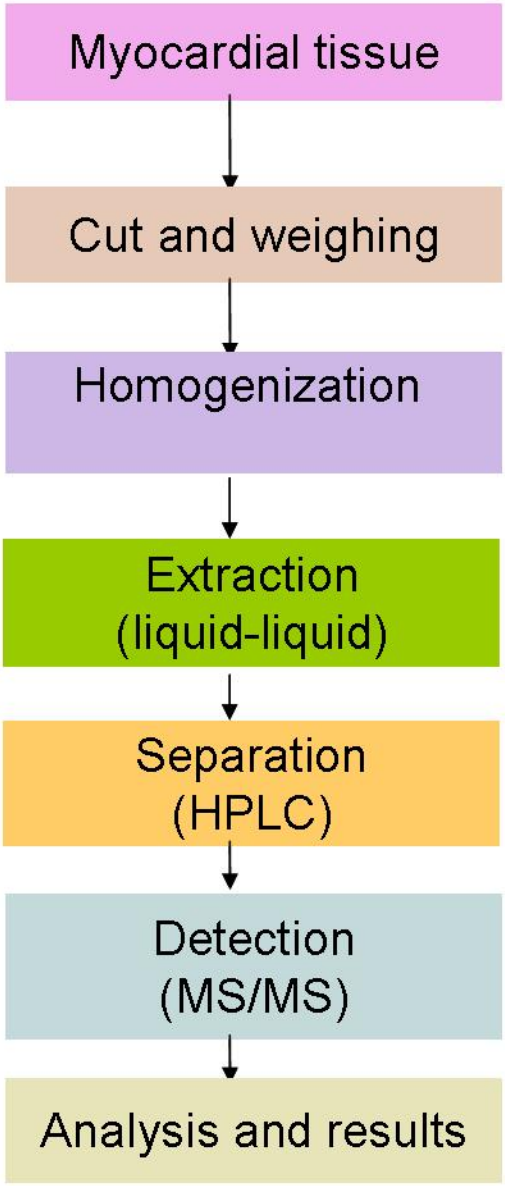

Figure 2.3
Workflow for analysis of doxorubicin and doxorubicinol in rat heart tissue. HPLC = high pressure liquid chromatography, MS/MS = mass spectrometry

2.5 Measurement of HSPs

2.5.1 Immunohistochemical staining procedure

Sections 9 µm thick from the SAL-DOX treated rats were cut on a microtome (Leica cm3050, Nussloch, Germany) and mounted on poly-lysine-treated microscope slides. Immunohistochemistry for HSP27, HSP47, GRP78, GRP94 and SERCA was done with polyclonal antibodies (Santa Cruz Biotechnology, INC, Canada). By staining for SERCA, (Sarcoplasmic Reticulum Ca^{2+}ATPase) which is an ATP-consuming calcium uptake pump located in the sarcoplasmic reticulum (SR) within the muscle cells, direct spread of SERCA can be localized, and it will also indirectly mark the myofibrils which are surrounded by SR and negatively stained. FITC anti-goat (Dako Cytomation A/S, Glostrup, Denmark) was used as secondary antibody for SERCA staining, and FITC anti-rabbit antibody (Dako Cytomation A/S, Glostrup, Denmark) was used as secondary antibody on the remaining ones.

Three different protocols were tested including fixation with formaldehyde, fixation with acetone and omission of fixation. Except from the fixation part, the three different protocols were almost identical with exception in the glycine incubation time which had duration of one hour for the formaldehyde fixation. The sections were first fixated with either formaldehyde for (30 min) or acetone for (20 min). Further the sections were rinsed in 0.1M phosphate buffered saline (PBS). Then the tissue were immersed in a solution of PBS and 0.1% Triton-X followed by a new washing in a solution of BPS, 0.05% Tween and 0.02% Triton- X. Slides were then incubated with 0.2 M glycine for 30 minutes, followed by washing twice in the solution of PBS, 0.05% Tween and 0.02% Triton-X. The sections were then incubated with blocking serum (PBS solution with 5% swine serum with 0.8% BSA, 15mM Na-acid, 0.05%Tween and 0.02% Triton-X) for 30 minutes in order to block nonspecific binding. Slides were further incubated with primary antibody at a dilution of 1:5, and 1:10 of blocking serum for 30 minutes in a humid chamber. Further the slides were washed in PBS solution (0.05% Tween and 0.02% Triton-X) for 10 minutes followed by incubation with labelled secondary antibody (FITC) for 30 minutes. Washing with the PBS solution (0.05% Tween and 0.02% Triton-X) was then repeated followed by rinsing in distilled water. The slides were finally mounted in a mounting media, covered with a coverslip and sealed with nail varnish. Omission of the primary antibody was used as a negative control.

Staining Protocol

1. Fixate with formaldehyde (30 min) or acetone (20min), or omit.
2. Immerse the slides three times in PBS (3x3min).
3. Immerse the slides once in PBS containing 0.1% Triton-X (5 min)
4. Immerse the slides three times in PBS containing 0.05% Tween and 0.02% Triton-X (3x3) min.
5. Incubate slides in PBS containing 0.2 M glycine [1] for 30 min.
6. Immerse in PBS containing 0.05% Tween and 0.02% Triton-X (10 min).
7. Incubate in PBS containing 5% swine serum, 0.8% BSA, 15mM Na-azid, 0.05% Tween and 0.02% Triton-X (30 min).
8. For the primary antibody incubation, incubate the slides with primary antibody[2] optimally diluted in PBS containing 5% swine serum, 0.8% BSA, 15mM Na-azid, 0.05% Tween and 0.02% Triton-X (40min).
9. Immerse the slides for in PBS containing 0.05% Tween and 0.02% Triton-X (10min).
10. For the secondary antibody incubation, incubate the slides with FITC (fluorescein isothiocyanate) fragments raised against the species of the primary antibody, optimally diluted in 5% swine serum, 0.8% BSA (Bovine Serum Albumin), 15mM Na-azid, 0.05% Tween and 0.02% Triton-X for 40 min at room temperature.
11. Immerse in PBS containing 0.05% Tween and 0.02% Triton-X
12. Immerse three times in distilled water.
13. Mount in antifiadant mounting media[3], coverslip, and seal with nail varnish.

HSP27, HSP 47, GRP78 and GRP94:

The primary antibody is raised in rabbit; the secondary is raised in swine and is against rabbit.

SERCA:

The primary antibody is raised in goat; the secondary is raised in rabbit and is against goat.

[1] Glycine reduces background fluorescence by binding to free aldehyde groups
[2] HSP 27, HSP 47,GRP78,GRP94, Serca
[3] Fluor Save™Reagent, CAlbiochem (U.S and Canada)

Microscopy

Expression of the different proteins was studied using a fluorescence microscopy (Leitz Aristoplan, Leica Microsystems, Bensheim, Germany) with objective 40/1.30 oil and photographed by a Leica DC 300 F camera (Leica Microsystems, Bensheim, Germany).

2.6 Statistics

All results are reported as mean values ± standard deviation (SD) in tables, and mean values ± standard error of the mean (SEM) in figures. Groups were compared with regard to physiological parameters with a Student t-test for independent groups with the exception of AoP during volume regulated flow which were compared with a linear model with repeated measurements statistics. SPSS for Windows (version 15.0) was used and $p < 0.05$ were considered as statistically significant.

3. RESULTS

3.1 Treatment with doxorubicin, saline and morphine

Doxorubicin treatment induced significant total body weight loss in both the MO-DOX and the SAL-DOX groups, but there was no significant difference in weight loss between the different groups (fig. 3.1) as well as with regard to the side effect nose bleeding. Development of fibrosis in the abdominal region due to repeated i.p. injections was evident in both groups. Seventy five percent of the animals died before the end of the experiment in the MO-DOX group. Mortality was evident in the group by day 9 (n = 1) and by day 10-11 (n = 5) after start of the treatment period. The remaining two rats were moribund, and it was decided to put them to death. Autopsy revealed signs of pleural and peritoneal oedema, but no other obvious damage causing death. Thus, physiological parameters from rats in the MO-DOX group were not available.

All animals in the SAL-DOX group survived throughout the duration of protocol.

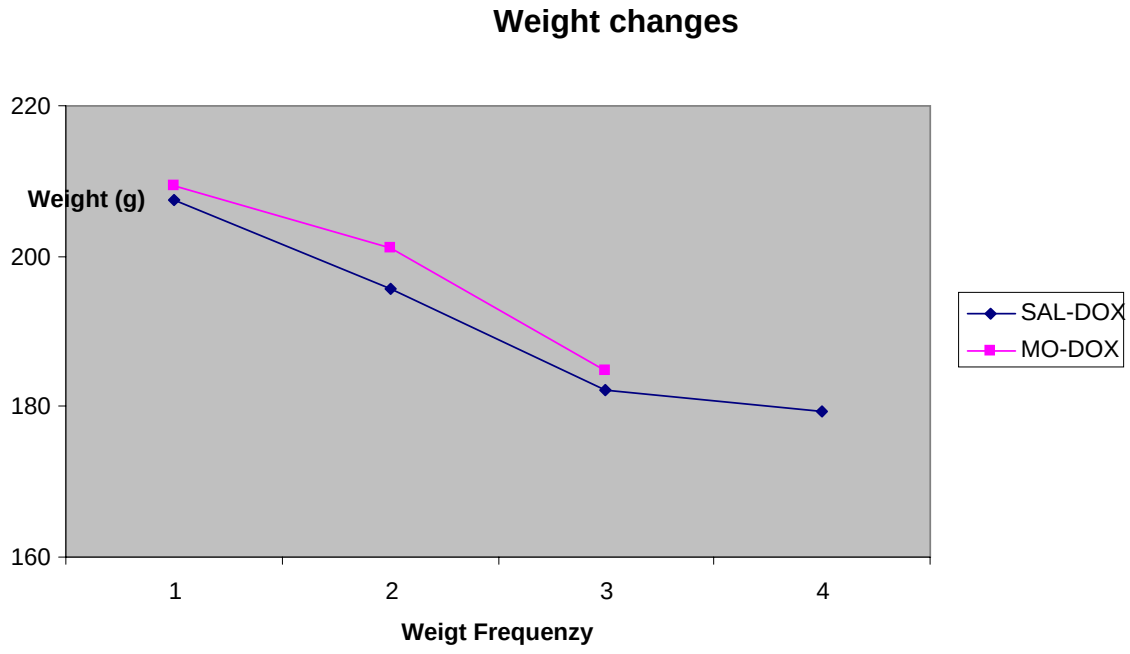

Figure 3.1 Mean changes in body weight of rats in the MO-DOX (n = 8) and SAL-DOX (n =8) group, respectively. Weights were measured on day 1, 4 and 8 in the MO-DOX group, and on day 1, 4 and 9 in the SAL-DOX group. Total follow up period was 11 days.

3.2 Physiological variables

Four and two hearts were excluded from physiological measurements in the SAL-DOX group and among control hearts, respectively. The results for physiological parameters are presented in table 3.1, except for measurements of AoP during volume regulated flow presented in figure 3.2. Treatment with DOX significantly ($p < 0.05$) reduced physiological parameters (40-60%) compared to control hearts without affecting LVEDP or HR. This was observed both during pressure and volume regulated flow (Table 3.1). Furthermore, treatment with DOX showed a tendency for increased AoP (40%) during volume regulated flow compared to control hearts. There was a significant ($p < 0.05$) difference between the two groups after 3 minutes of volume regulated flow by use of a Student t-test. However, by use of a repeated linear model with repeated AoP measurements the results failed to reach significance ($p < 0.07$). Left ventricular developed pressure increased in both groups during volume regulated flow.

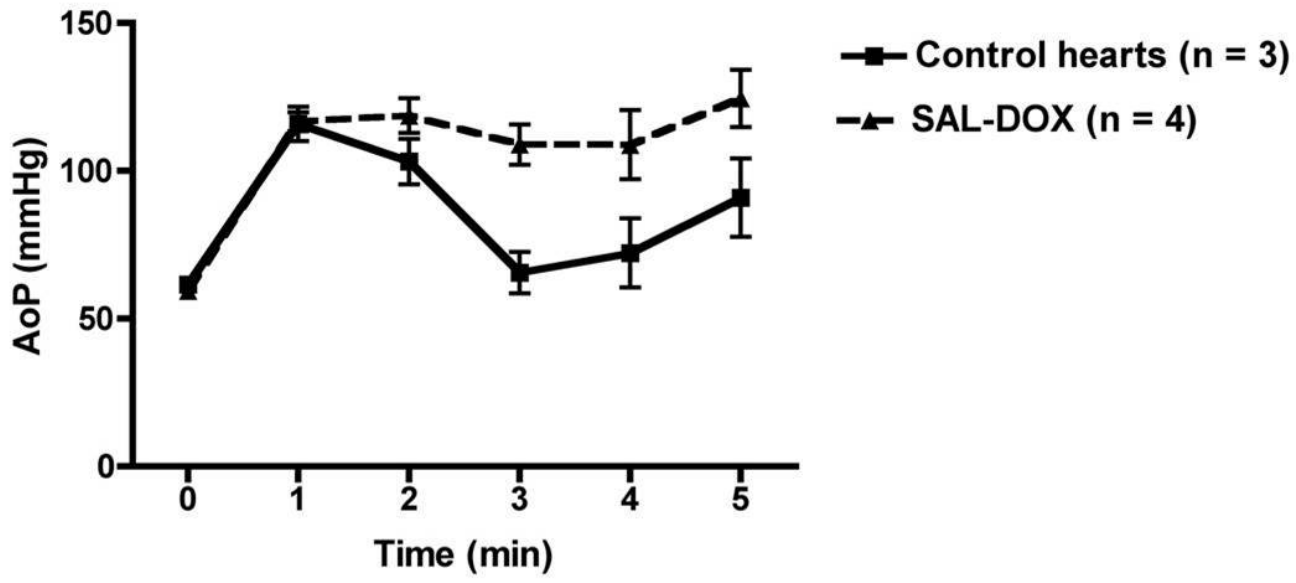

Figure 3.2
Aortic pressure (AoP) during volume regulated perfusion in SAL-DOX and control hearts. Time = 0 represents end of pressure regulated perfusion. Data presented as mean + SEM

TABLE 3.1
Physiological variables of isolated rat hearts at the end of pressure- and volume regulated perfusion, respectively.

	PRESSURE REGULATED PERFUSION		VOLUME REGULATED PERFUSION	
	CONTROL HEARTS (n=3)	SAL-DOX (n=4)	CONTROL HEARTS (n=3)	SAL-DOX (n=4)
LVDP (mmHg)	97.5± 12.7	39.4± 16.6*	120.3± 7.4	69.5± 16.8*
AoP (mmHg)	61.5± 0.4	59.4± 0.7*	87.8± 30.0	124.8± 19.4
dP/dtmax (mmHg/sec)	3716.4 ± 748.7	1377.1 ± 560.4*	4853.1±469.30	2423.7 ±913.7*
dP/dtmin (mmHg/sec)	-2078.9± 395.5	-771.3 ± 274.9*	-2514.9 ± 65.5	-1266.1 ± 503.2*
LVDEP (mmHg)	6.5± 0.3	9.6± 10.1	9.1± 1.8	13.5± 13.8
HR (beats/ minute)	293.1± 45.9	274.7± 89.8	314.6± 45.1	254.2± 84.8

SD Standard deviation
LVDP Left ventricular developed pressure = left ventricular systolic pressure (LVSP) - left ventricular end-diastolic pressure (LVEDP)
AoP Aortic pressure
dp/dt_{max} Maximum value of 1st derivative of LVDP as a function of time
dp/dt_{min} Minimum value of 1st derivative of LVDP as a function of time
LVEDP Left ventricular end-diastolic pressure
HR Heart rate
* Significantly different from Control hearts, $p < 0.05$

3.3 Tissue accumulations of doxorubicin and doxorubicinol

Myocardial content of doxorubicin and its major metabolite doxorubicinol are listed in table 3.2. The concentration of measured content of both DOX and DOX_{ol} varied among the rat hearts. The concentration of DOX_{ol} was in some of the hearts lower than the lowest calibrator in the method which means that the measured concentration is less accurate. Normally the measured concentration is within the calibration area, but from the shape of the calibration curves (figure3.4) great deviation is not expected.

TABLE 3.2
Tissue accumulations of doxorubicin and doxorubicinol

	Tissue (gram)	Saline (ml)	Doxorubicin (nmol/g)	Doxorubicinol (nmol/g)
Rat heart 1	0.10424	2	3.086	0.166
Rat heart 2	0.10225	2	2.743	0.169
Rat heart 3	0.10299	2	2.641	0.096
Rat heart 4	0.10186	2	1.511	0.049
Rat heart 5	0.09908	2	4.275	0.311
Rat heart 6	0.10046	2	2.164	0.087
Rat heart 7	0.10191	2	2.265	0.118
Rat heart 8	0.10143	2	2.904	0.129
		Mean:	2.699	0.141
		SD:	0.808	0.080
		RSD:	29.9%	56.6%

SD Standard deviation
RSD Relative standard deviation

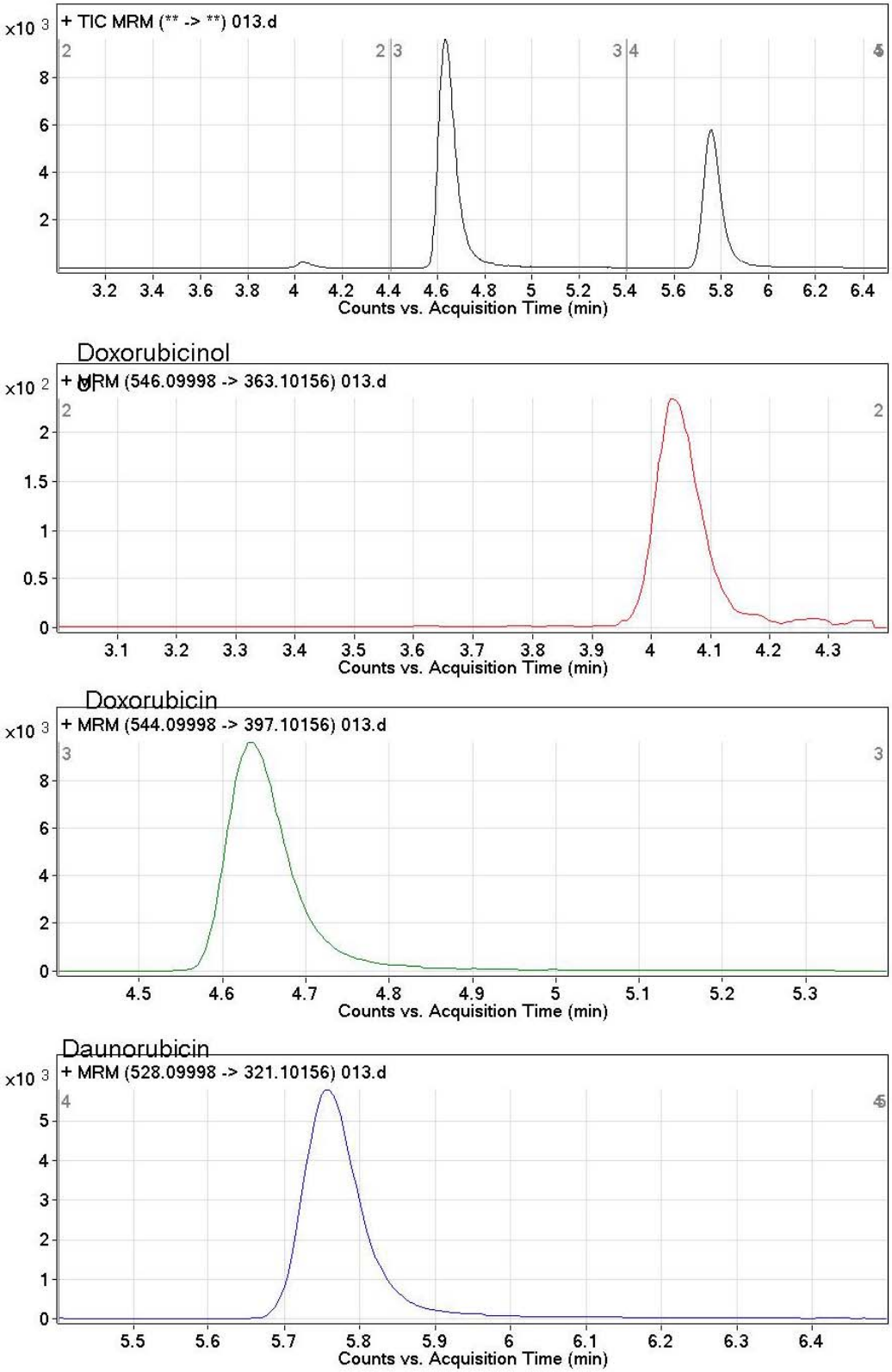

Figure 3.4
Calibration curves for doxorubicinol, doxorubicin and the internal standard daunorubicin respectively. The figure at the top of the page, which shows the three different substances in one diagram, illustrates the small amount of the metabolite doxorubicinol compared with the content of doxorubicin.

3.3 Expression of HSPs

Fixation with both formaldehyde and acetone resulted in poor staining. The acetone fixated tissue dissolved during staining. Omission of the fixation step gave much better results compared to fixation, and was therefore the method used.

Positive staining for HSP27, GRP78 and SERCA was detected in myocardial tissue from both groups (table 3.3). Positive staining for GRP94 was only detected in tissue from the SAL-DOX group, while staining for HSP47 was negative in both tissue groups. As expected SERCA staining was most evident owing the fact that SERCA is located in sarcoplasmic reticulum (SR), which is an internal membrane system in muscle responsible for Ca^{2+} regulation and contractility in heart muscle.

Examples of positive staining are presented in figure 3.4 and 3.5

TABLE 3.3
Expression of Sarcoplasmic Reticulum Ca^{2+}ATPase (SERCA) and Heat Schock Proteins (HSP) in myocardial tissue

	SAL-DOX Hearts	Control hearts
Serca (1:5)	POS	POS
Serca (1:10)	POS	POS
HSP 27 (1:5)	POS	POS
HSP 27 (1:10)	POS	POS
HSP 47 (1:5)	NEG	NEG
HSP 47 (1:10)	NEG	NEG
GRP 78 (1:5)	POS	POS
GRP 78 (1:10)	POS	POS
GRP 94 (1:5)	POS	NEG
GRP 94 (1:10)	POS	NEG

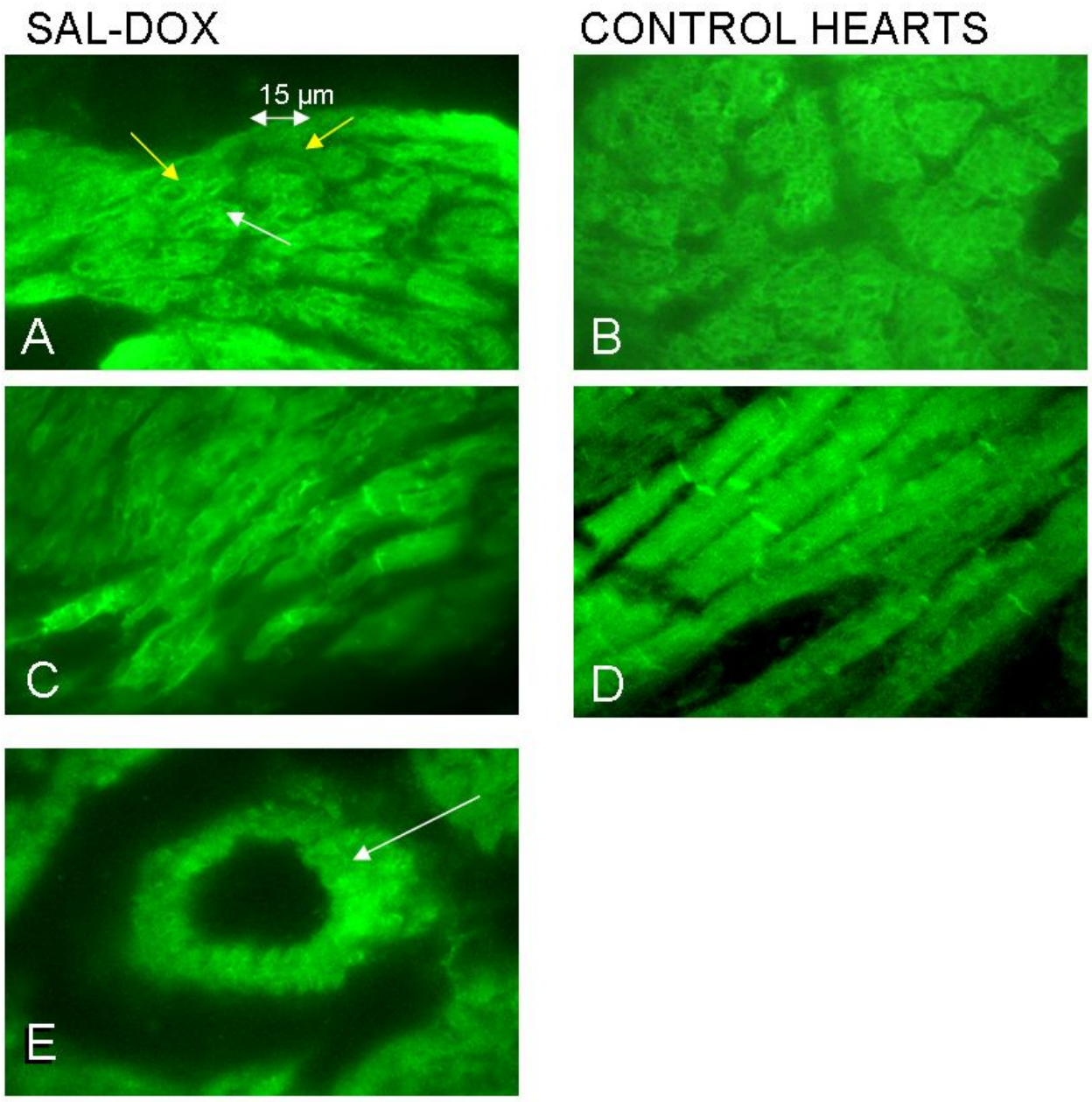

Figure 3.4
Figure shows images of rat myocardial tissue from the SAL-DOX and the control hearts. A and B represents tissue stained for SERCA, C, D and E are stained for HSP27.
A: Cross section showing nucleus (yellow arrow) and myofibrils (white arrow). B: Cross section showing several cells
C and D: Sections showing intercalated disk that connects adjacent ventricular myocytes. The disk serves to bind cells to each other, and to communicate between cells.
E: Arteriole surrounded by stained endothelium cells.

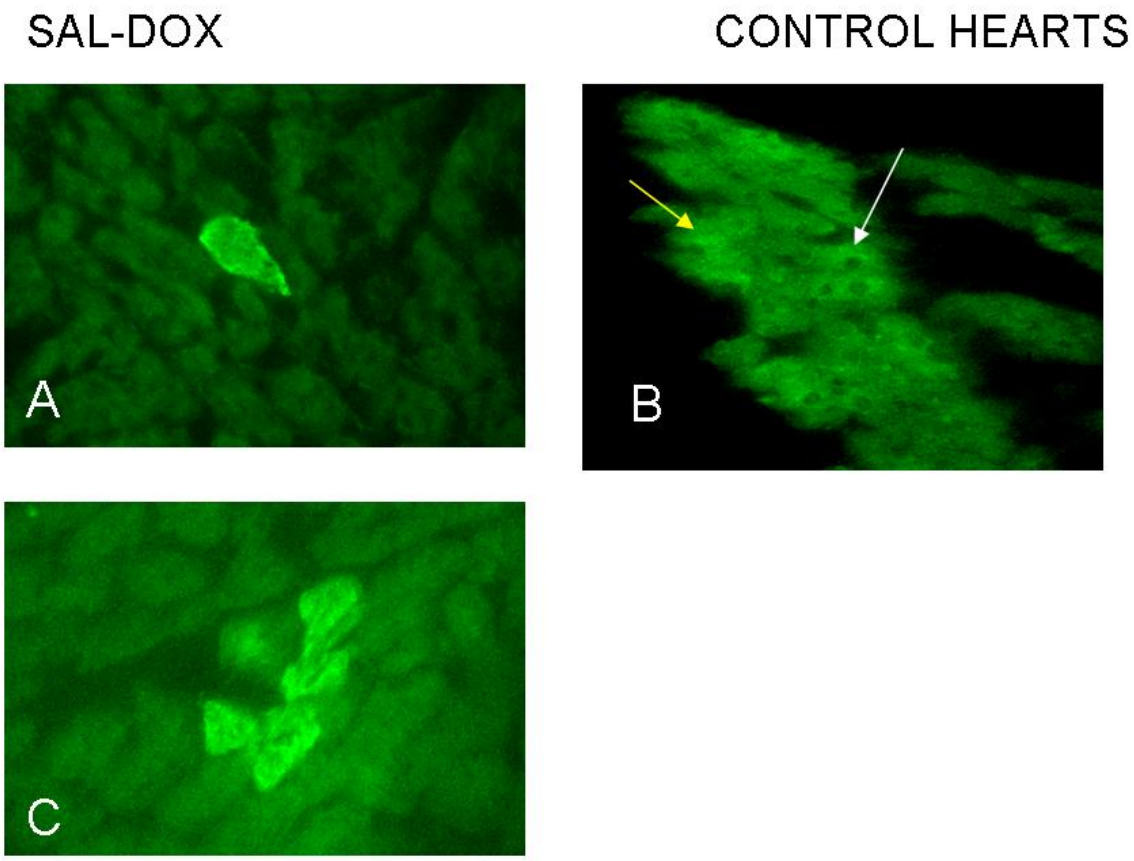

Figure 3.5
Images of myocardial tissue from rat stained for GRP 78 (A and B) and for GRP 94 (C).
A: Cross section demonstrating obvious staining of GRP78 in one cell.
B: The short arrow shows an area with stained endothelium cells while the long arrow point out nucleus in a myocyte.
C: Four cells are stained with GRP94.

4. DISCUSSION

4.1 Evaluation of the model and comparison with previous studies

The final results show that the proposed model is suitable to evaluate cardiotoxicity of anthracyclines. This finding is in accordance with previous studies with anthracycline associated cardiotoxicity using animal models (Platel, Pouna et al. 2000; Robert 2007).

The main finding in this thesis is that the model could is to assess interventions aimed at reducing cardiotoxicity of anthracyclines. The experimental model has a short duration (pre-treatment for 11 days) and rats treated with DOX in accordance with the current protocol are suitable for subsequent isolated heart perfusion for measurement of physiological parameters. These are significantly impaired, but not to an extent that the effect of interventions are masked. The significant loss of weight in both the doxorubicin treated groups is mainly associated with reduced food and water consumption due to starvation (Sridhar, Dwivedi et al. 1992).

The project findings are comparable to others that have tested similar models in a more elaborate design (Platel, Pouna et al. 2000; Robert 2007). LVDP in doxorubicin treated rats were significantly reduced compared with the control rats during both pressure regulated- and volume regulated perfusion. A similar short term model illustrated comparable results (Robert 2007). They studied the cardiac performances of isolated perfused hearts of rats that had been treated with various anthracyclines within 12 days administrated by repetitive injections. One group administered doxorubicin (3mg/kg) every other day for 12 days up to a total cumulative dose of 18 mg/kg. Even though they used a higher cumulative dose compared with this study, they found that their model was able to predict correctly what was already known concerning the cardiotoxicity of anthracycklines, as well as demonstrating protective effects from among others liposomal encapsulation. They referred to a significant reduction ($p < 0.05$) of LVDP values of 67.8 ± 13 mmHg which is similar to the dose's used in this study (69.5 ± 17 mmHg). This suggests that the model is reproducible. Due to difficulties mounting the heart and exclusion criteria we only have data from four rats in the SAL-DOX group. It is unclear from the article how many rats Robert (2007) used in each experimental group, and direct comparison of the selection of animals is therefore impossible.

In another study (Platel, Pouna et al. 2000), the cardiotoxicity of taxanes and anthracyclines in combination versus anthracyclines alone using the model of isolated perfused

rat heart combined with an *in vivo* treatment period were similar to the present results. Different anthracyclines were administered every other day for 11 days and was followed by isolated perfused rat heart by the Langendorff technique. Doxorubicin treatment (3 mg/kg/day) induced mortality, significant weight loss and side effects like nose blood and diarrhoea. The doxorubicin treatment also induced significant alterations in cardiac function parameters which reflect the cardiotoxic effects; there was a 20% reduction in heart contractility ($LVdP/dt_{max}$) and a 33% reduction in heart relaxation ($LVdP/dt_{min}$) which is similar to our results. A slightly larger reduction in heart contractility and heart relaxation was observed compared with the results above, though the study group was smaller. The $LVdP/dt_{max}$ and $_{min}$ in the treated hearts were significantly reduced during pressure regulated- as well as volume regulated perfusion. This indicates that the project model is suitable for the evaluation of cardiotoxicity irrespective of the two different perfusion modes.

The AoP variables during volume regulated flow were significantly higher in the SAL-DOX treated animals than in the control hearts. This probably reflects the increased resistance in the coronary arteries due to doxorubicin treatment. See figure 4.1.

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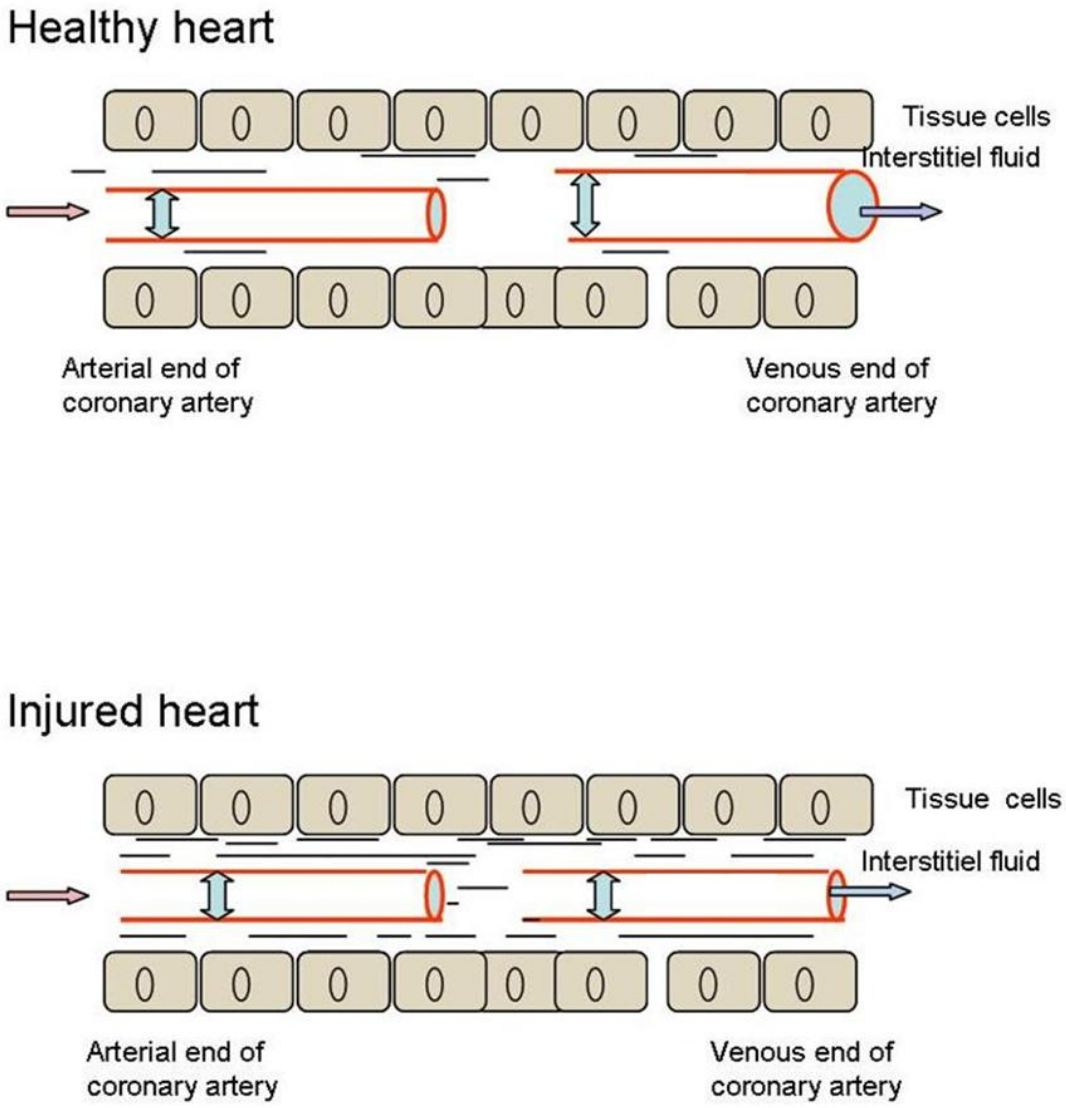

Figure 4.1
The figure illustrates the relation between AoP and the rigidity of the coronary arteries in the injured versus the healthy heart. In the healthy heart the coronary arteries will dilate and expand due to increased flow and AoP will stabilize and decrease. The coronary arteries in the injured heart are rigid due to exposure to doxorubicin, and will not dilute at the same extent with the consequence that AoP only decreases slightly. Another aspect is the density of capillaries in the normal heart (> 2000 per m^2) of which only 60-80% are open and functioning. As flow increases there will be a recruitment of capillaries and AoP will decrease. The injured heart may not be able to recruit the "resting" capillaries. Increased pressure in the tissue due to oedema (illustrated with black lines) may also explain why the injured heart does not manage to reduce AoP.

4.2 Content of doxorubicin and doxorubicinol

LC-MS/MS measurements of doxorubicin and its metabolite doxorubicinol showed significant accumulation of anthracyclines in the myocardial tissue. The results are comparable to those of others. A content of doxorubicin and doxorubicinol of 21.0 μg/g protein and 1.8 μg/g protein, respectively, has been reported in rat heart tissue of rats treated with a cumulative dose of DOX 9 mg/kg over 10 days (Olson, Mushlin et al. 1988). Our results were 2.66 nmol/g (DOX) and 0.141 nmol/g (DOX_{ol}). Olson et al. (1988) state the weight per gram protein, while we state weight per gram tissue which includes water. However, the protein content in a raw heart is 18% (Matvareportalen Access year 2008), so our data does not differ much from their results.

Doxorubicinol is present in a much lower concentration than doxorubicin, though it is proposed to be more toxic compared with doxorubicin (Olson, Mushlin et al. 1988). It has been established that doxorubicinol is nearly 30 times more potent than doxorubicin at depressing contractility and systolic myocardial function in isolated rabbit papillary muscle (Olson, Mushlin et al. 1988).

The dose of doxorubicin used to induce cardiotoxicity varies between different laboratories. In general, injections of 2.0-4.0 mg/kg are used, up to cumulative doses between 10-20 mg/kg, but higher doses have been reported. In this study, we used 3.0 mg/kg doxorubicin up to a cumulative dose of 12 mg/kg to induce cardiotoxicity. Selection of dose and concentration of doxorubicin was done with accordance to similar studies. It was important to give the rats a cumulative dose high enough to cause myocardial damage, but at the same time low enough to keep them alive. The dosage- and concentration frame of doxorubicin used in present study has been used in earlier studies, and has yield comparable results (Arola, Saraste et al. 2000; Platel, Pouna et al. 2000; Robert 2007).

It is desirable to reduce the cardiotoxicity of doxorubicin and its main metabolite doxorubicinol while retaining its anticancer effect. Thus, interventions that reduce accumulation of anthtracyclines and/or metabolites in myocardial tissue are of interest, and can be measured directly in the model. Furthermore, an inhibitor of the enzymes responsible for the conversion of doxorubicin to doxorubicinol could be of interest, however it has been implied that doxorubicinol also play an important role in the angineoplastic effect.

4.3 Evaluation of the protective effects of Morphine

The second finding is that pre-treatment with morphine increased mortality when combined with DOX. Thus, intervention with morphine could increase the cardiotoxicity of DOX rather than reduce it. Due to the increased morbidity and mortality in the MO-DOX group, evaluation of effects on myocardial function with isolated heart perfusion was impossible. However, it is not clear from the pilot study if the increased mortality when morphine combined with doxorubicin was due to increased cardiotoxicity or some other mechanism. Further studies are needed to evaluate this, and a possibility is to test the interaction of morphine and doxorubicin in an *ex vivo* isolated rat heart model.

The increased mortality associated with morphine was surprising. An article with quite opposite results concerning possible protective effects of morphine against DOX-induced cardiotoxicity was recently published (Kelishomi, Ejtemaeemehr et al. 2008). Male Sprague-Dawley rats (n = 6) were administered i.p. with MO (10 mg/kg) 30 minutes prior the administration of DOX (1.25 mg/kg). Drugs were administered four days a week for four weeks to a maximal cumulative dose of DOX, 20 mg/kg. A second group (n = 6) received DOX only (1.25 mg/kg) following the same protocol. According to their results fifty percent of the animals in the DOX only treated group died before end of experiment, while in the MO pre-treated group all animals survived. The different protocols cannot fully explain the contradictory results. A speculation is that pre-treatment with MO increases DOX's concentration in the myocardium due to a pharmacokinetic interaction. Morphine has systemic vasodilatory effects, and could thereby enhance DOX-induced cardiotoxicity by changing rate of distribution of the anthracycline in the rat. Kelishomi et al. (2008) injected MO doses three times higher than what was used in the present study, but all their animals pre-treated with MO survived. Morphine was administrated 30 minutes prior to DOX compared to 60 minutes in this study. A hypothesis is that the systemic effect of MO is different at 60 minutes compared to 30, and that the lower dose of DOX in their study could be of importance. Morphine administered s.c. or i.m. achieve maximum effect after 45-90 minutes in man. Intraperitoneal absorption is faster than from i.m. injection, and we therefore decided to administer MO 60 minutes prior to DOX. However, the plasma half-life time of MO is 30 minutes in rats (Frassdorf, Weber et al. 2005) which may be the reason why Kelishomi et al. (2008) injected MO 30 minutes prior to DOX. The dosage of MO used in our experiment is less than half of the dosage Kelishoma et al. (2008) used. However, rodents generally need larger doses of analgesic compared with humans. For analgesic purpose 10 mg/kg is recommended for rats (Jenkins 1987), so our dosage is in the lower limit for rodents. It is not clear at present why the results are contradictory, but Schultz et al. (1995) suggested that δ_1-opioid receptor activation

serve not only as a trigger of the preconditioning (PC) response, but also as a mediator of the memory phase of PC in the rat myocardium. Administration 60 minutes prior to DOX could involve a triggering phase for protection, but it is not clear if protection against DOX injury requires a continuous high level of morphine. Further studies with morphine and DOX are needed to find if there is a threshold dosage for protection (or damage), if the protection involves preconditioning and if the protection needs morphine in the mediator phase.

Kelishomi et al. (2008) did not report about fibrotic nor necrotic tissue in the injection area even though the animals were injected more frequently then in the present model. They injected each rat 32 times over a four week period i.p. while our rats were injected 8 times in total. Doxorubicin is toxic to tissue in the case of extravasations, and can result in serious necroses which are difficult to treat with a high risk of infections. If the injection area becomes inflamed, necrotic or fibrotic it is painful to be injected. Furthermore, correct administration and distribution of drugs could be more variable. However, this could not explain the high mortality in the MO-DOX group as we would expect this effect would be randomized to all experimental groups. However, our observations suggest that repeated i.p. injections should be replaced by other modes of administration.

4.4 HSPs detection

The measurement of HSPs associated with anthracyclines has previously been associated with more extensive protocols including treatment of rats for several weeks (Gabrielson, Bedja et al. 2007).The study does not show any significant variation of HSP expression in the two groups, and the staining results were synonymous with exception of GRP94 (1:5, and1:10) which was only positive in the SAL-DOX group. This may indicate an up-regulation of GRP94 in the injured hearts compared with the healthy hearts, but we did not perform a quantitative analyse, thus it is difficult to point out any clear differences between the two groups. Beyond that, the gross appearance of the cell seems to be unaltered. The rats were only treated for 11 days, and this duration is probably too short to detect elevation of doxorubicin induced HSPs. From yhe results we are not able to say whether doxorubicin induces HSPs or not. The poor staining results due to fixation with formaldehyde may be a result of increased background staining due to increased hydrophobicity. Therefore it is recommended to omit fixation of the tissue in future corresponding studies.

Compared with other studies we were not able to establish a certain doxorubicin induced up- regulation of any of the HSPs probed for. An up-regulation of HSP70 and HSP90 has been demonstrated, though by Western blotting (gel electophoresis) and not by immunofluorescence staining (Gabrielson, Bedja et al. 2007). The study method should ideally have contained a quantitative analyse of the HSPs expression.

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5. LIMITATIONS

There are several limitations associated with the study. The dose and protocols are not immediately comparable to a clinical setting, and animal data can not be directly extrapolated to humans. Furthermore, presence of heart failure *in vivo* was not tested. The fact that rat and man are not identical as species suggests that the present results should be interpreted with caution. There could be pharmacokinetic differences with regard to the drugs in rats and humans, and the toxicodynamic effects could be different. A particular question is if the impaired heart function induced by DOX in the present results is representative of cardiotoxicity observed in the clinic.

An important limitation is the experience of the person performing the experiments. In the present case it was limited time to practice animal handling, to obtain good injection technique, to become a proper Langendorff perfusionist and learn tissue sectioning as well as staining for immunohistochemistry. Thus, this could have clearly influenced the results, and the reduced numbers of hearts available for measurement of physiology reflects this. However, these effects would have been randomized to both groups, and cannot explain the differences between the groups with or without pre-treatment with morphine.

6. CONCLUSIONS

- The present rat model is suitable to assess interventions to reduce cardiotoxicity of anthracyclines.
- Pre-treatment with morphine increases mortality of doxorubicin in the present *in vivo* model.

7. FURTHER STUDIES

Due to contradictory results in the study compared to the findings of in particular those of Kelishomi et al. (2008), further investigations based on this model are recommended. The study should be repeated by more experienced scientists. In particular, it could be of interest to study the *in vivo* interaction of MO and DOX with regard to both pharmacokinetic parameters and cardiovascular responses. This assessment should include a model were morphine is administrated *in vivo* by another technique than i.p. or alternatively prior to doxorubicin in the *ex vivo* perfused heart model via a drug injection port. Thus, direct effects on the heart can be dissociated from systemic effects when studying the interaction of the two drugs.

With regard to doxorubicin induced HSPs it could be of interest to make a time-based overview of HSP up-regulation. It would also be of interest to examine the degree of DOX-induced apoptosis by an appropriate staining technique.

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