Subcellular Basis of Vitamin C Protection Against Doxorubicin-Induced Changes in Cardiomyocytes and Sca-1 Positive Cells

by

Ana Ludke

A Thesis submitted to the Faculty of Graduate Studies of

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Department of Physiology
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Abstract

Understanding the molecular basis of doxorubicin (Dox)-induced oxidative stress leading to cardiomyopathy is crucial to finding cardioprotective strategies to manage this important clinical problem. Improving the antioxidant defenses of cardiac cells could be one strategy for cardioprotection. The role of oxidative stress in Dox-induced cardiotoxicity as well as testing the efficacy of antioxidant Vitamin C (Vit C) in offering protection to cardiomyocytes was investigated. As stem cells have been suggested to play a role in this cardiotoxicity, Dox-mediated oxidative stress effects, with and without Vit C, on the stem cell antigen-1 positive (Sca-1 positive) cells from heart as well as bone marrow were also examined.

Our time-course studies of the effects of Dox on the isolated cardiomyocytes showed that the phosphorylation of mitogen-activated protein kinases and p53 followed the rise in reactive oxygen species (ROS) production. Dox also downregulated the Sodium-dependent Vit C Transporter-2 (SVCT-2) and this may have enhanced Dox-induced increase in oxidative stress. Pro-apoptotic markers Bax/Bcl-xL ratio and caspase 3 cleavage were higher after the activation of stress-induced pathways and viability of cells was decreased. Dox-induced increase in apoptosis and decrease in cell viability depended in part on the activation of p38/JNK and p53 proteins, but not on the ERK protein. Exposure to Dox, increased membrane leakage, autophagy and lipid peroxidation. On the other hand, Dox exposed cardiomyocytes had decreased overall antioxidant capacity as well as

expression of the endogenous antioxidant enzymes glutathione peroxidase, Cu/Zn superoxide dismutase and catalase.

Dox affected Sca-1 positive cells in a prominent manner which was marked by a dose-dependent increase in cell loss, cell leakage and ROS levels as well as decrease in cellular ATP levels.

Vit C pre-treatment prior to the addition of Dox delayed and reduced Dox-induced ROS production, activation of stress-induced pathways and the rise in apoptotic and autophagic markers, partially rescuing cardiomyocyte viability. Vit C was able to blunt the decrease in SVCT-2 as well as Dox-induced oxidative stress. Vit C also offered protection to Sca-1 positive cells by partially preventing Dox-induced changes to these cells. The data presented in this thesis improves our knowledge of the molecular mechanisms leading to Dox-induced cardiotoxicity as well as suggest cardioprotection by Vit C for a better management of cancer patients.

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List of Abbreviations

ABTS	2,2'-azino-di-[3-ethylbenzthiazoline sulphonate]
ACE	angiotensin-converting-enzyme
ADP	adenosine diphosphate
AK	adenylate kinase
AMP	adenosine monophosphate
ANP	atrial natriuretic peptide
APAF1	apoptotic protease activating factor 1
Ask 1	apoptosis signal-regulating kinase 1
Atg	autophagy-related genes
ATP	adenosine triphosphate
Bad	Bcl-2-associated death promoter
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2 protein containing BH1, BH2 and BH3 domains
Bcl-2	B-cell lymphoma-2
Bcl-w	B-cell lymphoma
Bcl-xL	B-cell lymphoma-extra large
Bid	BH3 interacting-domain
Bim	Bcl-2 family containing BH3-only protein
BNP	brain natriuretic peptide
BNip3	Bcl-2 /adenovirus E1B 19 kDa protein-interacting protein 3
BSA	bovine serum albumin
$C_6H_8O_6$	ascorbate
Ca^{+2}	calcium
CAT	catalase
CK	creatine kinase
c-kit	stem cell factor receptor
Cu/Zn SOD	superoxide dismutase using copper and zinc as metal cofactor
DCFDA	5-(6)-chloromethyl-dichlorofluorescin diacetate
DNA	deoxyribonucleic acid
Dox	doxorubicin
ECG	electrocardiography
EDTA	ethylenediamine tetraacetic acid
EGTA	ethylene glycol tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
ERK 1/2	extracellular signal-regulated kinase 1/2
FACS	fluorescence-activated cell sorting
Fas	homotrimeric type II transmembrane protein
FDXR	ferredoxin reductase
Fe^{2+}	reduced iron
FITC	fluorescein isothiocyanate
GAPDH	glyceraldehyde 3-phosphate dehydrogenase

GATA4	family of zinc finger transcription factors
Glu	glutamate
GLUT	glucose transporter
Gly	glycine
GSH-Px	glutathione peroxidase
GSH	reduced glutathione
H_2O_2	hydrogen peroxide
H9c2	cell line from embryonic BD1X rat heart tissue by B. Kimes and B. Brandt
HepG2	human hepatoma cell line
HER2	human epidermal growth factor receptor 2
HO-1	heme oxygenase-1
IGF-BP3	insulin-like growth factor-binding protein 3
IgG	immunoglobulin G
Islet-1	LIM-Homeodomain transcription factor
JNK	c-Jun NH2-terminal protein kinase
KILLER/DR5	DNA damage−inducible p53−regulated death receptor gene
LC3	microtubule-associated protein light chain 3
LLC-PK1	pig kidney epithelial cell line
LPO	lipid peroxides
MAPK	mitogen-activated protein kinase
MAPKK	mitogen-activated protein kinase kinase
MAPKKK	mitogen-activated protein kinase kinase kinase
MDM2	murine double minute 2
Mef2c	myocyte-specific enhancer factor 2C
MEKK	same as mitogen-activated protein kinase kinase
MKK	same as mitogen-activated protein kinase kinase
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MUGA	multigated radionuclide angiography
NAC	N-Acetyl Cysteine
NADPH	nicotinamide adenine dinucleotide phosphate
NYHA	New York Heart Association
Nix	Bcl-2 family containing BH3-only protein
Nkx2.5	homeobox protein
NOS	nitric oxide synthases
NOXA	Bcl-2 family containing BH3-only protein (latin word for damage)
$O_2^{\bullet-}$	superoxide
$OH^{\bullet}$	hydroxyl
p 38	class of mitogen-activated protein kinase
p 53	tumor suppressor p53
PARP	poly (ADP-ribose) polymerase
PBS	phosphate buffered saline
PD98059	ERK MAPK inhibitor
PFT	pifithrin
PI	propidium iodide
PIDD	p53-induced protein with a death domain

Pro	proline
PTEN	phosphatase and tensin homolog
Puma	p53 upregulated modulator of apoptosis
PVDF	polyvinylidene difluoride
Raf	rapidly accelerated fibrosarcoma
Ras	rat sarcoma small GTPase protein
RIPA	radioimmunoprecipitation assay buffer
RLU	relative luminescence unit
ROS	reactive oxygen species
SAPK	stress-activated protein kinase
SB203580	p38/JNK MAPK inhibitor
Sca-1	stem cell antigen-1
Sca-1 positive	stem cell antigen-1 positive
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Ser	serine
SOD	superoxide dismutase
SVCT	sodium-dependent vitamin C transporter
TAK1	TGFbeta Activated Kinase 1
Thr	threonine
TNF-α	tumor necrosis factor alfa
top II	topoisomerase II
Tyr	tyrosine
UV	ultraviolet
Vit C	vitamin C
Vit E	vitamin E

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Publications include:

1) Ludke A, Sharma AK, Bagchi AK, Singal PK. Subcellular basis of vitamin C protection against doxorubicin-induced changes in rat cardiomyocytes. Mol Cell Biochem. 360(1-2):215-24, 2012. - with kind permission of Springer Science and Business Media.

2) Ludke AR, Sharma AK, Akolkar G, Bajpai G, Singal PK.Downregulation of vitamin C transporter SVCT-2 in doxorubicin-induced cardiomyocyte injury. Am J Physiol Cell Physiol. 303(6):C645-53, 2012.

I. Introduction

Anthracyclines (e.g. doxorubicin - Dox) are the most effective drugs available for the treatment of neoplastic diseases. However, a major factor limiting their use is a cumulative, dose-dependent cardiotoxicity. Dox-induced cardiomyopathy and heart failure result from a permanent loss of cardiac cells due to cell necrosis and apoptosis (Lefrak et al. 1973; Singal and Iliskovic 1998; Chatterjee et al. 2010). Use of Dox as a part of the anticancer cocktail has been associated with a significant increase in the number of long-term cancer survivors (Yeh et al. 2004). Thus, the life threatening side effect, the Dox-induced cardiomyopathy, still remains an important clinical problem, which can develop concurrent with the therapy or may have a late onset. Unfortunately, no satisfactory clinically applicable preventive treatment is presently available. Therefore, understanding the mechanism of this cardiomyopathy as well as its prevention remains an important issue. There is increasing evidence that oxidative stress plays a role in the cardiomyocyte death seen in Dox-induced cardiomyopathy (Myers et al. 1977; Singal et al. 1997; Kumar et al. 2001; Lou et al. 2006) and some of the antioxidants have been shown to mitigate these changes (Siveski-Iliskovic et al. 1995; Cvetkovic and Scott 2005; Ludke et al. 2009).

This thesis comprises the study of distinct but interconnected lines of investigation to tease out the role of oxidative stress as well as the beneficial effects of antioxidant Vitamin C (Vit C) in Dox-induced cardiotoxicity.

Stress-induced pathways have been associated with Dox-induced cardiotoxicity. Members of the mitogen-activated protein kinase (MAPK) family are activated in various types of stress-induced apoptosis (Matsuzawa and Ichijo 2008) including Dox-induced cardiomyopathy (Kumar et al. 2001; Lou et al. 2005). Different patterns of activation have been shown for the three main MAPKs in response to Dox treatment (Lou et al. 2005; Lou at al. 2006). Both p38 kinase (p38) and c-Jun NH2-terminal protein kinase (JNK) MAPK pathways are typically related to apoptosis, whereas extracellular signal-regulated kinase 1/2 (ERK1/2) was shown to be downregulated in different cardiac stress conditions (Lou et al. 2005; Matsuzawa and Ichijo 2008). Another protein, tumor suppressor p53, has also been shown to be upregulated in Dox-induced cardiotoxicity (Liu et al. 2004; Chua et al. 2006). Upon activation, p53 is phosphorylated at Ser15 and translocated into the nucleus to induce the expression of genes associated with cell arrest, DNA repair, and apoptosis (Suzuki et al. 2007). Whether inhibition of any one or all of those signaling proteins (p38/JNK, ERK, and p53) can interfere with Dox-induced damage in cardiomyocytes remains elusive. As oxidative stress has been suggested to be the major cause of Dox-induced cardiotoxicity, the beneficial effects of Vitamin C (Vit C) which is a diet-derived antioxidant known to quench reactive oxygen species (ROS) (Gutteridge and Halliwell 2000) are appealing as a cardioprotective strategy. In a recent review, it has been highlighted that most of the Western populations are not likely to benefit from Vit C supplements because of their adequate nutritional status (Lykkesfeldt and Poulsen 2010). However, in the same study it was suggested that there is a need to examine the effect of Vit C as a single supplement in subpopulations which could

really benefit from its supplementation. One such subpopulation may be comprised of cancer patients which are generally depleted of Vit C (Mayland et al. 2005; Lykkesfeldt and Poulsen 2010), making them more vulnerable to oxidative stress-mediated injury. Although experimental studies have shown that Vit C does not interfere with the antitumor activity of Dox and it significantly prolongs the lives of mice, rats and guinea pigs treated with Dox by reducing cardiotoxicity (Fujita et al. 1982; Shimpo et al. 1991; Wold et al. 2005; Santos et al. 2007), there is no adequate information available whether the downstream pathways involving MAPKs and p53 are the basis of such improvement in Dox-induced cardiotoxicity.

Vit C can enter cells both in its reduced and oxidized forms, as ascorbic acid and dehydroascorbate, utilizing respectively sodium-dependent transporters (SVCT) or glucose transporters (GLUT) (Corti et al. 2010). Two Sodium-dependent Vit C Transporters (SVCT-1 and SVCT-2) have been identified to mediate the uptake of L-ascorbate (Tsukaguchi et al. 1999). SVCT-2 has a 10-fold higher affinity for ascorbate (Tsukaguchi et al. 1999), and SVCT-2 expression in many tissues including the heart has been documented (Rajan et al. 1999). Data on how different drugs affect Vit C transport systems is lacking, but modulation of SVCT expression and function by cytokines and steroids have been described (Berger et al. 2003; Savini et al. 2007; Chi and May 2009). Although no data are available for this sodium transporter in cardiomyocytes, isolated adult cardiomyocytes have been shown to accumulate Vit C after hypoxic stress in vitro resulting in enhanced resistance against oxidative stress (Guaiquil et al. 2004). Increased expression of SVCT-2 and accumulation of Vit C were also observed in heart tissue homogenates of failing hearts which may represent a protective mechanism

against increased oxidative stress in heart failure (Rohrbach et al. 2008). The study of Vit C transporters in isolated cardiomyocytes is needed for a complete understanding of Vit C cardioprotection in Dox-induced cardiomyopathy.

It has been documented that cardiac dysfunction after cumulative doses of Dox is not only due to the loss of cardiomyocytes, but other cardiac cells as well (De Angelis et al. 2010; Huang et al. 2010). More recently, progenitor cells in the adult heart have been identified by their expression of cell surface markers such as c-Kit or stem cell antigen-1 (Sca-1) (Beltrami et al. 2003; Oh et al. 2003; Matsuura et al. 2004; Kajstura et al. 2008). Although, their role in heart structure and function is far from being understood, two new studies raised the possibility that Dox-induced cardiomyopathy may also be a stem cell disease, mediated by a severe loss of not only functional cardiomyocytes but resident cardiac progenitor cells as well (De Angelis et al. 2010; Huang et al. 2010). Thus investigation of the involvement of oxidative stress in Dox-induced injury to cardiac and bone marrow Sca-1 positive cells is a promising area of research.

The current work addresses the role of oxidative stress in Dox-induced damage to cardiomyocytes and Sca-1 positive cells. We also examined potential mechanisms involved in cardiac protection due to Vit C. Using various techniques, we have tracked the time course of changes in oxidative stress, stress-induced proteins, apoptosis and cell viability due to Dox. In order to examine the role of MAPKs and p53 in Dox-induced cardiomyocyte apoptosis, we made use of pharmacological inhibition. Further we have indentified the increase in ROS as well as oxidative stress as the key events leading to Dox-induced changes to cardiomyocytes. Using antioxidant Vit C to counteract Dox-induced oxidative

stress, we begin to characterize cardioprotective mechanisms against this drug-induced cardiomyopathy. By using immunofluorescence, we have identified the localization of Vit C transporters and their influence on the beneficial effects of Vit C after Dox exposure in cardiomyocytes. We have also noticed that Sca-1 positive cells are more sensitive than cardiomyocytes to Dox-induced damage.

Based on the data obtained, it is suggested that Dox-induced oxidative stress has a causal role in the injury to cardiomyocytes and Sca-1 expressing cells. Furthermore, Vit C offered protection by mitigating the oxidative stress due to Dox.

II. Literature Review

1. Doxorubicin

1.1 Brief history

Doxorubicin (Dox) is an anthracycline antibiotic isolated from the pigment-producing *Streptomyces peucetius* and was introduced in cancer therapy in the late 1960's (Weiss 1992). It is among the most effective anticancer agents introduced in the past 50 years (Minotti et al. 2004). Dox is administered as a single agent or in combination with other chemotherapeutic drugs. In fact, it is an essential component of the cocktail used for the treatment of breast cancer, childhood solid tumours, soft tissue sarcomas, and aggressive lymphomas. Dox is a frontline chemotherapeutic agent because it improves overall survival in cancer patients (Ferlay et al. 2008). Such an increase in survival rate became evident after the introduction of anthracyclines in the chemotherapy regimens (Chen et al. 2011). Particularly among childhood cancers, the 5-year survival rate increased from 30% in the 1960s to about 80% more recently (Jemal et al. 2010; Lipshultz and Adams 2010; Trachtenberg et al. 2011). However, the clinical use of this chemotherapeutic agent is hampered by its side-effects such as hematopoietic suppression, nausea, vomiting, extravasation, and alopecia, in addition to the life-threatening cardiotoxicity (Lefrak et al. 1973; Singal et al. 1997). Cardiac complications are the leading noncancerous causes of severe complications affecting mortality, morbidity and quality of life of cancer patients in the years subsequent to chemotherapy (Oeffinger et al. 2006; Trachtenberg et al. 2011). Since Dox is still included in most chemotherapeutic regimens, the risks and

benefits of eliminating the tumor must be considered along with preservation of cardiac function to optimize patient outcomes.

1.2 Structure, characteristics and mechanisms of action

Anthracyclines are cationic, lipophilic drugs, allowing them to freely penetrate the cellular membranes including cytoplasmic organelles and vesicles. The molecular structure of Dox is represented in Figure 1 and it is composed of aglyconic and sugar moieties. The aglycone consists of a planar tetracyclic ring with quinone-hydroquinone groups in rings B-C, a methoxy substituent at C-4 in ring D, and a short side chain at C-9 with a carbonyl at C-13. The amino sugar is attached by a glycosidic bond to the C-7 of ring A and consists of a 3-amino-2,3,6-trideoxy-L-fucosyl moiety. The side chain of Dox terminates with a primary alcohol.

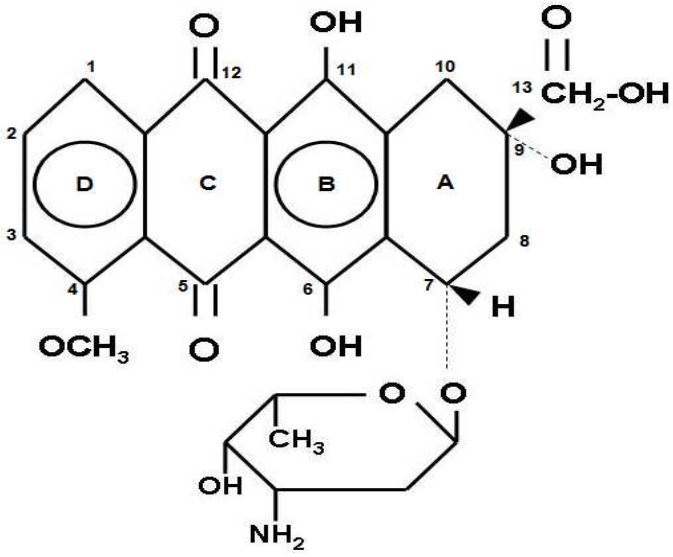

Figure 1: Chemical structure of doxorubicin.

Due to its lipophilic characteristics, this drug has a relatively long half-life in the body, and in the heart, it can be about 48 hours (Singal et al. 2000; Minotti et al. 2004). Dox is cleared from plasma through liver (Danesi et al. 2002) and excreted primarily through the hepatobiliary route (Wildiers et al. 2003). The pharmacokinetics of Dox may exhibit large interpatient and intrapatient variations that account for the individual antitumor response and Dox-related side effects including cardiotoxicity (Robert and Gianni 1993; Lal et al. 2010). In this regard, the accumulation of anthracyclines also differs among various tissues, and it has been already reported to be higher in the liver, heart, white blood cells and bone marrow (Lal et al. 2010).

Despite extensive progress, the molecular mechanisms responsible for Dox anticancer activity as well as Dox-induced cardiotoxicity are not completely understood (Gewirtz 1999; Minotti et al. 2004; Chen et al. 2007; Mordente et al. 2009; Peng et al. 2010; Menna et al. 2012).

1.3 Antitumor effects

The potential antitumor mechanisms of Dox are diverse (reviewed in Gewirtz 1999), though these are commonly attributed to an intercalation of the drug in the DNA base pairs and blocking of cell replication by inhibiting topoisomerase II (top II) (Nitiss 2009; Pommier et al. 2010). Inhibition of top II will trigger cell death of rapidly dividing cancer cells (Cutts et al. 2005; Swift et al. 2006 ; Coldwell et al. 2008; Nitiss 2009; Pommier et al. 2010) since this enzyme catalyzes the cleavage and re-ligation of DNA to regulate DNA topology repressing replication, leading to

inhibited synthesis of macromolecules (Li and Liu, 2001). Among topoisomerases, topII alfa is considered to be the primary molecular target for anthracycline antibiotics. Thus the high efficacy of Dox in tumour cells can be associated to the highly elevated expression of top II alfa in cancer cells (Mukherjee et al. 2010; Desmedt et al. 2011). Interestingly, top II beta is expressed in adult heart (Capranico et al. 1992). However, role of top II beta in Dox-induced cardiotoxicity has not been described thus far. Although the use of this antineoplastic drug is associated with cardiomyopathy, dissociation of antitumor activity from the cardiotoxic effects of the drug has been documented (Silveski-Iliskovic et al. 1995; Singal et al. 1997).

1.4 Cardiotoxicity

Several mechanisms underlying Dox-induced cardiotoxicity have been suggested. The list includes: overproduction of reactive oxygen species (ROS) causing oxidative stress, mitochondrial dysfunction and impairment of energy metabolism with loss in the high-energy phosphate pool, disturbance of Ca^{+2} handling and contraction, disturbances of myocardial adrenergic signaling, cell necrosis and activation of intrinsic and extrinsic pathways of apoptotis leading to cardiomyocyte loss (Singal and Iliskovic 1998; Gewirtz 1999; Minotti et al. 2004; Tokarska-Schlattner et al. 2006; Menna et al. 2008; Ludke et al. 2009; Octavia et al. 2012).

Despite the long list, an increase in cardiac oxidative stress is the most accepted mediator of Dox-induced cardiomyopathy. Various chemical processes

may cumulatively contribute to the generation of oxidative stress. In this regard, the quinone moiety of Dox in ring C (Fig. 1) can be reduced by oxi-reductases producing a semiquinone radical associated with a redox cycling leading to reactive forms of oxygen (Davies and Doroshow 1986; Gewirtz 1999; Menna et al. 2008). Doxorubicin reduction in the presence of free iron was also shown to promote redox recycling in cardiomyocytes (Xu et al. 2005).

Dox is known to cause a reduction in different antioxidant enzymes (Lim et al. 2004; Aries et al. 2004; Timolati et al. 2006). Furthermore, cancer patients treated with Dox show a decline in non-enzymatic antioxidants such as Vit C (Bodansky et al. 1952; Gonçalves et al. 2005). The activation of a number of focal signaling pathways has also been identified as critical transducers leading to oxidative stress (Garrington and Johnson 1999; Baines and Molkentin 2005). Furthermore, enzymes such as nitric oxide synthases (NOS) and NADPH oxidase were also shown to potentiate the production of ROS first initiated by redox cycling of Dox (Octavia et al. 2012).

A cardiac biopsy can be used for diagnosis of Dox-induced cardiotoxicity and cardiomyopathy. Electron microscopy studies have shown myofibrillar disarray, dilation of the sarcoplasmic reticulum, cytoplasmic vacuolization, mitochondrial swelling, disordered nuclear chromatin, and increased lysosomal number (Singal et al. 1987; Peng et al. 2005). Particularly, cytoplasmic vacuolization is universally seen in patients and laboratory animals that developed Dox-induced cardiotoxicity (Robert 2007).

1.4.1. Acute, early and late-onset cardiotoxicity

According to the time of onset, Dox-induced cardiomyopathy has been categorized in three distinct forms: acute, early and late-onset of chronic progressive cardiotoxicity (Figure 2) (Takemura and Fujiwara 2007; Zuppinger et al. 2007, Franco et al. 2011).

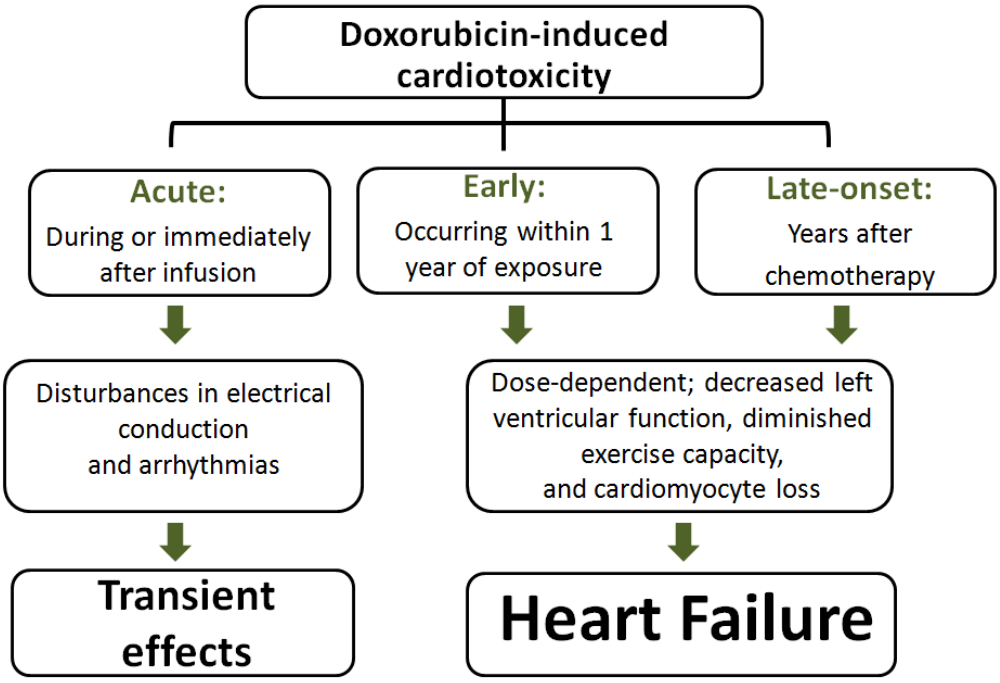

Figure 2: Acute, early and chronic cardiac effects of doxorubicin.

The acute cardiotoxicity generally occurs in <1% of patients immediately after infusion of the anthracycline, but it was shown to be as high as in 11% of patients after 2-3 days of drug administration (Swain et al. 2003; Takemura and Fujiwara, 2007). It presents as an acute, transient decline in myocardial contractility. Typically, it may also include arrhythmias, ECG changes, and infrequently pericarditis and myocarditis (Swain et al. 2003). Most of these changes

are usually reversible (reviewed in Wouters et al. 2005). Although in some cases of acute dilated cardiomyopathy, sudden cardiac death has also been reported (Floyd et al. 2005).

The early-onset cardiomyopathy may start during the treatment period or within the first year after treatment (Krischer et al. 1997; Kremer et al. 2001). Early cardiotoxicity is presumably related to the damage or loss of cardiomyocytes, which results in depressed left ventricle contractility (Lipshultz et al. 1991). In both children and adults, the risk of early cardiotoxicity increases with the cumulative dose of anthracycline (Von Hoff et al. 1979; Swain et al. 2003).

The late-onset cardiomyopathy is diagnosed when the cardiomyopathic changes occur at least 1 year or later after the therapy is complete. At 6 years after anthracycline therapy, about 65% of survivors of childhood cancer have abnormalities of cardiac structure and function (Lipshultz et al. 1991; Lipshultz et al. 1995). These abnormalities appeared to be progressive in most patients (Lipshultz et al. 1991; Lipshultz et al. 2005). The risk of anthracycline-induced clinical heart failure was estimated to be 4-5% after 15–20 years of the start of therapy (Green et al. 2001; Kremer et al. 2001).

Both early and late-onset of cardiotoxicity are characterized by a dose-dependent, symptomatic or asymptomatic, progressive decrease in left ventricular function often resulting in congestive heart failure (Swain et al. 2003; Pinder et al. 2007; van Dalen et al. 2008). The incidence of Dox-induced cardiomyopathy is significantly influenced by several well defined risk factors including cumulative dose, age of the patient, previous radiation therapy, concomitant administration of other anticancer drugs, and a pre-existing history of heart disease (Von Hoff et al.

1979; Singal and Iliskovic 1998; Ludke et al. 2009). Data obtained from longitudinal studies in anthracycline-treated survivors of childhood cancer reveal that asymptomatic cardiac abnormalities were demonstrated at lower (45 mg/m^2) cumulative doses of Dox when other risk factors are present (Lipshultz et al. 2005). Moreover, the incidence of cardiotoxicity was shown to increase in female patients (Silber et al. 1993; Lipshultz et al., 1995). This has been shown to be determined at least partially by differences in drug clearance (Dobbs et al. 1995) due to body fat composition (Silber et al. 1993). Therefore, the greater systemic exposure to the active drug in female patients (Wade et al 1992; Dobbs et al. 1995) implies that dose of exposure remains the underlying risk factor. Additional studies are needed to further define if gender plays a specific role in Dox-induced cardiotoxicity.

More prominently, anthracycline cardiotoxicity is exponentially dose-dependent, with an average incidence of 5.1% at 400 mg/m^2 which becomes much higher when the cumulative dose reaches above 500 mg/m^2, albeit with substantial individual variation (Lefrak et al. 1973; Von Hoff et al. 1979; Swain et al. 2003). These findings define a strong basis embraced by the Clinical Practice Guidelines of the American Society of Clinical Oncology regarding the use of cardioprotective agents when a cumulative dose of 300 mg/m^2 of Dox is achieved (Schuchter et al. 2002). Conversely, there are patients who can tolerate a higher dose of Dox in the absence of risk factors, emphasizing the importance of defining the underlying mechanism of Dox mediated cardiac dysfunction. These observations suggest that there is no completely “safe” dose of anthracyclines to avoid this cardiomyopathy (Lipshultz et al. 2005).

2. Oxidative stress

The term oxidative stress refers to an imbalance between ROS production and antioxidant defences. ROS are oxygen-derived chemical species characterized by their high reactivity. They include free radicals i.e. species with unpaired electrons, such as superoxide ($O_2^{\bullet -}$) and hydroxyl ($OH^{\bullet}$) and non-radicals, species capable of generating free radicals such as hydrogen peroxide (H_2O_2). Regardless of the aetiology, there is increasing evidence recognizing that ROS and oxidative stress could be both a contributor to and product of many cardiovascular disease processes, such that a positive feedback relationship can exist (Giordano 2005). Furthermore, ROS levels are generally observed to rise with the severity of disease (Nagayoshi et al. 2009; Amir et al. 2009).

Under pathophysiological conditions, ROS have the potential to cause cellular damage and dysfunction. The detrimental effects will depend upon site, source and amount of ROS produced, and the overall redox status of the cell (Seddon et al. 2007). Cardiomyocytes have a constant use of high energy phosphates, thus rely on oxidative phosphorylation. The balance between oxidant and antioxidant systems may be tight in the normal heart. Thus, this condition will be further worsened by Dox-induced cardiomyocyte ROS production leading to increase in oxidative stress, cell damage and heart failure.

2.1 Cardiac Antioxidant reserve

The myocardium is equipped with a variety of endogenous nonenzymatic and enzymatic antioxidant systems responsible for prevention or removal of ROS.

Due to the high oxidative metabolism in cardiac tissue, these antioxidant systems may be just sufficient enough to metabolize ROS generated during normal cellular activity (Doroshow et al. 1980). Nonenzymatic antioxidant reserve includes intracellular antioxidants such as the vitamins C, E, and β-carotene (a precursor to vitamin A), ubiquinone, lipoic acid, urate, and reduced glutathione (GSH). As for enzymatic antioxidant defenses, the best-characterized are catalase (CAT) and glutathione peroxidase (GSH-Px), which coordinate the catalysis of H_2O_2 to water, and the superoxide dismutase (SOD), which facilitates the dismutation of $O_2^{\bullet-}$ (Halliwell and Gutteridge 2007). The cardiac antioxidant defenses also include hemeoxygenase-1 (HO-1), an enzyme that maintains reduced iron in heme substrate in cardiomyocytes, converting potentially toxic heme, into the antioxidant biliverdin (Maines 1988).

Dox-induced cardiotoxicity was already shown to disturb antioxidant defense systems as well as repair pathways (Swift et al. 2007; Kang 2007). Dox decreases the activity of the well known antioxidant enzymes CAT, Cu/Zn SOD, and GSH-Px (Lim et al. 2004; Aries et al. 2004; Timolati et al. 2006). More recently, a decrease in HO-1 has been shown to play a role in Dox-induced cardiotoxicity (Suliman et al. 2007; Bernuzzi et al. 2009). All of these findings suggest an increase in oxidative stress caused by an increase in free radical production and a decrease in diverse antioxidant enzymes supporting the cardiac damage caused by Dox.

2.2 Sources of oxidative stress

Excessive ROS derived from mitochondria have been shown in failing hearts (Tsutsui et al. 2008). Cardiomyocytes have the highest density of

mitochondria compared to most other cells to meet the demand for synthesis of ATP by oxidative metabolism (Tsutsui et al. 2009.). In failing myocardium, mitochondrial electron transport enzymes (complexes I and III) are an important source of large amount of ROS. Mitochondrial ROS generation has been reported as an important contributor to heart failure (Giordano 2005; Nicolson 2007). In addition, the mitochondria are among the most studied targeted organelles of Dox-induced cardiotoxicity (Octavia et al. 2012). Doxorubicin was shown to penetrate the mitochondria (Sokolove 1994; Ashley and Poulton 2009) affecting multienzyme complexes of the electron transport chain/oxidative phosphorylation system (Bianchi et al. 1987; Chandran et al. 2009). Disruption of the cardiolipin–protein interface by Dox in the mitochondria was also already described as a process that enhances the formation of $O_2^{\bullet-}$ (Goormaghtigh et al. 1980; Schlame et al 2000). Thus, mitochondria are important targeted sites for Dox to induce oxidative stress.

The transmembrane enzymes NADPH oxidases have been found to be increased in experimental models of heart failure (Qin et al. 2005) as well as in end-stage failing human myocardium (Cameron et al. 2006; Murdoch et al. 2006; Kuroda and Sadoshima 2010). These enzymes catalyze electron transfer from NADPH to molecular oxygen, resulting in the formation of $O_2^{\bullet-}$. In addition, ROS produced by NADPH oxidases can promote ROS generation by other sources, thereby amplifying total levels of ROS. Furthermore, these enzymes can be activated by a variety of important stimuli associated with heart failure, including angiotensin II, norepinephrine, endothelin, insulin, inflammatory cytokines and growth factors, mechanical stretch, or hypoxia/reoxygenation (Lambeth 2004). Activation of NADPH was already demonstrated after Dox exposure (Deng et al.

2007). In this regard, in vitro studies using NADPH complex inhibitors have presented cardiac protection (Gilleron et al. 2009).

Nitric oxide synthase (NOS) enzymes normally generate nitric oxide, but may instead generate $O_2^{\bullet-}$ and H_2O_2 if they become ‘‘uncoupled’’, a state that is especially likely to occur during stress conditions (Vásquez-Vivar 2009). NOS uncoupling and subsequent $O_2^{\bullet-}$ production have implicated in the genesis of vascular disorders including hypertension and atherosclerosis as well as subsequent heart failure (Moens and Kass 2007). There is some evidence that NOS uncoupling leading to ROS formation may also play a role in Dox-induced myocardial dysfunction (Duquaine et al. 2003; Neilan et al. 2007). Regardless of the source, an excess of ROS in the myocardium is clearly detrimental.

ROS-mediated damage largely occurs via lipid peroxidation as well as reduction in thiol groups (Kaul et al. 1993). Lipid peroxidation results in changes in membrane architecture and permeability as well as alterations in membrane bound enzymes activities. Every compartment of the cell surrounded by membranes can be affected. Along with the Dox-induced mitochondrial defects described erlier, ROS-mediated by Dox exposure in cardiomyocytes was already shown to disrupt normal sarcoplasmic reticulum function with impairement of normal calcium homeostasis (Holmberg and Williams 1990; Arai et al. 2000). In addition, the list of targets for ROS is not exhaustive and also includes DNA and protein oxidation (Mukhopadhyay et al. 2009). Amino acids like cysteine can undergo covalent modifications of their free thiol group by ROS which may lead to impaired protein signaling (Foster et al. 2009). The major consequences of these ROS targets for the heart are: impairment in contractile function, dysregulation in protein

production, and activation of stress-induced pathways leading to apoptotic and necrotic cell death (Seddonet al. 2006).

3. Stress-induced pathways in Dox treatment

Studies on Dox-induced cardiomyocyte damage have shown that multiple signaling pathways are involved. Among these pathways, recent studies have shown that Dox-induced cardiomyocyte loss is associated with increased expression and activation of p53 tumor suppressor protein (Liu et al. 2004; L'Ecuyer et al. 2006; Liu et al. 2008a) and the major mitogen-activated protein kinases (MAPK) families (Zhu et al. 1999; Kawamura et al. 2004; Lou et al. 2005; Lou et al. 2006; Fan et al. 2008; Liu et al. 2008a). The three best characterized effector MAPK subfamilies are the extracellular signal-regulated protein kinases (ERK 1/2), the c-Jun NH2-terminal kinases (JNK), and p38 (Garrington and Johnson 1999; Widmann et al. 1999). In regard to MAPKs, different activation patterns are described for the three kinase members following Dox treatment in cardiac tissue, but the underlying mechanism for their activation and targets remain poorly understood.

3.1 Cell response to stress stimuli: p53 and mitogen-activated protein kinases

The tumor suppressor p53 is part of a small family of related proteins that includes p63 and p73 where p53 is responsible to prevent tumour development and maintain genomic integrity (Vousden and Lu 2002). Several stress signals

activate p53 including DNA damage, genotoxic damage, oncogenes, certain drugs and toxic agents, oxidative stress, hypoxia/anoxia resulting in apoptosis, senescence, cell cycle arrest, as well as differentiation which prevent propagation of damaged DNA (Liu and Chen 2006; Hornand and Vousden 2007). Under normal conditions, p53 is expressed at low levels because of negative feedback regulation by MDM2 – one of the main regulators of p53 stability and activity (Haupt et al., 1997; Kubbutat et al., 1997; Roxburgh et al. 2012). Upon cellular stress, p53 is stabilized and phosphorylated which allows the activation of numerous gene targets. Multiple phosphorylation sites have been identified on the NH2-terminal end of p53. However, the most well-studied phosphorylation site is Ser 15, which is known to be essential for the transactivation of p53 (Vousden and Lu 2002). Genes associated with p53 activation comprise: death-receptor pathways (KILLER/DR5, FAS, PIDD), inhibition of survival signals (IGF-BP3, PTEN), and mitochondrial pathways (APAF1, BAX, FDXR, NOXA, PUMA) (Vousden and Lu, 2002; Mirza et al. 2003; Rahman et al. 2005). Furthermore, p53 transcription-independent pro-apoptotic functions have been already described (Green and Kroemer, 2009). The different cellular responses after p53 activation appear to be related to cell type, source of stress, and intensity of stimuli.

In the heart, p53 has been shown to be upregulated by ischemia (Long et al. 1997; Toth et al. 2006), oxidative stress (Cesselli et al. 2001), mechanical stress (Leri et al 1998a), tachycardia caused by pacing (Leri et al. 1998b), and anthracyclines (Liu et al. 2008a). In addition, involvement of telomere dysfunction induced by p53 upregulation in the development of heart failure has been suggested (Oh et al. 2003; Torella et al. 2004). Thus, increasing evidence indicates

that p53 plays an important role in stress-induced cell death leading to heart failure.

Following Dox treatment of cardiomyocytes *in vitro* and administration *in vivo*, this drug rapidly accumulates in the nucleus (Kumar et al. 1999) interfering with DNA stability. Dox-induced oxidative stress is also responsible for damaged DNA. In fact, inhibition of p53 by pifithrin (PFT)-α, the cell-permeable p53 inhibitor (Lin et al. 2003), has been shown to inhibit the pro-apoptotic effects of Dox *in vitro* (Chua et al. 2006). Genetic deletion of p53 in mice receiving Dox showed a survival effect (Shizukuda et al. 2005) suggesting a critical role for p53 in Dox-induced apoptosis (Spallarossa et al. 2009).

Mitogen-activated protein kinases (MAPKs) received considerable attention over the recent years as a family of proteins that play an integral role in the signaling events in response to stress. Activation of MAPKs requires dual phosphorylation of a Thr-X-Tyr motif (where X is either a Gly, Pro, or Glu) in the regulatory loop (Baines and Molkentin 2005). All subfamilies have a clearly delineated activation cascade constructed as a hierarchy beginning at the upstream MAPK kinase kinase - MAPKKK (Raf, MEKK1-4, TAK1, and Ask-1), to the MAPK kinase - MAPKK (MEK1/2, MKK4/7, MKK3/6, and MKK5), which in turn activates the MAPKs (ERK 1/2, JNK 1/2/3, p38 α/β/γ/δ) through serial phosphorylation (Ferrell 1996). Once activated, these terminal effector kinases directly phosphorylate a diverse selection of cytoplasmic, nuclear, and mitochondrial proteins that modulate gene expression, cellular metabolism, growth and death (Lenormand et al. 1993).

The canonical activation of these pathways is triggered in response to a variety of stimuli that include: growth factors; physical, chemical and physiological stressors such as ultraviolet light, oxidative stress, osmotic shock, infection, and cytokines (Ferrel 1996; Kyriakis and Avruch 2001; Ramos 2008). Involvement of oxidative stress is commonly reported in MAPK activation (Kyriakis and Avruch 2001; Knock and Ward 2011). It appears that the effectors MAPKs are not directly redox-sensitive but oxidative stress modulates the activation of upstream pathways (Knock and Ward 2011). These upstream targets include redox modulation of transmembrane protein tyrosine phosphatase, or direct redox activation of ras or Ask 1, thus stimulating the MAPK activation cascades (Lander et al. 1997; Lee and Esselman 2002; Adachi et al. 2004; Clavreul et al. 2006).

Although the highly conserved family of MAPKs are ubiquitously expressed and are activated in a variety of cell types, their mechanisms of activation, their substrates, and the consequences for the cell have been demonstrated to be cell-type specific. This is particularly important in highly specialized cells such as the terminally differentiated cardiomyocytes (Rose et al. 2010).

First identified in the early 1980s, ERK1/2 is suggested to phosphorylate over 100 possible substrates (Kolch 2000; Yoon and Seger 2006) regulating cell cycle progression, proliferation, cytokinesis, transcription, differentiation, senescence, cell death, migration, GAP junction formation, actin and microtubule networks, and cell adhesion (Yoon and Seger 2006). This pathway is strongly activated by growth factors, serum, and phorbol esters, but it can also be activated by G protein-coupled receptors, cytokines, microtubule disorganization, and oxidative stress (Goldsmith and Dhanasekaran 2007; Menick et al. 2007). In

cardiomyocytes, members of the ERK MAPK family have been implicated in survival signaling in response to ischemia-reperfusion, oxidative stress, hypoxia, β-adrenergic stimulation, and anthracycline exposure (Aikawa et al. 1997; Yue et al. 2000; Lou et al. 2005; Kennedy et al. 2006; Li et al. 2006; Kovacs et al. 2009). Inhibition of ERK1/2 with PD98059 increases the rate of myocyte apoptosis induced by oxidative stress or hypoxia/reoxygenation (Aikawa et al. 1997; Yue et al. 2000), indicating that, in addition to their role in myocyte growth, ERK activation constitutes a cytoprotective element in cardiomyocytes. *In vivo*, cardiospecific activation of this pathway produces concentric cardiac hypertrophy with increased contractile function where the isolated cardiomyocytes are more resistant to cell death stimuli, which implies a cytoprotective role for ERK1/2 (Bueno et al. 2000). The direct molecular mechanism whereby ERK signaling mediates cardiomyocyte protection is poorly understood, but ERK1/2 activation has generally been shown to antagonize the apoptotic pathway (Kennedy et al. 2006; Li et al. 2006; Kovacs et al. 2009).In Dox-induced cardiotoxicity, it has been suggested that ERKs, indeed, are upregulated early (this activation is considered an adaptive and/or protective response), but transiently downregulated during the heart failure stage (Lou et al. 2005).

JNK was discovered in the 1990s as a second subfamily of MAPKs (Hibi et al. 1993). JNK also plays a role in a number of different biological processes including cell proliferation, differentiation, apoptosis, cell survival, actin reorganization, cell mobility, metabolism, and cytokine production (Davis 2000; Bogoyevitch and Kobe 2006). As a stress-activated protein kinase (SAPK), JNK is strongly activated by inflammatory cytokines and cellular stresses such as heat

shock, hyperosmolarity, ischemia-reperfusion, ultraviolet (UV) radiation, oxidative stress, DNA damage, and endoplasmic reticulum stress (Bogoyevitch 2006).

p38, another subfamily of SAPKs from MAPKs was also identified in the 1990s (Han et al. 1993; Han et al. 1994). In response to stress-stimuli, p38 is activated to exert its biological functions including apoptosis, cell survival, cell cycle regulation, differentiation, senescence, cell growth and migration (Zarubin and Han 2005; Thornton and Rincón 2006). p38 was already described to respond to over 60 different extracellular and intracellular stimuli including UV radiation, heat, osmotic shock, pathogens, inflammatory cytokines, growth factors, and oxidative stress (Ono and Han 2000).

JNKs and p38-MAPKs have been shown to be activated in cardiomyocytes in response to oxidative stress and pro-inflammatory cytokines (Sugden and Clerk 1998ab; Clerk et al. 1999). SB203580 inhibits the activation of MAPK activated protein kinase 2 (MAPKAPK2) (Sugden and Clerck 1998; Clerck et al. 1998). Concentrations of SB203580 up to ~1 μM are relatively selective for p38-MAPK *in vitro*, and higher concentrations inhibit certain JNK isoforms as well (Clerk and Sugden 1998). This inhibition has been demonstrated to be cardioprotective, placing JNK and p38-MAPKs in a pro-apoptotic role (Ma et al. 1999; Barancik et al 2000; Martin et al. 2001; Milano et al. 2007).

After Dox exposure, phosphorylation of p38 MAPKs and JNKs increases early and persists until the heart failure stage, suggesting that these kinases may play a dominant role in the progression of anthracycline-induced cardiomyopathy and heart failure (Lou et al. 2005; Lou et al. 2006).

Conflicting results have also been reported for the activation of p53 and MAPKs family in response to Dox treatment (Spallarossa et al. 2006; Liu et al. 2008a; Tokarska-Schlattner et al. 2010). Therefore, a better understanding of the roles of p53 and MAPK pathways as potential therapies for heart failure require major progress in this area. Their specific role in Dox-induced cardiotoxicity needs further investigation in order to elucidate whether they can be the targeted candidates for therapeutic intervention.

4. Dox-induced cell death

All the cardiotoxic events leading to cardiomyocyte loss will contribute to Dox-induced cardiomyopathy and heart failure. Cardiac cell fate may be determined by different types of cell death including apoptosis, necrosis, and excess autophagy (Figure 3). Activation of the complex mechanisms that include oxidative stress, mitochondrial damage, DNA damage, and cell membrane injury will finally culminate in cell death. *In vitro* and *in vivo* studies on Dox-induced cardiotoxicity have associated the loss of cardiomyocytes with apoptotic and necrotic cell death (Singal et al. 1997; Kumar et al. 2001; Liu et al. 2004; Ikegami et al. 2007; Liu et al. 2008b; Riad et al. 2009). In addition, autophagy contributing to cell death was also recently reported (Lu et al. 2009; Kobayashi et al. 2010).

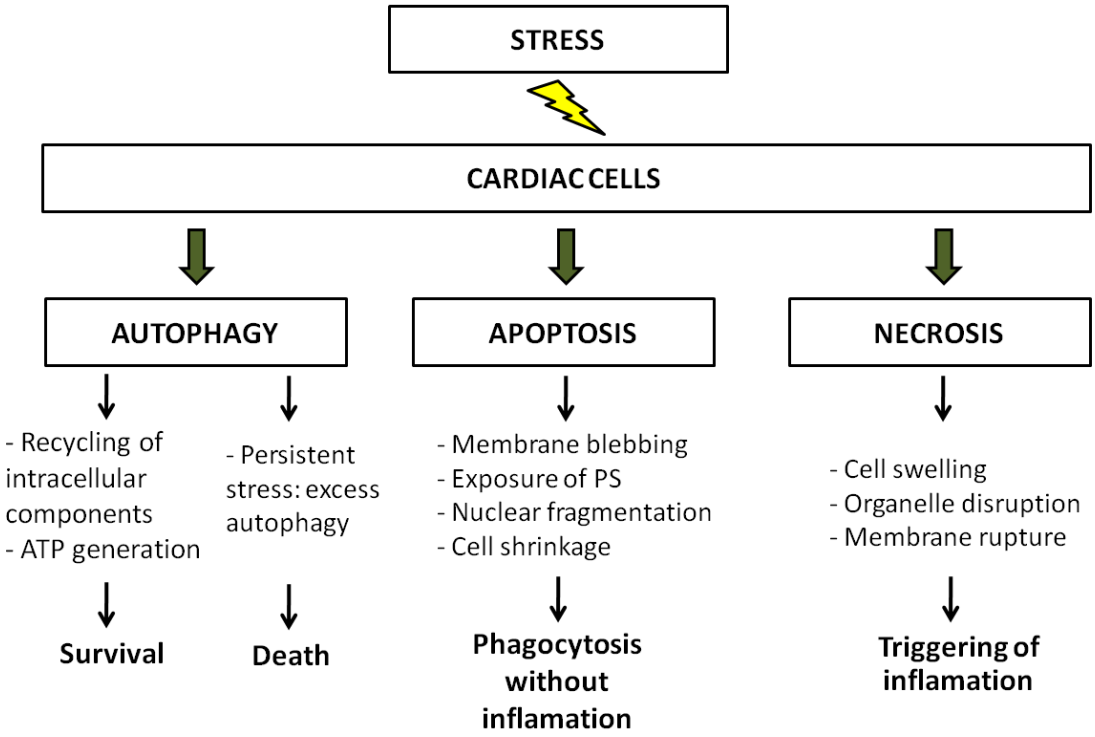

Figure 3: Stress-induced activation of cardiac cell death involves autophagy, apoptosis and necrosis with their characteristic changes. PS, phosphatidylserine.

Apoptosis: There is increasing evidence linking oxidative stress and Dox-induced cardiomyocyte apoptosis (Singal et al. 1997; Nakamura et al. 2005; Kumar et al. 2002; Lou et al. 2005). Dox-induced cardiomyocyte apoptosis has been studied in both acute and chronic cardiotoxicity (Arola et al. 2000; Kotamraju et al. 2000; Kumar et al. 2001; Bennink et al. 2004; Kawamura et al. 2004; Fisher et al. 2005; Lou et al. 2005). Apoptosis is a highly regulated and energy-dependent active form of cell death (Kerr 2002). Physiologically, apoptosis is crucial for normal development and cell homeostasis (Du Pasquier et al. 2006). Despite the homeostatic role during development, it plays a detrimental role in disease conditions. The characteristic factors underlying apoptosis are membrane blebbing

without the loss of membrane integrity, cell shrinkage, DNA fragmentation, chromatin condensation and formation of apoptotic bodies that allow their removal by phagocytosis without inflammatory process (Walker et al. 1988). In addition, caspase activity is another exclusive hallmark of apoptosis (Denault and Salvesen 2008). Apoptosis is considered to be a process that can start from two canonical signaling pathways, responding to external and internal stimuli (Regula and Kirshenbaum 2005).

In the extrinsic pathway, external death ligands, such as Fas ligand, tumor necrosis factor (TNF)-α, TNF-related apoptosis-inducing ligand with their receptors induce recruitment and activation of cytosolic adaptor molecules which trigger the death-inducing signaling complex (Robaye et al. 1991; Chinnaiyan et al. 1995; Pan et al. 1997; Sheridan et al. 1997). Initiator caspases (caspase 8) are activated, with subsequent activation of downstream effector caspases such as caspase 3 (Salvesen and Dixit 1997; Denault and Salvesen 2008). Compared with the extrinsic pathway, the intrinsic pathway can be activated by a variety of stimuli including ultraviolet radiation, oxidative stress, drug treatment, hypoxic conditions and genetic damage. This pathway is mediated by the mitochondria that undergo specific changes to release pro-apoptotic factors (Petronilli et al. 2001). Mitochondrial disturbances involve opening of the mitochondrial permeability transition pore contributing to the initial stages of apoptotic cell death (Marchetti et al. 1996). When mitochondria release intermembrane proteins such as Cytochrome C to the cytoplasm, there is formation of the apoptosome responsible for activating downstream caspases (Susin et al. 1999). This process is regulated by the members of the B-cell lymphoma-2 (Bcl-2) family (Kirshenbaum and de

Moissac 1997; Gustafsson and Gottlieb 2007), which includes the anti-apoptotic and pro-survival members Bcl-2, Bcl-xL, Bcl-w, and the pro-apoptotic members Bax, Bak, Bad, Bid, Nix, Bim, Puma and BNip3 that enhance apoptosis via inhibition of anti-apoptotic Bcl-2 proteins or activation of pro-apoptotic proteins resulting in mitochondrial damage (Van Delft and Huang 2006). The ultimate balance between pro- and anti-apoptotic proteins within a cell is considered to play a role in determining the cell fate to activate or restrain apoptosis. Both the extrinsic and intrinsic apoptotic pathways converge on the activation of the downstream executioner caspases 3, 6, and 7 (Denault and Salvesen 2008).

Necrosis: Activation of necrotic cell death is morphologically distinct from apoptosis and autophagy. Necrosis is presented by organelle and cytoplasmic swelling, in particular the mitochondria and early rupture of the plasma membrane (Majno and Joris 1995). This membrane rupture releases cellular contents at injury site, inducing inflammation, being triggered by severe damage and energy depletion (Vanlangenakker et al. 2008). Recent studies have shown that necrotic cell death can also be well controlled and programmed (Galluzzi and Kroemer 2008; Dorn 2009; Papatriantafyllou 2012). Experimental studies showed that cardiac expression of proinflammatory cytokine, inflammatory cell infiltration, and necrosis are increased in Dox-treated mouse hearts (Li et al. 2006; Ikegami et al. 2007; Riad et al. 2009). Oxidative stress has also been associated with the necrotic cardiomyocyte death. The use of free radical scavengers was shown to protect cardiomyocytes from anthracycline-induced necrosis (Ikegami et al. 2007). It is speculated that increased ROS leads to mitochondrial calcium overloading, causing mitochondrial swelling and ATP depletion, triggering necrotic cell death

(Dorn 2009; Gustafsson and Gottlieb 2008). Dox-induced mitochondrial DNA damage, mitochondrial respiration mutilation, mitochondrial dysfunction, and ATP depletion have all been shown to contribute to necrosis (Lebrecht and Walker 2007; Solem et al. 1996; Wallace 2003; Wallace 2007; Zhou et al. 2001). In addition, ROS-induced lipid peroxidation may also contribute to cardiomyocyte necrosis (Casey et al. 2007).

Autophagy: This is a highly conserved catabolic process in which unwanted or damaged cellular components are degraded by lysosomal enzymatic action (Yorimitsu and Klionsky 2005). Cytosolic proteins and organelle degradation are engulfed into double-membraned vesicles called autophagosomes, which then fuse with lysosomes and subsequently degrade the unwanted material to maintain cellular homeostasis (Terman and Brunk 2005). Autophagy plays an important role in organelle biogenesis and turnover, controlling the precise balance between protein synthesis and degradation. However, excessive autophagy may also lead to cell death (Rubinsztein et al. 2005; Tsujimoto and Shimizu 2005; Yorimitsu and Klionsky 2005; Schmid and Munz 2007).

Autophagy is regulated by autophagy-related genes (Atg) (Azad et al. 2008) that are regulators of the four stages of autophagy: induction, elongation and autophagosome formation, docking/fusion and lysosomal degradation (Abeliovich et al. 2000). The initial stage involves the recruitment of microtubule light chain-3 (LC3). Proteolytic cleavage of LC3 produces LC3-I which is converted to the membrane-associated form, LC3-II forming the autophagosome (Juhasz and Neufeld 2008). LC3 II is currently used as an indicator of the number of autophagosomes (Itakura and Mizushima 2010). The contents of the fused

autophagosome-lysosome thereby provide the cell with small molecules that can be utilized for ATP production or recycling reactions (Luzio et al. 2007).

Basal autophagy normally occurs in the myocardium and it represents a prevalent renewal mechanism of cellular components, though it is substantially potentiated under pathological conditions, including cardiac hypertrophy, cardiomyopathy, and heart failure (Aviv et al. 2011; Hill 2011). Studies have indicated that autophagy serves as a double-edged sword in the heart under stress; on one hand, it functions by removing protein aggregates and damaged organelles as a pro-survival pathway maintaining energy homeostasis, while on the other, excessive autophagy can lead to apoptosis (Terman and Brunk 2005; Matsui et al. 2008; Rothermel and Hill 2008; De Meyer and Martinet 2008; Gustafsson and Gottlieb 2009). In Dox-induced cardiotoxicity, some studies have demonstrated that Dox injures the mitochondria, resulting in energy depletion, which induces autophagy associated with apoptosis in cardiomyocytes leading to heart failure (Lu et al. 2009; Kobayashi et al. 2010).

4.1 Cell death pathways crosstalk

Although apoptosis, necrosis and autophagy have independent pathways, there is growing evidence that crosstalk among these pathways also takes place (Zhang and Ney 2009; Gustafsson 2011). In this regard, mitochondria have been considered a point of convergence and the Bcl-2 family has been implicated in the crosstalk between apoptosis and autophagy (Tsujimoto and Shimizu 2005; Hoyer-Hansen et al. 2007; Ghavami et al 2010). It has also been demonstrated that ROS-

induced increase in intracellular calcium not only triggers apoptosis and necrosis, but also induces autophagy by activation of calmodulin-dependent kinase kinase and AMP-activated protein kinase (Hoyer-Hansen et al. 2007). Other apoptosis-related proteins such as p53 have also been shown to play a role in autophagy (Crighton et al. 2007).

Furthermore, different types of cell death may be involved in Dox-induced cardiomyopathy. The activation of such forms of cell death may result from the activation of various pathways that include oxidative stress, mitochondrial damage, DNA damage, and induction of pro-apoptotic proteins and cell membrane injury.

5. Cardiac stem cells - New insight for late onset cardiomyopathy

From the three distinct types of Dox-induced cardiotoxicity described earlier: acute, early-onset, and late-onset, the delayed cardiotoxicity presents a particularly challenging puzzle in terms of defining the molecular mechanisms underlying this important clinical problem. Although high ROS production, and consequent oxidative stress, can probably explain several aspects of Dox-induced cardiotoxicity, it is not clear how it can be related to late occurrence of cardiomyopathy (Olson and Mushlin 1990; Minotti et al. 1999). The frequency of cardiotoxic effects has been reported to be as high as 57% among survivors of childhood cancer (Paulides et al. 2006), and a survey by the Pediatric Cardiomyopathy Registry shows that 15% of all adult patients with cardiomyopathy were previously treated for cancer during childhood or adolescence (Giantris et al.

1998). This cardiotoxicity is characterized by a latent period during which cardiac function appears normal and the patient is asymptomatic until the onset of the cardiac dysfunction severe enough to warrant heart transplantation. This kind of heart failure has been shown to be precipitated by events such as exercise, pregnancy, and acute viral infection (Ali et al. 1994; Pai and Nahata 2000).

One of the explanations for the late onset of cardiac dysfunction is based on studies showing that exposure to Dox impairs normal cardiac growth leaving the heart with chronically increased hemodynamic stress (Lipshultz et al. 1991). The loss of cardiomyocytes documented in children exposed to Dox (Lipshultz et al. 2005) might also contribute to this reduction in ventricular mass and increase in cardiac stress. Indeed, cardiomyocyte loss was already shown in clinical and experimental studies using treatments with anthracyclines (Singal et al. 1987; Sawyer et al. 1999; Lipshultz et al. 2004; Lim et al. 2004). Recent studies have demonstrated for the first time that anthracyclines markedly diminish the number of cardiac progenitor cells (De Angelis et al. 2010; Huang et al. 2010). A study from Huang et al. (2010) concluded that the decreased number and accelerated senescence of cardiac progenitor cells in young “juvenile” mice is part of the cause for the Dox-induced heart dysfunction after a subsequent stress in the adult stage.

The idea that the heart has some regenerative capacity and perhaps capacity for cardiomyocyte division is in fact very recent and still in debate (Bergmann et al. 2009). Several current studies have recognized a subset source of cells within the adult heart with cardiogenic potential (Oh et al. 2003; Beltrami et al. 2003; Martin et al. 2004; Laugwitz et al. 2005; Rosenblatt-Velin et al. 2005; Moretti et al. 2006; Cai et al. 2008). The regenerative capacity of the mammalian

heart appears to be not sufficient to cope with the loss of cardiomyocytes seen after myocardial infarction or Dox-induced cardiomyopathy (Sturzu and Wu 2011).

These recent studies have identified a pool of primitive cells residing in the myocardium that upon stimulation become committed to form cells of cardiac lineage such as cardiomyocytes, smooth muscle cells, and endothelial cells (Oh et al. 2003; Beltrami et al. 2003; Urbanek et al. 2005; Linke et al. 2005). These primitive cell populations have been identified by the expression of markers expressed by hematopoietic stem cells, in particular c-kit (stem cell factor receptor) and the Sca-1 (stem cell antigen-1) as well as early cardiac markers, such as Nkx2.5, GATA4, Mef2c, and Islet-1 (Oh et al. 2003; Beltrami et al. 2003; Martin et al. 2004; Laugwitz et al. 2005; Rosenblatt-Velin et al. 2005; Moretti et al. 2006; Cai et al. 2008). They have been shown to represent a small fraction of the total cellular content of the heart (0.005% to 2%). However, the origin and relationship of these different cardiac stem cell populations remain to be determined (Anversa et al. 2007).

Although recent findings raised the possibility that Dox-induced cardiotoxicity may also be a “stem cell disease” (De Angelis et al. 2010, Huang et al. 2010), many aspects of Dox-induced damage in these cells warrant further investigation. For example, as has been discussed earlier, oxidative stress has been suggested to play a role in Dox-induced cardiomyocyte injury which appears to be mitigated by certain antioxidants. However, such detailed analysis of Dox-induced changes in cardiac or circulating stem cells is still missing. We used Sca-1 positive cells isolated from heart and bone marrow for our studies. Sca-1 is a glycosyl

phosphatidylinositol-anchored cell surface protein that has been widely used as a marker to isolate hematopoietic stem cells (Yutoku et al. 1974).

6. Strategies to reduce cardiotoxicity

6.1 Early detection: Usage of Dox, even with the greatest care, carries a risk of developing cardiac dysfunction. Early detection of left ventricular systolic dysfunction is one strategy for addressing the cardiac safety profile of Dox, potentially avoiding the detrimental effects of heart failure. Standard clinical practice for monitoring Dox-induced cardiotoxicity includes noninvasive assessment of baseline left ventricular systolic function before the start of Dox therapy, regular monitoring during treatment and follow-up after the therapy has been completed (Singal and Iliskovic 1998).

The list of diagnostic procedures commonly used for serial monitoring of Dox-induced cardiomyopathy include: physical examination, electrocardiography, serial echocardiography, scintigraphy, multigated radionuclide angiography (MUGA) using different radiolabeled antibodies including 123I-MIBG, 123I-BMIPP, 111In-antimyosin antibody, positron emission tomography, endomyocardial biopsy, measurement of humoral factors, ANP, BNP, endothelin, and troponin T (Takemura and Fujiwara 2007). Hence, patients treated with Dox may need a constant follow-up of cardiac parameters, at the discretion of the physician, before late cardiomyopathy has already manifested.

6.2 Heart failure management: Poor responsiveness to inotropic agents and cardiac assist devices has been reported (Lefrak et al. 1973; Singal et al. 1992). Nevertheless, with the advent and wide use of new standard medical therapy for heart failure, the treatment for Dox-mediated cardiomypathy has improved survival rates (Pfeiffer 1992; Pitt et al. 1999; Hjalmarson et al. 2000).

The response of Dox-induced cardiomyopathy to the current standard medical therapy, especially in patients treated with beta-blockers as well as rennin-angiotensin system inhibitors has improved (Tallaj et al. 2005). Institution of angiotensin converting enzyme (ACE) inhibition in patients with all stages of heart failure results in a 20-25% relative risk reduction in all causes of mortality (Al-Mallah et al. 2006). Additionally, institution of beta-blockers in patients with heart failure results in a 30-35% relative risk reduction in all causes of mortality. In the treatment of congestive heart failure, including anthracycline-induced cardiomyopathy, ACE and beta-blockers are proven Class I medications as per the American College of Cardiology/American Heart Association and Canadian Cardiovascular Society guidelines i.e. there is evidence for and/or general agreement that these treatments are beneficial, useful, and effective. (Malcom et al. 2008; Hunt 2009).

In addition to the strategies reported above, there could be three major strategies to protect the heart from Dox-induced cardiotoxicity: 1) finding a better analog in order to eliminate this toxic effect; 2) minimizing the total amount of drug administered retaining its antitumor efficacy; and/or 3) finding adjuvant therapies with the ability to prevent the cardiomyopathy.

6.3 Finding a better analogue: Structural modifications of anthracyclines have resulted in approximately 2000 analogues of the two original drugs doxorubicin and daunorubicin. Only a few of them have reached clinical approval including epirubicin and idarubicin (Weiss 1992). Although none of them has shown stronger antitumor efficacy than the original drugs, some studies have suggested modest decrease in cardiotoxicity (Phase III Italian Multicentre Breast Study 1988; A prospective randomized French Epirubicin Study 1988). However, a recent meta-analysis by van Dalen et al. (2010) found no significant difference in the frequency of clinical heart failure between patients treated with either epirubicin or Dox. For this reason, Dox still remains the major chemotherapeutic drug in use.

6.4 Decrease drug concentration: The most used strategy to minimize the total cumulative dose of Dox is the combined administration of other antitumor drugs in the form of "cocktails" selected for different types of tumors. Other chemotherapeutic agents that are commonly used synergistically with Dox include: vincristine, procarbazine, cisplatin, and cyclophosphamide (Cass et al. 1996; Zancanella et al. 2006). This alternative, however, could lower its efficacy as an antitumor drug as the optimal dose may not be achieved.

Dox in combination with more targeted therapy has also been used in clinical practice. In this manner, studies combining Dox with a taxane were evaluated in the setting of metastatic breast cancer. Taxanes, including paclitaxel and docetaxel, are microtubular inhibitors that induce apoptosis in breast cancer and inhibit tumor angiogenesis. Adding a taxane to anthracycline-based adjuvant chemotherapy prolongs survival in node-positive patients (Bonneterre et al. 1999),

and taxanes are already approved in this indication. Unfortunetelly, congestive heart failure has also been reported with this combination (Gianni et al. 1995). Thus, in combination with a taxane, an upper limit of 360 mg/m^2 of Dox defines a safer range for the application of the active two-drug combination (Biganzoli et al. 2003). Ongoing clinical trials are being conducted to determine optimal dose and clarify drug mechanisms and cardiac protection (Piedbois et al. 2007).

Combination of Dox and trastuzumab is also being used to treat a particular subpopulation of breast cancer patients. Trastuzumab is a humanized monoclonal antibody against the extracellular domain of human epidermal growth factor receptor 2 protein (HER2) which belongs to a family of four transmembrane receptor tyrosine kinases that mediate the growth, differentiation, and survival of cells (Gschwind et al. 2004). Overexpression of HER2 protein, amplification of the HER2 gene, or both may account for approximately 25% to 30% of breast cancer cases and is associated with a poorer prognosis (Slamon et al. 1987). It is also associated with aggressive behavior in the tumor (Slamon et al. 1989). For this reason, trastuzumab is indicated for cancer patients with HER2 overexpression and has a successful therapeutic index, with supporting evidence for improvement of the efficacy of available therapies, such as Dox, in all stages of breast cancer (Marty et al. 2005). Cardiotoxicity (principally congestive heart failure) still remains a concern when trastuzumab is used either in the adjuvant or metastatic setting following the administration of anthracycline (Seidman et al. 2002). The cardiotoxic mechanism of trastuzumab alone is not known but has been shown to be different from that of Dox and may be secondary to a sequential stress mechanism (Zeglinski et al. 2011). Considering the established central role of anthracyclines in

anticancer treatment, the enhanced cardiotoxic concern of combination therapy with trastuzumab should be taken into account in the design and conduct of more studies involving this adjuvant therapy.

6.5 Controlling plasma peak concentration: The majority of anticancer drugs are administered as intravenous infusions leading to an initial peak followed by a decay of drug concentrations. Observational studies have implied that continuous infusion of conventional anthracyclines is less cardiotoxic than bolus infusion due to lowering the peak serum exposure of cardiac cells (Storm et al. 1989; Ishisaka et al. 2006). It is generally believed that long-term exposure to drugs at modest concentrations would be safer than a pulsed supply of the drug at higher concentrations. In a large study, however, continuous anthracycline infusion compared with a bolus infusion showed no cardioprotective benefit with similar rates of subclinical cardiotoxicity in both treatment groups (Cortes-Funes and Coronado 2007).

A more promising approach to limit peak concentrations is to employ a drug-delivery system such as liposomes and nanoparticles in order to target preferentially the tumor. Hence increasing the efficacy and lowering the side effects of Dox.

Liposome-encapsulated anthracyclines are new drug carrier systems that can modify Dox's biodistribution, with two formulations approved for clinical use by the US Food and Drug Administration - Doxil or Caelyx and Myocet. Encapsulation in lipid-based (liposome) formulations improves the half-life of Dox in the system and the drug may selectively accumulate in tumor tissue that contains more

fenestrations in the capillary structure (Abraham et al. 2005). Approval was based, in part, on data suggesting that the decreased Dox-induced cardiotoxicity occurred in the absence of decreased therapeutic activity (Safra et al. 2000; Gabizon et al. 2004). Although the two liposomal formulations of Dox have been shown to considerably reduce cardiotoxicity (Johnston and Gore 2001; Batist et al. 2001), it does not eliminate it. The easier access to conventional Dox continues to make this option more feasible in cocktails used in the management of most solid tumors.

Nanotechnology is also part of new drug-delivery systems. Nanoparticles made of biodegradable polymers, for example, are particles with sizes in the range of 20–200 nm that can bind hydrophilic or hydrophobic drugs leading to prolonged time in the circulation (Farokhzad et al. 2006). These particles are being designed to link with tumor targeting ligands, such as peptides and small molecules, being able to target tumor antigens as well as tumor vasculature with high affinity and specificity (Xiao et al. 2012). Currently many substances are under investigation for this type of drug delivery for cancer therapy. Experimental studies have shown promise in improving oral bioavailability and reducing cardiotoxicity with the use of Dox nanoparticles (Italia et al. 2006; Kalaria et al. 2009). Many aspects such as tissue distribution and toxicity of these particles remain to be investigated before advancing to clinical setting.

Taken together, these findings raise questions about whether targeting tumor delivery of a given anthracycline could be more productive than changing its structure and mode of action in cancer or cardiac cells.

6.6 Stem cell therapy: The only report using stem cell therapy in Dox-induced cardiomyopathy was a pre-clinical study done by De Angelis et al (2010). These authors demonstrated that infusion of cardiac progenitor cells after anthracycline exposure provides some degree of cardiac rescue. More studies in this field are needed to determine if stem cell therapy could enhance regenerative capacity of the young heart and guarantee better recovery from the anthracycline exposure.

6.7 Use of cardioprotective drugs: Adjuvant cancer therapy with dexrazoxane is the only agent currently recommended by the American Society of Clinical Oncology to prevent cardiotoxicity in specific adult cancer treatment protocols (Wouters et al. 2005). It is a valuable option for the prevention of cardiotoxicity in drug-induced cardiomyopathy. Dexrazoxane has been clinically shown to reduce cardiac damage, decreasing the risk of Dox-induced cardiotoxicity compared with no treatment; and does not increase the relative risk of chemotherapy failure or decrease survival (Hensley et al. 2009). Dexrazoxane is a cyclic derivative of ethylenediamine tetraacetic acid (EDTA), which provides cardiac protection from anthracyclines primarily through its hydrolytic products which have the ability to chelate free and bound intracellular iron, including iron that is bound in anthracycline complexes, thereby reducing the formation of ROS (Cvetkovic and Scott 2005). Current guidelines support the use of dexrazoxane to protect against the cardiotoxicity associated with conventional dose of Dox in patients with advanced but anthracycline-sensitive cancer (Hensley et al. 2009). Since dexrazoxane may potentiate hematological toxicity induced by chemotherapy or

radiation (van Dalen et al. 2008), there is some concern that dexrazoxane contributes to more adverse effects and potentially limits the tumor response. Therefore, for each individual patient, clinicians should balance the cardioprotective effect of dexrazoxane against the possible risk of adverse known side effects of worsening thrombocytopenia and granulocytopenia.

6.8 Antioxidant therapy: Antioxidants are recognized as beneficial therapeutic strategies when oxidative damage contributes significantly to the disease pathology (Siveski-Iliskovic et al. 1995; Halliwell 2001; Liu et al. 2003). In this regard, there is now sufficient evidence linking free radical generation, oxidative stress, subcellular changes and apoptosis to the development of Dox-induced cardiomyopathy and failure.

Protective strategies, therefore, have focused on administering drugs or natural compounds that improve the antioxidant defenses of cardiomyocytes against anthracycline-induced oxidative stress. Significant body of evidence from experimental studies has shown cardioprotective antioxidant therapy in different models of heart failure (Dhalla and Singal 1994; Khaper and Singal 2001) including Dox-induced cardiomyopathy (Siveski-Iliskovic et al. 1994). Conversely, some clinical studies did not find significant positive results with antioxidant supplementation with respect to a reduction in cardiovascular diseases (Losonczy et al. 1996; Kushi et al. 1996). These studies have raised skepticism for the use of antioxidants in heart disease. Nevertheless, experts in the oxidative stress field have shown that the reason for failure is likely to be related to the fact that these studies were performed in well-nourished populations and/or it could be simply due

to the failure of these compounds to effectively decrease oxidative damage in the subjects tested (Halliwell 2009).

In Dox-induced cardiomyopathy, promising cardioprotective effects were reported with the use of probucol, a lipid lowering drug with strong antioxidant properties (Siveski-Iliskovic et al. 1994; Iliskovic and Singal 1997). The combination did not compromise the antitumor benefits of the drug (Siveski-Iliskovic et al. 1995). Many different natural products with antioxidant capacity were also shown to reduce acute or chronic cardiotoxicity in animal models of Dox-induced heart failure (Quiles et al. 2002).

Detailed reviews (Injac and Strukelj 2008; Granados-Principal et al. 2010) have gathered data about Dox exposure with combined treatments using different cardioprotective compounds such as natural products, different drugs and approaches including exercise and diet as cardioprotective strategies. It was concluded that there is plenty of reliable information regarding cardiac protection by enhacing antioxidant capacity. The most commonly used and investigated compounds are vitamins, coenzyme Q, flavonoids, polyphenols, herbal antioxidants, selenium and virgin olive oil (Quiles et al. 2002; Granados-Principal et al. 2010). Such adjunctive and protective therapies, though promising, remain to be tested in clinical trials.

In order to lay out the molecular mechanisms of non-enzymatic antioxidant therapy, this thesis examined the potential role of well known antioxidant vitamin C (Vit C). In general, Vit C is often used as a gold standard antioxidant to compare the protective activity of other compounds against the toxic effects of Dox (Chularojmontri et al. 2005; Kim et al. 2006; Choi et al. 2007).

7. Vitamin C

7.1 Brief History

The isolation and identification of Vit C was reported in 1933 (Svirbely and Szent-Gyorgyi 1933). Szent-Györgyi was awarded the 1937 Nobel Prize in Medicine for his accomplishments in this field. Nonetheless, the link between a dietary deficiency leading to scurvy and a “vital” compound has been known for many centuries (McBride 1991). This disease particularly affected seamen during lengthy voyages in the age of sail. Its prevention was discovered soonafter to be simple by eating citrus fruit and vegetables (Sauberlich 1997). In the same report that confirmed Vit C identification, a series of experiments conducted confirmed its antiscorbutic activity, and since then scurvy was ascribed to Vit C deficiency (Svirbely and Szent-Gyorgyi 1933). Several individuals and organizations were also important in the history of Vit C, especially Nobel Prize winner Linus Pauling, with great scientific contributions by advocating the use of mega doses of this vitamin for disease prevention, in particular cancer (Pauling 1970).

7.2 Vit C synthesis, structure and functions

Vit C is present in high quantities (typically 10-100 mg/100 g) in various foods, particularly of plant origin. The high Vit C content in plants is attributed to the fact that it is formed from sugars, through a sequence of four enzyme-driven steps, which convert glucose to Vit C (Padayatty et al. 2003). For this reason, L-Ascorbate is considered to be a carbohydrate-like substance with weak acid properties, $C_6H_8O_6$, structurally related to glucose that naturally occurs attached either to a hydrogen ion, forming ascorbic acid, or to a metal ion, forming a mineral ascorbate (Hancock et al 2000). Vit C, or ascorbate, can also react with ROS by donating hydrogen. ROS take electrons from ascorbate, which is oxidized first to monodehydroascorbate and then to dehydroascorbate (DHA) (Figure 4).

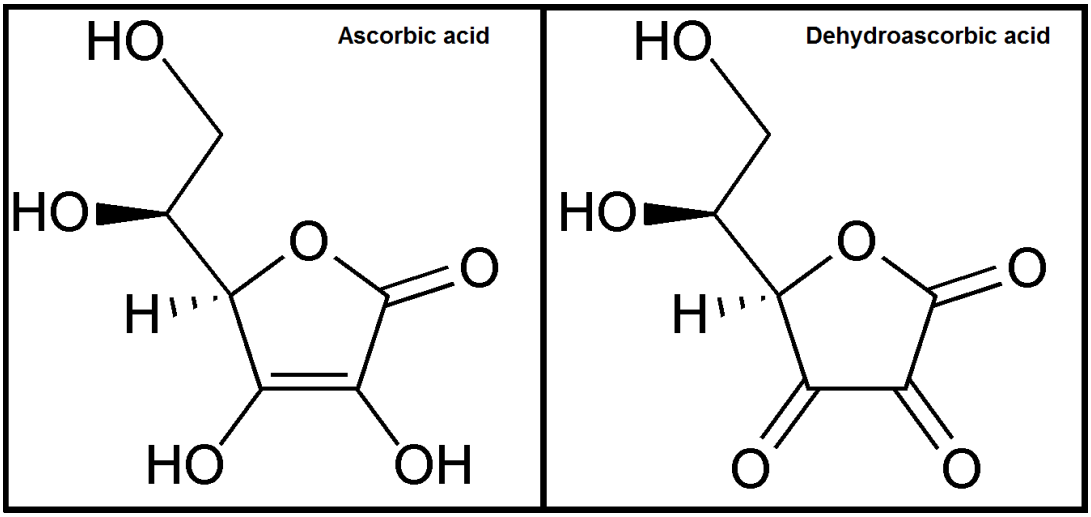

Figure 4: Chemical structure of reduced (Ascorbic acid) and oxidized (Dehydroascorbic acid) forms of Vitamin C.

Vit C is considered an essential vitamin for some of the vertebrate species due to the loss in its synthesis e.g. bats, guinea pigs, capybaras, some species of

birds and fish, tarsiers, monkeys, apes, and human beings (Drouin et al. 2011). Animals that are not able to produce Vit C, lack the active enzyme L-Gulonolactone oxidase which is a microsomal enzyme that catalyzes aerobically the conversion of L-gulonolactone to L-ascorbate with the production of H_2O_2 (Chatterjee et al. 1960).

It is suggested that uric acid has taken over some of the functions of ascorbate as a compensatory mechanism for the loss of Vit C synthesis in animals that lack its production. In this view, primates have lost the ability to break down uric acid parallelly with the loss of the ability to produce ascorbate, and both are considered strong reducing agents (Proctor 1970). In humans, Vit C is an essential dietary component. It functions as a central hydrophilic antioxidant to maintain lower levels of ROS as well as participates in enzymatic reactions as a cofactor by donating an electron for important enzymes (Linster and van Schaftingem 2007).

As an antioxidant, Vit C is considered to be one of the most potent and least toxic antioxidants for humans (Sauberlich 1994). It has the ability to scavenge reactive oxygen, nitrogen, and chlorine species, thereby effectively protecting cellular substrates from oxidative damage. Vit C is also known to regenerate vitamin E (Vit E) from its reduced form (Hediger 2002). Vit E in turn serves as an antioxidant in the lipid phase due its solubility in lipids (Gutteridge and Halliwell 2000). As a reducing agent, Vit C is important in a variety of biochemical reactions in which it maintains metal ions, such as Fe^{2+}, in their reduced forms. Examples of other enzymatic reactions that require Vit C include those involved in collagen synthesis, carnitine synthesis, catecholamine synthesis, peptide amidation and tyrosine metabolism (Hediger 2002). Although Vit C is also able to increase the

pro-oxidant chemistry of copper and iron (Podmore et al 1998), it mostly acts as an antioxidant rather than as a pro-oxidant (Halliwell and Gutteridge 1990; Mühlhöfer et al 2004).

7.3 Plasma levels and transport

The Recommended Dietary Allowance for Vit C is 90 and 75 mg/day for males and females, respectively. This recommendation continues to be based primarily on the prevention of deficiency diseases such as scurvy, rather than promotion of optimum health or the prevention of chronic diseases. Complete plasma saturation occurs at oral doses of ≥400 mg daily, achieving physiological blood concentrations of 60–160 μM and any excess of the vitamin is excreted by the kidney (Padayatty et al 2004). However, pharmacologic concentrations up to 20 mM in blood were shown to be achieved with intravenous and intraperitoneal administration (Padayatty et al 2004; Verrax and Calderon, 2009). In adults, there is significant evidence that Vit C is safe at the oral dose of 1 g/day (Meadows et al 1991). It has already been reported that sustained high physiological levels of Vit C start to decrease in about 2 hours after high dietary intake (Gonçalves et al 2005). This suggests that frequent oral intake of physiological doses may be one alternative to maintain higher plasma physiological levels of Vit C. Vit C is widely distributed in tissues and can be found in reduced (ascorbate) or oxidized forms (DHA) forms (Sauberlich 1994).

DHA is structurally similar to glucose, and it has been proposed that entry of DHA is mediated by glucose transporters (Vera et al 1994; Vera et al. 1995).

However, such an entry into cells can be competitively inhibited by sugars (Rose 1988, Dhariwal et al. 1991). Thus, an active transport via sodium-dependent Vit C co-transporters (SVCTs) appears to be the predominant system for Vit C transport in the body.

Since SVCTs have been identified (Tsukaguchi et al, 1999), two subtypes of SVCT (SVCT-1 and SVCT-2) have been cloned (Daruwala et al 1999; Rajan et al, 1999). SVCT-1 and SVCT-2 mediate the uptake of L-ascorbate (Tsukaguchi et al, 1999). The expression of SVCT-1 is reported to be mainly in the intestinal epithelium and in the liver, whereas SVCT-2 is ubiquitously expressed in various organs, including the myocardium (Goldenberg and Schweinzer 1994). Considering the fact that Vit C levels change in different tissues, increased Vit C transport could explain enhanced resistance to oxidative stress in a particular tissue. Although such studies are lacking in the isolated cardiomyocyte, accumulation of Vit C and increased expression of SVCT-2 in heart failure have been documented as a protective mechanism against increased oxidative stress (Guaiquil et al. 2004; Rohrbach et al. 2008). These findings highlight the importance of the understanding of Vit C transport under other cardiac conditions such as Dox-induced cardiomyopathy.

8. Dox and Vit C – Clinical and Animal Studies

Experimental studies have shown that Vit C does not interfere with the antitumor activity of Dox and it significantly prolongs the life span of mice, rats and guinea pigs treated with Dox (Fujita et al. 1982, Shimpo et al. 1991, Wold et al.

2005; Santos et al. 2007). Considering these beneficial effects, cancer patients who generally have low concentrations of ascorbic acid in plasma (Bodansky et al. 1952; Gonçalves et al. 2005) are more vulnerable to oxidative stress-mediated injury and could benefit from supplementation. Additional dietary source or supplement should be considered to maintain high physiological plasma levels of this vitamin in the body (Yoshihara et al. 2010).

8.1 Clinical studies: There is still a great debate whether antioxidants compromise anticancer ability of drugs. In the analysis of 280 peer-reviewed articles including randomized clinical trials where various antioxidants were used along with chemotherapy, it was consistently reported that the antioxidant supplementation does not interfere with therapeutic modalities for cancer (Simone et al. 2007). Thus, some evidence has been provided that antioxidants in the clinical practice do not interfere with toxic effects of chemotherapy on cancer cells.

Furthermore, when breast cancer patients received a combined adjuvant therapy with Vit C and E along with the chemotherapeutic cocktail containing Dox, the beneficial effects analyzed in patient's lymphocytes showed preservation of overall antioxidant status, reduced lipid peroxidation and DNA damage (Suhail et al. 2012). Information is lacking on randomized clinical trials and epidemiological studies designed to evaluate whether patients receiving antioxidant therapy concomitantly with chemotherapy are less prone to Dox-induced cardiotoxicity.

8.2 Animal studies: Experimental studies suggest that Vit C alone or in combination with Vit E may delay general toxicity of Dox and prevent the cardiotoxicity. Vit C was also shown to prolong the life of tumor-bearing animals,

alleviating cardiac lipid peroxidation and decreasing the earliest alterations seen after Dox-treatment in cardiomyocytes (Shimpo et al. 1991; Viswanatha-Swamy et al. 2011). Improvements in cell structure included a reduction in the dilation of sarcoplasmic reticulum and transverse tubular system and the appearance of a large number of cytoplasmic fat droplets (Shimpo et al. 1991). In addition, vitamin-supplemented groups presented the ability to decrease toxic effects of Dox on the elevation of the plasma levels of alanine aminotransferase, lactate dehydrogenase, urea, and creatinine (Santos el al. 2007; Swamy et al. 2011). Furthermore, an increase in cardiac antioxidant enzymes glutathione peroxidase, catalase, and superoxide dismutase was also seen (Swamy et al. 2011).

The administration of antioxidant Vit C in H9c2 cardiomyoblasts or neonatal cardiomyocytes exposed to Dox, was able to counteract ROS production coupled with the blunting of Dox-induced injury and preserving cell viability. The cardioprotective effects of Vit C have been primarily associated with a decrease in oxidative stress and oxidative stress-induced changes (Yamanaka et al. 2003; Wold et al. 2005; Chularojmontri et al. 2005; Kim et al. 2006; Choi et al. 2007). Another aspect of the protective effect of Vit C is the intramitochondrial accumulation of the vitamin, which inhibits oxidative damage of mitochondrial DNA (KC et al. 2005). Vit C may also mitigate peroxynitrite-induced oxidation reactions, which occur partially by an inhibition of the interaction between nitric oxide and superoxide, and partially by repairing the peroxynitrite-induced damage (Jackson et al. 1998). Although treatment of neonatal rat cardiomyocytes or cardiomyocyte-like cells with Dox and Vit C may potentially provide a good *in vitro* heart cell model for probing the mechanism of clinical cardiotoxicity,there is very little information on

the beneficial effects of Vit C on adult cardiomyocytes, a more relevant cell model to investigate Dox-induced cardiotoxicity. Furthermore data is also lacking on this matter regarding the study ofcardiac or bone marrow stem cells exposed to Dox.

III. Rationale, hypothesis and objectives.

Rationale: Based on the literature reviewed here, it is apparent that oxidative stress is one of the major causes of Dox-induced cardiotoxicity. Vit C which is the least toxic antioxidant available and an essential dietary component for humans is found to be low in cancer patients. It is likely that this population could benefit from Vit C supplementation as a potential vital antioxidant that would effectively reduce Dox-induced oxidative stress and subsequent cardiac damage. However, there is little information with regard to the subcellular basis of any benefitial effect of Vit C. Furthermore, delayed cardiomyopathy seen in some of the Dox-treated patients may in fact be due to Dox-induced injury to cardiac and/or circulating stem cells. Therefore, we sought to study the role of oxidative stress in Dox-induced cardiotoxicity using adult cardiomyocytes as well as Sca-1 positive cells derived from heart and bone marrow.

Hypothesis: Vit C will reduce Dox-induced cell death in cardiomyocytes and Sca-1 positive cells by mitigating oxidative stress.

Approach: We used *ex vivo* studies designed to understand Dox-induced cardiotoxicity at the cellular level using isolated adult cardiomyocytes, a relevant cell model to this drug-induced disease as well as Sca-1 positive cells isolated from heart or bone marrow due to their possible association with cardiac progenitor cells. The cells were exposed to Dox with and without Vit C. For some studies N-Acetyl Cysteine was also used as a positive antioxidant control. Different endpoints were analyzed: cell viability by using trypan blue and MTT assays; cell leakage by measuring creatine kinase and adenylate kinase released in the culture media;

ATP levels by using vialight assay; oxidative stress assessed by using DCFDA fluorescence, lipid peroxidation assay; overall antioxidant status and antioxidant enzymes protein expression; Vit C Transporters (sodium-dependent Vit C transporter-2 and glucose transporter 4) by using western blot and immunofluorescence; stress-induced signaling pathways including p38, ERK 1/2, JNK, and p53 by using western blot and specific pharmacologic inhibitors; autophagy by using western blot for LC3 protein expression; apoptosis by using Hoescht staining, pro and anti-apoptotic proteins including Bax, Bcl-xL, Caspase 3 and PARP and early apoptosis by annexin-propidium iodide assay.

The following **objectives** have been addressed:

1) **Establish the dose (Dox, 0.01-20 µM; Vit C, 5-100 µM) and time (1 to 48 hours) of exposure for different treatments.**
2) **At the established optimal dose and time of exposure to Dox (10 µM for 24 hours), with and without Vit C (25 µM) in cardiomyocytes:**
    a. **Investigate the effect of Dox on oxidative stress parameters and the antioxidant effects of Vit C;**
    b. **Examine the effect of Dox on cell injury and viability with and without Vit C;**
    c. **Examine the effects of Dox on some of the key stress-induced signaling proteins: p38, ERK 1/2, JNK, and p53 as well as the influence of Vit C on the Dox-induced changes;**
    d. **Investigate the effect of Dox on apoptosis and autophagy with and without Vit C;**

e. **Investigate the effects of Dox on Vit C transporters as well as Vit C effects on the Dox-induced changes at these transporters.**

3) **Investigate the effects of different concentrations of Dox with and without Vit C on the Sca-1 positive cells derived from heart and bone marrow and compare with that on adult cardiomyocytes.**

IV. Materials and Methods

The study conforms to the Guidelines for the Care and Use of Laboratory Animals approved by the University of Manitoba Animal Care Committee following the guidelines established by the Canadian Council on Animal Care.

1. Cardiomyocyte and Sca-1 positive cell isolations

Cardiomyocytes were isolated from normal adult male Sprague–Dawley rats (250–300 g body weight). Hearts were excised and mounted on a modified Langendorff perfusion apparatus. The perfusate (Ca^{2+} free buffer) contained 110 mM NaCl, 2.6 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25mM $NaHCO_3$, and 11 mM Glucose (pH 7.4). The Ca^{2+} free perfusion was then switched to recirculating mode with the buffer containing 25 µM calcium, 0.1% w/v collagenase, and 0.25% w/v bovine serum albumin, for 30 min. The collagenase-digested ventricles were chopped into small pieces and gently passed through pipettes with progressively smaller tip diameters and with an increasing concentration of $CaCl_2$. The suspension was filtered through a nylon mesh (200 µm) and was allowed to settle for 10 min. The supernatant was discarded and the cell pellet was resuspended in medium 199 (M199) containing $CaCl_2$ (1.8 mM). Cardiomyocytes (10^6 per 60mm dish) were plated in laminin-coated (20 µg/ml) polystyrene tissue culture dishes. Plated cells were incubated in serum-free culture medium M199 supplemented with antibiotics (100 U/ml penicillin + 100 µg/ml streptomycin) at 37℃ under a 5% CO_2, 95% O_2 air atmosphere. Two hours after plating, the culture medium was

changed to remove unattached dead cells, and the viable cardiomyocytes were incubated overnight.

Cardiac Sca-1 positive cells: Sca-1 positive cells were isolated from the supernatant of the collagenase-digested ventricles. Sca-1 positive cells were separated from the cardiomyocytes by centrifugation using a Magnetic Cell Sorting System (Miltenyi Biotech), following the manufacturer's protocol, with slight modification. Small cells obtained from the supernatant, containing most of the Sca-1 positive population were resuspended in the separation buffer, which consisted of 0.5% BSA in phosphate-buffered saline (PBS) and centrifuged at 300×g for 10 minutes at 4–8 ℃. 10 µL of anti-sca-1-FITC (Miltenyi Biotec) was added per 10^7 total cells, incubated for 10 minutes at 2−8 ℃ and washed with buffer. Cell pellet was then resuspended in buffer containing 20 µL of anti-FITC microbeads (Miltenyi Biotec) per 10^7 total cells and incubated for 15 minutes at 2–8 ℃. Cells were washed and resuspended up to 10^7 cells in 500 µL of buffer. Separation was performed utilizing a MiniMACS Column (Miltenyi Biotec) and sorted populations of cells were subsequently analyzed by fluorescence-activated cell sorting (FACS) to determine the purity of the Sca-1 positive cells. Gated cells up to 10^4 were counted on a BD FACSCalibur and the data were analyzed using CellQuest Pro software (BD Biosciences) and quantified using Origin 8 software.

Bone marrow Sca-1 positive cells: Femur as well as tibia from both legs from normal adult male Sprague–Dawley rats (250–300 g) were excised under sterile conditions. All the attached connective tissue was removed from the bones. Bone marrow was extracted by flushing the bone cavity with sterile PBS. Cells were

disaggregated by gentle pipetting and passed through 30 µm nylon mesh to remove cell debri. Cells were washed and centrifuged at 300×g for 10 minutes at 4–8 ℃. Sca-1 positive cell separation as well as their purity was determined following the same procedure described above.

2. Cell treatments

After the initial 24 hour incubation that allowed cells to adjust to the culture conditions, cardiomyocytes and Sca-1 positive cells (95% viability) were exposed to different concentrations of Dox (Adriamycin®, Pfizer) (0.01-20 µM) alone or in combination with different concentrations of Vit C (5-100 µM) (Sigma) to establish an optimal concentration of Dox as well as Vit C for further studies. For studies on the role of different cell signaling proteins, cardiomyocytes were incubated with 25 µM of the following inhibitors (Sigma): p38/JNK inhibitor (SB203580); ERK1/2 inhibitor (PD98059); and p53 inhibitor (pifitrin-alfa). In addition, in some studies, cardiomyocytes were incubated with N-Acetyl Cysteine (NAC) (Fisher Scientific) (50 µM), a water soluble antioxidant used as a positive antioxidant control for Vit C. Table 1 and 2 describe different treatments used in cardiomyocytes and Sca-1 positive cells, respectively.

Table 1: Treatment groups for Cardiomyocytes

Group *	Medium 199 supplemented with*:
Control	Cells in the culture medium only
Dox	Doxorubicin, 0.01, 0.1, 1.0, 10 and 20 μM
Vit C	Vitamin C, 5,10, 25, 50, 100 μM
Vit C + Dox	Vit C, 25 μM + Doxorubicin, 10 μM
p38/JNK inhibitor	25 μM SB203580
p38/JNK inhibitor + Dox	SB203580, 25 μM + Doxorubicin, 10 μM
ERK inhibitor	25 μM PD98059
ERK inhibitor + Dox	PD98059, 25 μM + Doxorubicin, 10 μM
p53 inhibitor	25 μM pifithrin-alfa
p53 inhibitor + Dox	pifithrin-alfa, 25 μM + Doxorubicin, 10 μM
NAC	N-Acetyl Cysteine 10, 25, 50, 100 and 150 μM
NAC + Dox	N-Acetyl Cysteine, 50μM + Doxorubicin, 10 μM

∗ In each group, antioxidants and inhibitors were added in the culture media 1 hour prior to addition of Dox for up to 24 hours.

Table 2: Treatment groups for Sca-1 positive cells

Group	Medium 199 supplemented with:
Control	Cells in the culture medium only
Dox	Doxorubicin, 0.01, 0.1, 1, 10 and 20 μM
Vit C	Vitamin C, 25 μM
Vit C 25 μM + Dox	Vit C, 25 μM + Doxorubicin, 10 μM
Vit C 50 μM + Dox	Vit C, 50 μM + Doxorubicin, 10 μM

∗ In each group, Vit C was added 1 hour prior to addition of Dox for 24 hours in the culture media.

3. Assessment of cell viability, ATP production and cell leakage

Viability of the cultured cardiomyocytes was determined by trypan blue staining and MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole) assay. **Trypan blue:** When cell membranes are irreversibly damaged, trypan blue, which is an anionic dye, is taken up by dead cells and binds to nuclei which appear blue in color. After different treatments, cardiomyocytes were stained with trypan blue (Sigma), 0.04% (w/v) for 5 minutes and cells were counted to quantify the percentage of rod shaped viable cells that did not stain blue.

MTT assay (Sigma): This assay was also performed to confirm viability of cardiomyocytes and Sca-1 positive cells. The yellow MTT is reduced to purple formazan in the mitochondria of living cells, thereby providing a measure of viability of cells. Cells were cultured in 96-well microplates with the same starting cell number per well (10^4 cells/well). After different treatments, 5 mg/ml MTT was added to the media in each well and further incubated at 37 ℃ for 2 h. All the remaining supernatant was removed carefully and 150 µl of dimethyl sulfoxide was added to each well and mixed thoroughly to dissolve the formed crystal formazan. After 10 min of incubation to ensure all crystal formazan were dissolved, the cytotoxicity of Dox was detected by measuring the absorbance of each well at 570 nm using an ELISA plate reader. Mean absorbance of all groups was quantified.

Adenosine triphosphate (ATP) was measured in cardiomyocytes and Sca-1 positive cells using ViaLight BioAssay kit (Lonza). Cardiomyocytes or Sca-1 positive cells (10^3 cells/well each) were cultured in a 96-well white walled laminin

coated tissue culture plates, and were allowed to settle overnight under normal culture conditions. After different treatments, ATP Monitoring Reagent Plus (100 µl) was added to each well and incubated for an additional 2 minutes. The plates were read on a Molecular Devices Lmax II microplate luminometer programmed to take a 1 second integrated reading of each well. Mean luminescence value (RLU) of all the treated groups was quantified.

Cellular leakage was evaluated by measurement of the release of creatine kinase (CK) or adenylate kinase (AK) in the culture medium following different treatments. **CK release** is an indication of damage to muscle and has been associated with injury, such as myocardial infarction, myocarditis, and other cardiac damage. The measurement in the culture media was performed using a spectrophotometric enzyme activity with a UV-Rate assay kit (Stanbio Laboratory, Boerne, TX). CK specifically catalyzes the conversion of creatine to phosphocreatine, consuming adenosine triphosphate (ATP) and generating adenosine diphosphate (ADP). Through a series of coupled enzymatic reactions, NADPH is produced and measured at 340 nm, being proportionate to the CK activity in the sample. After different treatments, 20 µl of cell supernatant was transferred to clear-bottom 96-well plate and mixed with 100 µL of water and 100 µL of enzyme reagent reconstituted in PBS buffer. This kinetic reaction was read at 10 min and again at 40 min and the mean values were calculated for each sample using a pre-determined equation. **AK release** in the culture media was performed using Toxilight Non-destructive Cytotoxicity BioAssay Kit (Lonza). AK is a robust protein that is released into the cell culture media when cells die. AK actively

phosphorylates ADP to form ATP, which is then measured on a luminometer using the bioluminescent firefly luciferase reaction. As the level of cytolysis increases, the amount of AK in the supernatant increases, resulting in the emission of a higher light intensity. Cardiomyocytes or Sca-1 positive cells (10^3 cells/well each) were seeded into a 96-well laminin coated tissue culture plate, and allowed to settle overnight under normal culture conditions. After different treatments, 20 µl of cell supernatant was transferred to 96-well white walled plate. AK detection reagent (100 µl) was added to each well and incubated for an additional 5 minutes. The plate was read on a Molecular Devices Lmax II microplate luminometer programmed to take a 1 second integrated reading of each well. Mean luminescence value (RLU) of all the treated groups was quantified.

4. Protein isolation and quantification

Culture dishes (60mm) with treated and control cardiomyocytes were removed from the incubator and washed in PBS for the protein extraction and quantification, and these culture dishes were then placed on ice. For cell lysis, RIPA lysis buffer (pH 7.6) containing 150 mM NaCl, 1.0% nonidet P-40 (NP-40), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 50 mM Tris, phosphatase inhibitors (10 mM NaF, 1 mM Na_3VO_4, and 1 mM EGTA), and protease inhibitors (4 µM leupeptin, 1 µM pepstatin A, and 0.3 µM aprotinin) were added to the culture dishes and the material was scraped using a rubber cell scraper. The content was placed into 1.5 ml eppendorf tubes and left on a -80^{o}C freezer for 1 h. The tubes containing the samples were thawed and sonicated for 5-10 sec three times and

then centrifuged at 14000 rpm in a microcentrifuge for 15 min at 4^{o}C. After centrifugation, supernatant was transferred to new 1.5 ml eppendorf tubes and stored at -80^{o}C until protein assay was carried out.

The quantity of protein in the samples was measured using a dye-binding assay (Bio-Rad). Protein was thawed on ice after removing from -80^{o}C freezer, and protein dilutions were prepared using PBS. Protein standards (0 - 2.0 µg/µl) were prepared diluting bovine serum albumin (Sigma) in PBS. Standards and sample dilutions were loaded into wells in triplicate using 10 µl of standard/sample per well of a 96-well dish. An acidic solution (250 µL) diluted 5:1 was added to each well. The 96-well plate was incubated for 10 minutes at room temperature and the absorbance was measured at 595 nm with a microplate reader.

5. Mitochondrial and cytosolic fractions

Isolation of intact mitochondria from cultured cells was made using Mitochondria Isolation Kit for cells (Thermo Scientific). After treatments, cardiomyocytes were lysed in extraction buffer, supplied with the mitochondria isolation kit. The homogenate was centrifuged at 600 g for 5 min, and the supernatant so obtained was then centrifuged at 10,000 g for 10 min. The supernatant obtained in the later step was saved as the cytosolic fraction. The pellet containing the mitochondria was suspended in storage buffer. Both fractions were immediately frozen (-80^{o}C) until detection of Cytochrome C and Bax on both the fractions using Western blot analysis (described later in this section).

6. Western Blot

Protein lysates used for running sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) were prepared by combining aliquots of lysates with Laemmli buffer (125 mM Tris-HCl, pH 6.8, 5% glycerol, 2.5% SDS, 5% 2-mercaptoethanol, and 0.125% bromophenol blue) and boiled for 5 minutes. Equal amounts of protein (30-45 µg) were loaded to SDS-PAGE in a discontinuous system. The 5% gel was used for protein stacking phase while 12 % gel was used for the separation of isolated proteins. Proteins were separated at 120V for ~1.5 h and electrophoretically transferred at 100 V for 1.5 hour to 0.45 µM polyvinylidene difluoride (PVDF) membrane using a transfer buffer, which consisted of 20 mM Tris, 150 mM glycine, 20% methanol and 0.02% SDS. The nonspecific binding sites were blocked by overnight incubation at 4^{o}C with blocking buffer (Fisher Scientific).

Detection of PVDF membrane-bound proteins was performed using specific primary antibodies for the study of signalling pathways, receptors, antioxidant enzymes as well as autophagy and apoptotic markers described later in this section. Primary antibodies were detected using a goat anti-rabbit IgG horseradish peroxidase conjugate secondary antibody (Bio-Rad, Hercules, CA, USA). Molecular weights of the separated proteins were determined using a standard (Bio-Rad) and biotinylated (Cell Signaling Technology inc.) protein ladder molecular weight markers. The membrane-bound proteins were visualized using the BM Chemiluminiscence (POD) Western blotting system and developed on X-ray film. The bands were quantified by image analysis software (Quantity One, Bio-

Rad). Rabbit GAPDH and Cyclophilin D antibodies were used as general and mitochondrial loading controls, respectively.

Expression and activation of ERK1/2, JNK, p38, and p53 at different time points were quantified by Western blotting. The phosphorylated and total protein content were analyzed by using their respective anti-rabbit antibodies: phospho-p44/42 MAPK (Thr202/Tyr204), total 44/42 MAPK, phospho-SAPK/JNK (Thr183/Tyr185), total SAPK/JNK, phospho-p38 MAPK (Thr180/Tyr182), total p38 MAPK, phospho p53 (ser 15), and total p53 (Cell Signaling Technology inc.). Antibodies used to detect apoptotic markers, autophagy, and antioxidant enzymes are described later in this section.

7. Study of Oxidative stress

Overall analysis of oxidative stress was done by the quantification of reactive oxygen species (ROS); lipid hydroperoxides; total antioxidant capacity; and protein expression of antioxidant enzymes.

Reactive oxygen species: Direct measurement of ROS was performed by using the fluorescent dye 5-(6)-chloromethyl-dihydroflourescein diacetate probe (DCFDA) (Molecular Probes, Eugene, Oregon, USA). Cardiomyocytes or Sca-1 posotive cells from different treatment groups cultured on laminin coated coverslips were washed with PBS and incubated with 10 µM solution of fluorescent probe, DCFDA, at 37℃ for 30 min in a humidified chamber p rotected from light. After this incubation period, fluorescent images from multiple fields per dish were recorded with the Olympus BX 51 fluorescent microscope with an excitation and emission

wavelength of 488 and 525 nm, respectively. Fluorescence intensity was measured using digital image-processing software (Image Pro Plus).

Lipid hydroperoxides were analyzed by using a commercially available kit (LPO-CC assay, Kamiya Biomed Co.). Treated isolated cardiomyocytes were washed with PBS and homogenized in double distilled water. The homogenate was suspended in chloroform:methanol which was then clarified with 0.6 ml of 0.9% saline. After centrifugation at 30,000 g for 5 minutes, 1 ml of the bottom chloroform layer was completely dried under vacuum in a rotary evaporator and reconstituted in 100 µl of isopropanol. An aliquot of 45 µl (containing 30µg of protein) of this solution was used to quantify lipid hydroperoxides. The reaction produced methylene blue, which was read at 675 nm (ELISA reader) to obtain the mean absorbance values.

Total antioxidant capacity of cardiomyocytes was measured using Antioxidant Assay Kit (Cayman Chemical) and Trolox as the standard. An aliquot of 20 µl of cell lysates (containing 30µg of protein) from different treatment groups was used to assay the ability of antioxidants to inhibit the oxidation of ABTS (2,2'-azino-di-[3-ethylbenzthiazoline sulphonate]) to its oxidized form (ABTS•) in the samples. The mean absorbance from each sample was read at 405 nm (ELISA reader) and calculated as molar Trolox equivalents obtained from a standard Trolox curve.

Protein expression of **antioxidant enzymes:** GSHPx, Cu/Zn-SOD, catalase, and heme oxygenase-1 was studied by Western blotting using their specific anti-rabbit antibodies (Cell Signaling inc.).

8. Study of apoptosis

Occurrence of apoptosis in isolated cardiomyocytes was detected using a commercially available Annexin-V-FLUOS assay kit (Roche Diagnostics GmbH). After the cell treatments, isolated adult cardiomyocytes were washed with PBS. Immediately after the washing, cells were exposed to 20 µl of Annexin-V-FLUOS staining solution and 20 µl of propidium iodide (PI) in a total volume of 250 µl of PBS per dish. The cells, protected from light, were incubated in humidified chamber for 30 minutes at 37^{o}C. After the incubation, samples were washed twice with phosphate buffered saline (PBS). The cells were mounted for microscopy using a Floursave reagent (Calbiochem). After staining, the culture plates were examined using fluorescent microscope (Olympus BX 51). The rod shaped cardiomyocytes exhibiting the green fluorescence only (Annexin-V-FLUOS) with an excitation and emission wavelength of 488 and 525 nm, respectively were counted as the ones in the early apoptosis. The cells exhibiting no fluorescence at all were counted as the normal living cells. Rounded cardiomyocytes showing red nuclei (positive for PI) were counted as dead cells. The cells were counted by examining 10 random microscopic fields. Data were expressed as a count of apoptotic cells/100 cells.

Nuclear fragmentation was determined by Hoescht 33258 (Sigma) staining of nuclei. For this, cardiomyocytes from different treatment groups in culture dishes were washed three times with PBS and incubated with Hoescht 33258 (1 mg/mL) for 10 min in a humidified chamber, protected from light, at 37℃. After staining, the culture plates were examined using fluorescent microscope (Olympus BX 51) with

an excitation and emission wavelength of 346 and 460 nm, respectively. Percentage of cells containing fragmented nuclei were counted.

Pro-apoptotic (Bax, caspase-3 cleavage, PARP cleavage) as well as anti-apoptotic (Bcl-xl) proteins were studied in different treatment groups from isolated cardiomyocytes by Western blotting technique described above. The total protein content was examined using their respective anti-rabbit antibodies for: Bax, caspase-3 (8G10), PARP, Bcl-xL (Cell Signaling Technology inc). In addition, mitochondrial and cytosolic fractions were isolated as described earlier and Cytochrome C and Bax protein expressions were analyzed in both mitochondrial and cytosolic fractions using rabbit Cytochrome C and Bax antibodies (Cell Signaling Technology inc).

9. Study of autophagy

Conversion of LC3-I to LC3-II was used as a marker for autophagy. This was assessed by Western blot analysis as described earlier using anti-rabbit LC3A/B antibody (Cell Signaling Technology inc.).

10. Study of Vit C transporters

Sodium-dependent Vitamin C transporter-2 (SVCT-2) and glucose transporter 4 (GLUT4) were analyzed by immunofluorescence and Western blotting (as described earlier) using rabbit SVCT-2 and GLUT4 antibodies (Santa Cruz). In some of the experiments for GLUT 4, cardiomyocytes were also exposed to 1 µM insulin (Sigma-Aldrich) in K–H buffer (4% bovine serum albumin, pH 7.4),

for 1 h at 37°, prior to immunofluorescent study.

Cardiomyocytes from different treatment groups were grown on glass coverslips coated with laminin. Cells were washed with PBS, fixed in 4% paraformaldehyde, quenched in 100 mM glycine, permeabilized in 0.1% (v/v) Triton X-100 and blocked in 3% BSA. The cells were incubated with rabbit SVTC-2 or GLUT4 antibodies followed by incubation with FITC-goat anti-rabbit antibody. Cells were then washed in PBS and mounted for microscopy using a Floursave reagent. Images were obtained using fluorescent microscope (Olympus BX 51) with excitation and emission at the wavelength of 488 nm and 525 nm, respectively. Controls were set using untreated cells and autofluorescence was examined by tagging the treated cells with secondary antibody. Fluorescence intensity was quantified using Image Pro-Plus software (v 5.1.2).

11. Statistical analysis

All experiments were done in duplicates for each treatment group and repeated five times (n = 5). For microscopy studies i.e viability test using trypan blue and fluorescence techniques (DCFDA, Hoescht, Annexin-V-FLUOS, Vit C transporters), a total of 10 dishes (35 mm x 10 mm) containing cells grown in glass coverslips coated with laminin were prepared for each treatment group. Ten different fields per dish were counted for the quantitative analysis. Data are expressed as the mean ± S.E.M. Groups were compared by one-way analysis-of-variance (ANOVA) or two-way ANOVA when more than one variable was analyzed for different groups, and the *post-hoc* Student-Newman-Keuls test was performed to identify differences between groups. P value of ≤ 0.05 was considered significant.

V.Results

1. Dose-response studies

Dose as well as time-response studies were undertaken to establish both an optimal dose and time for Dox as well as Vit C. In some experiments, N-Acetyl Cysteine (NAC) was used as positive control for antioxidant. For dose-response, the effects of various doses of the selected treatments on cell viability, ATP levels and levels of reactive oxygen species (ROS) were studied.

For viability studies, the conversion of MTT substrate by isolated cardiomyocytes in presence of different concentrations of Dox (0.01 to 20 µM) was measured. The range of drug concentration used was carefully selected to cover subclinical, clinical and supraclinical levels. In this dose range, very low concentration of 0.01 µM is subclinical causing no cardiotoxicity whereas 0.1-1 µM are expected in patient's plasma after Dox administration being clinically relevant i.e antitumor efficacy coupled with little cardiotoxic effects. The higher doses (10 and 20 µM) of Dox are considered to be supraclinical with definite cardiotoxic effects (Greene et al. 1983; McHowat et al. 2001). Although not all statistical significance differences are presented in symbols in the figure, the exposure to different concentrations of Dox for 24h resulted in a dose-dependent significant decrease of the ability of the cells to convert the MTT substrate, shown by the decrease in absorbance (Figure 5 A) which is directly related to a reduction in cardiomyocyte viability.

ATP levels were also measured to evaluate energy level. For this, the highly sensitive ViaLight assay kit (Lonza) was used to provide accurate analysis with the advantage of the power of bioluminescent detection of ATP. ATP levels were significantly reduced in Dox group in a dose dependent manner (Figure 5 B). Unless stated otherwise, for further experiments, 10 µM of Dox was selected as the dose for inducing cardiotoxic effects which still maintained good percentage of viable cells. Moreover, 10 µM of Dox has been reported to be effective in its antitumor activity although more likely related to Dox-induced cardotoxicity (Greene et al. 1983; McHowat et al. 2001).

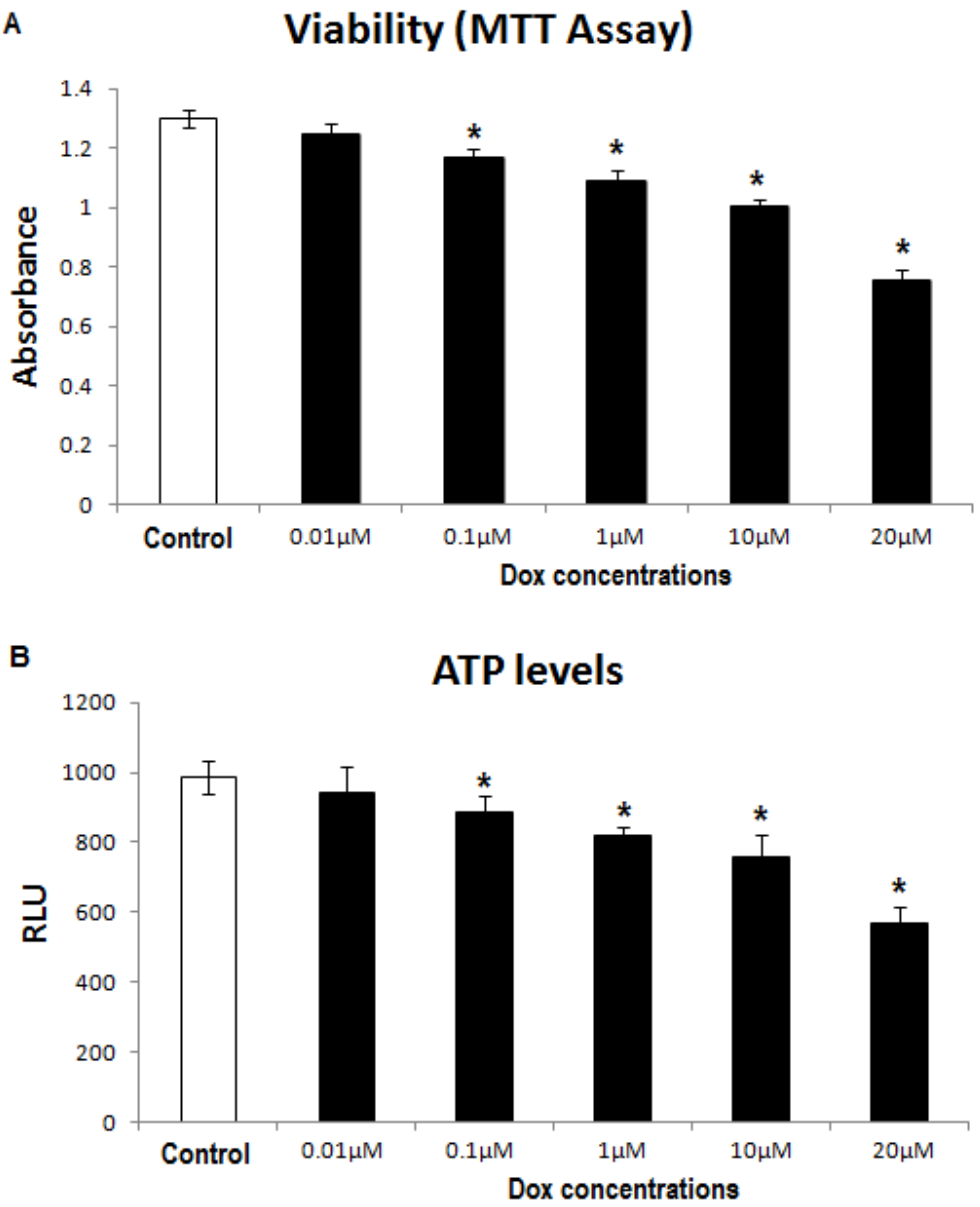

Figure 5 A and B. A) Viability of cells; and B) ATP levels in cardiomyocytes exposed to different concentrations of doxorubicin - Dox (0.01-20 µM). Mean absorbance and luminescence (RLU) values ± S.E.M. (n=5/group) as determined by MTT (viability) and Vialight (ATP level) assays. Cardiomyocytes were cultured in the presence of Dox for a total of 24 hours. *Significantly different (P ≤ 0.05) from Control.

In order to determine the most optimal concentration of Vit C and its positive antioxidant control NAC, viability of cells (using Trypan Blue) and ROS production (using DCFDA fluorescence) were measured for different physiological doses of these antioxidants. These results are shown in Figure 6 A and B and Figure 7 A and B. In these experiments, the culture media was supplemented with different concentrations of Vit C (5, 10, 25, 50 and 100 µM) or NAC (10, 25, 50, 100 and 150 µM) 1 hour prior to the addition of Dox (10 µM) for 24h. Doses of 5 and 10 µM of Vit C or 10 and 25 µM of NAC did not significantly influence the Dox-induced decrease in viability (Figure 6 A and B) or increase in ROS levels (Figure 7 A and B). The least doses that had significant beneficial effects on blunting Dox-induced decrease in viability and increase in ROS levels were seen at 25 µM for Vit C and 50 µM for NAC. Further increases to 50 and 100 µM of Vit C or 100 and 150 µM of NAC did not show any additional benefit. For this reason, 25 µM of Vit C and 50 µM of NAC were chosen as the working concentrations of Vit C and NAC for further studies.

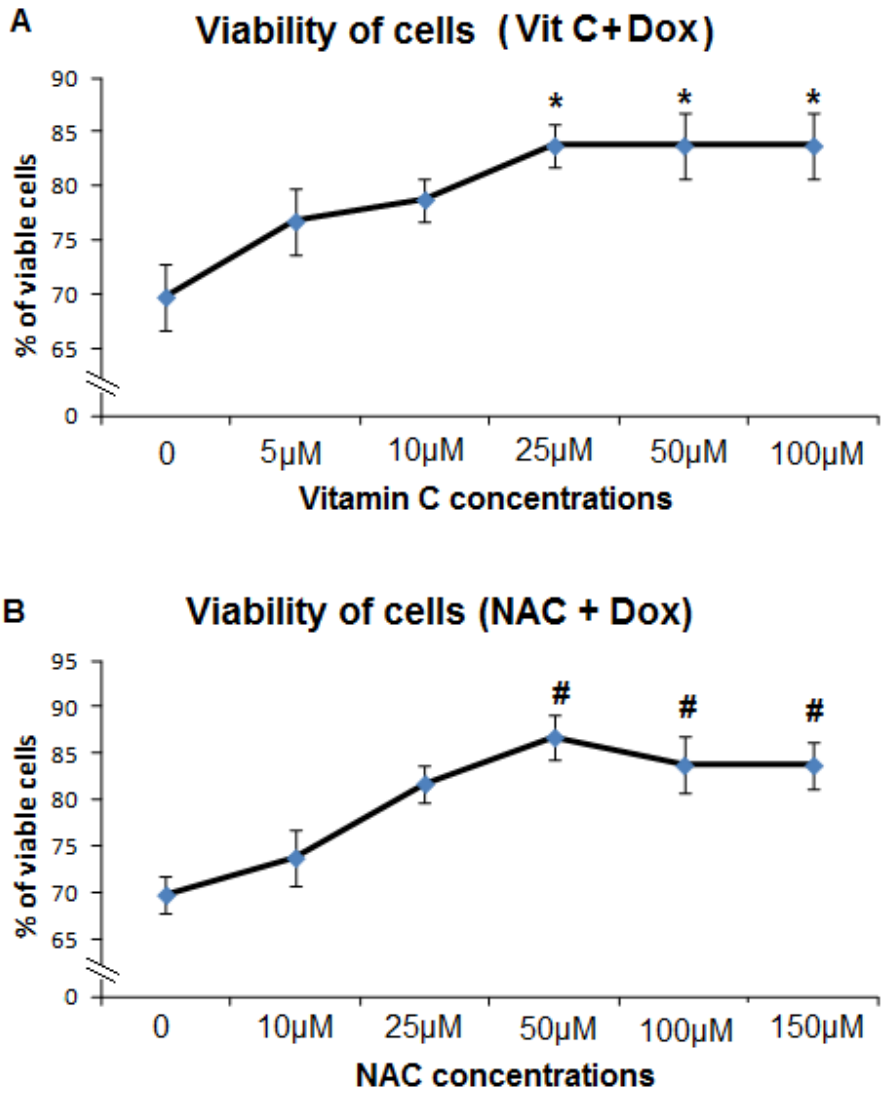

Figure 6 A and B. Effects of Vit C on Dox-induced changes in cardiomyocyte viability using trypan blue staining. N-acetylcysteine (NAC) was used as a positive antioxidant control. Cardiomyocytes were treated with different concentrations of A) Vit C (5-100 μM) or B) NAC (10-150 μM) 1 hour prior to the addition of Dox (10 μM) for 24h. Data expressed as mean ± S.E.M. of five different experiments. *Significantly different ($P \leq 0.05$) from 0, 5 and 10 μM of Vit C in the presence of Dox. #Significantly different ($P \leq 0.05$) from 0, 10 and 25 μM of NAC in the presence of Dox.

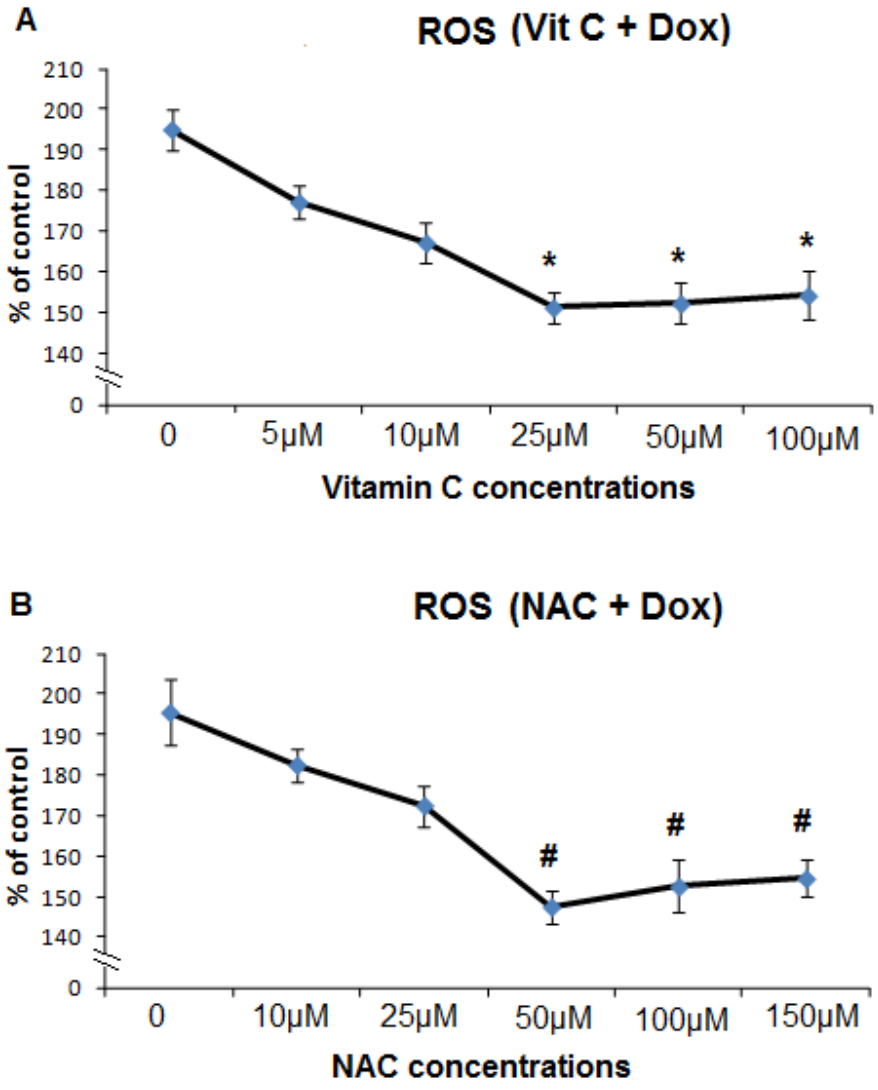

Figure 7 A and B. Effects of Vit C on Dox-induced changes in cardiomyocyte Reactive Oxygen Species (ROS) levels as analyzed by DCFDA fluorescence (A). N-acetylcysteine (NAC) was used as a positive antioxidant control (B). All treatments are the same as in Figure 2. Data expressed as mean ± S.E.M. of five different experiments. *Significantly different ($P \leq 0.05$) from 0, 5 and 10 μM of Vit C in the presence of Dox. #Significantly different ($P \leq 0.05$) from 0, 10 and 25 μM of NAC in the presence of Dox.

2. Time-course effects of Dox and Vit C in cardiomyocytes

In order to establish an optimal time of exposure, cardiomyocytes were incubated with Dox for 5 different time durations (1, 3, 6, 12, 24h) with and without pre-treatment with Vit C. In these time course studies, at all time points, cell viability (Figure 8 A and B), ROS levels (Figure 9 A and B), stress-induced pathways (Figure 10 A and B) and apoptotic markers (Figure 11 A and B) were analyzed. In pilot experiments, cardiomyocyte viability was assessed after 48h of Dox treatment (data not shown), however the number of viable cells obtained after this exposure time was below 50%. Furthermore, the reliability of data obtained in cultured adult cardiomyocytes is highly dependent on preservation of the original phenotype which can change after a prolonged culture as well as exposure time (Banyasz et al, 2007). For these reasons, the 24h mark was selected as the upper limit time point to present the time-course results.

2.1 Cell viability

In Figure 8 A and B, trypan blue was used to study cell viability through the visualization of dead cells. Dox treatment decreased viability of cardiomyocytes and this change was significant ($P \leq 0.05$) at 12 and 24h of treatment (Figure 8 A). Pre-treatment with Vit C significantly reduced the Dox-induced loss of cell viability. There was no cell loss due to Dox at 12h when Vit C was used as an adjuvant treatment, and cardiomyocyte loss was significantly blunted at 24h (Figure 8 A).

In Control and Vit C groups the majority of cardiomyocytes in medium did not stain with trypan blue (Figure 8 B), but in Dox-treated cardiomyocytes, there was a significant ($P \leq 0.05$) increase in trypan blue stained cells (Figure 8 B). At 24h, about 90% of cardiomyocytes were viable in the Control and Vit C groups. Cell viability was reduced to 73% with Dox alone. Vit C was able to significantly ($P \leq 0.05$) prevent this Dox-induced decrease in the cell viability.

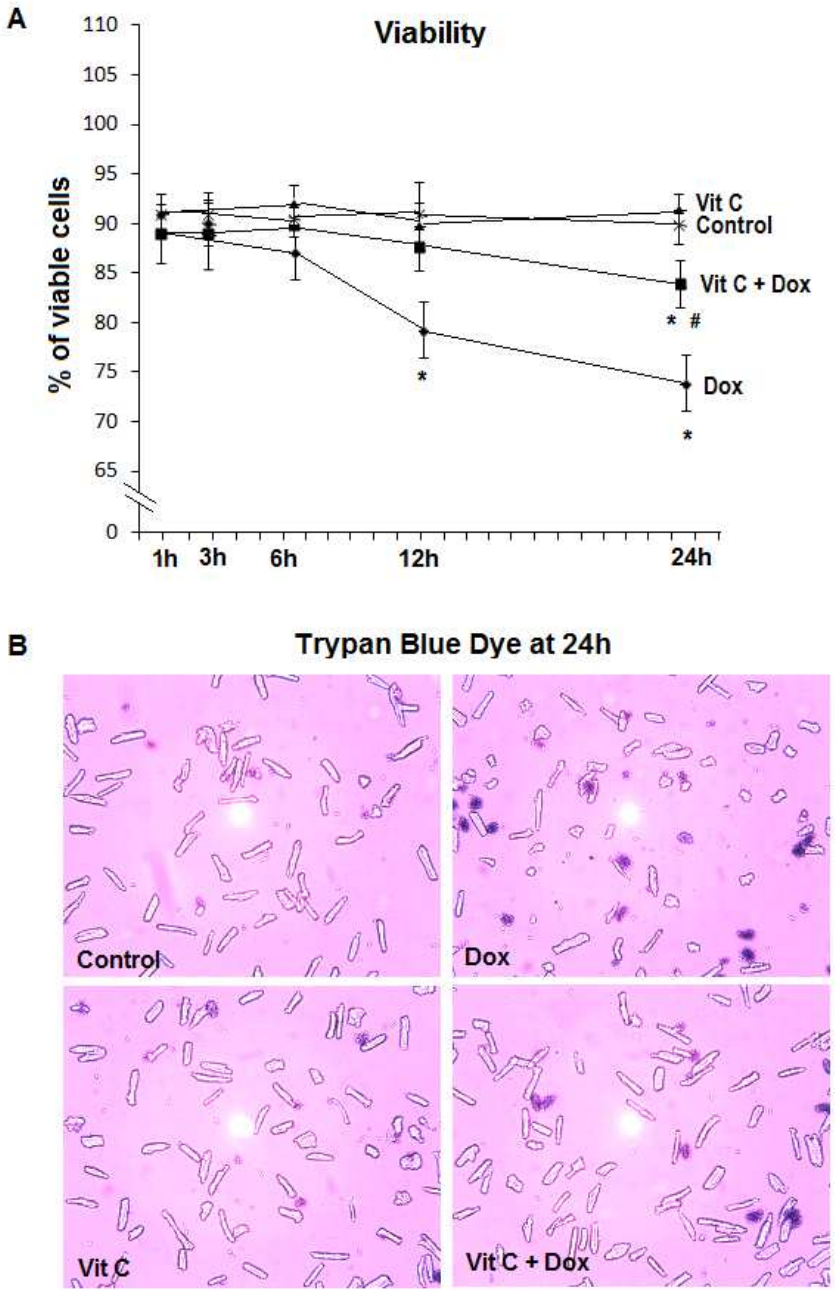

Figure 8 A and B. Dox-induced changes in cardiomyocyte viability and effects of Vit C as shown by trypan blue staining. A) Percentage of viable cardiomyocytes at different time points; B) representative images of cardiomyocytes at 24 h. Dead cells are stained in purple. Cardiomyocytes were treated with Vit C (25 µM) 1 hour prior to the addition of Dox (10 µM) for up to 24h. Data are expressed as mean ± S.E.M. of five different experiments. *Significantly different ($P \leq 0.05$) from Control and Vit C and # significantly different ($P \leq 0.05$) from the Dox group at the same time point.

2.2 Reactive oxygen species

ROS levels were determined by the intensity of green fluorescent dye (DCFDA) in adult cardiomyocytes after different treatments. Dox treatment increased ROS levels in a time-dependent manner, and these levels were significantly higher ($P \leq 0.05$) than Control as early as 3h in culture (Figure 9 A and B). Although not all statistical significance differences among groups and time points are presented in the figure, ROS production after Dox exposure progressively and significantly increased with time. Vit C was able to delay as well as blunt the sharp increase in ROS levels induced by Dox (Figure 9 A). Representative images of cardiomyocytes stained with DCFDA after 24h of different treatments are shown in Figure 9 B. The increase in green fluorescence seen in Dox group was significantly reduced by co-treatment with Vit C.

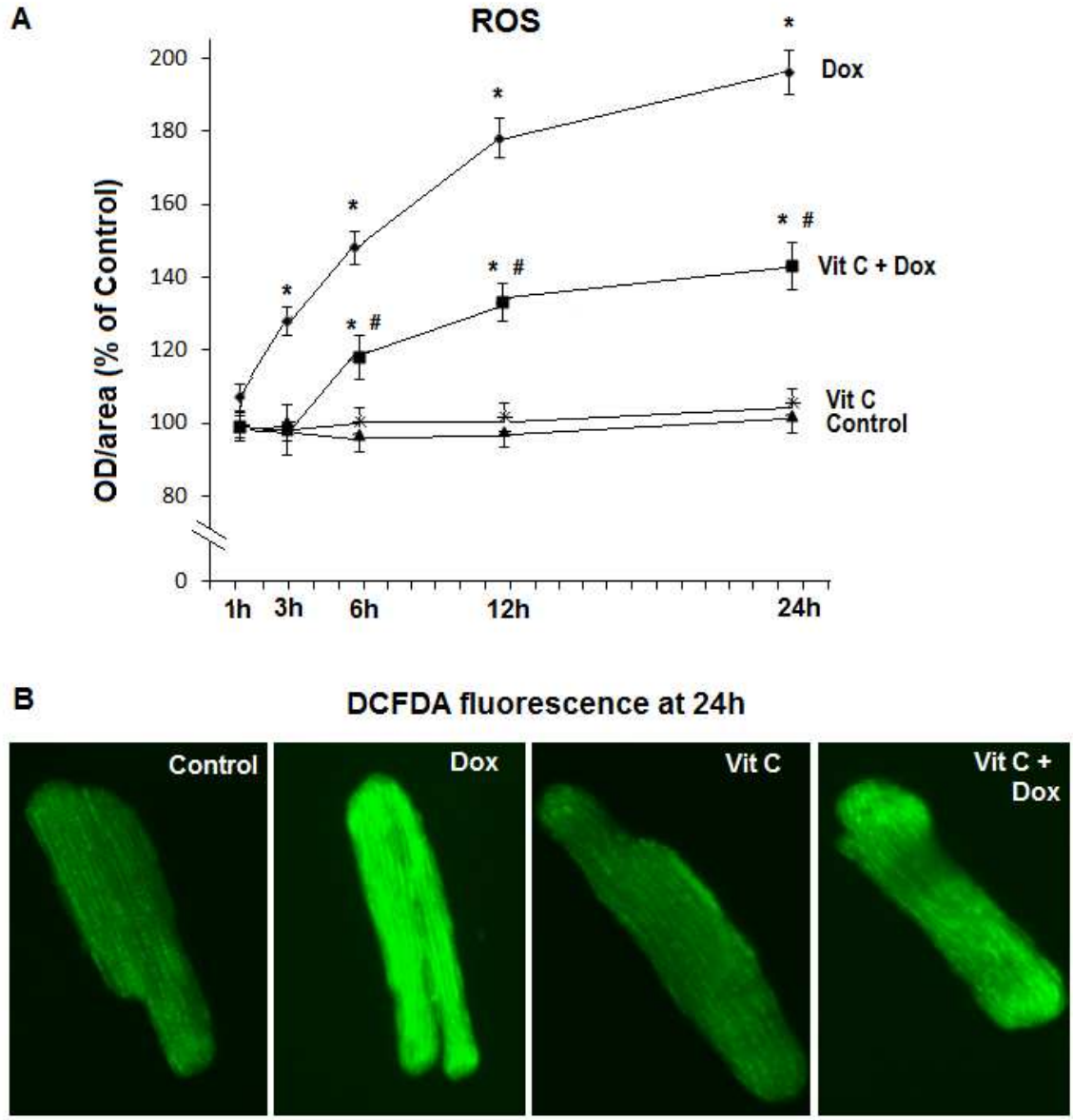

Figure 9 A and B. Levels of Dox-induced Reactive Oxygen Species (ROS) and effects of Vit C (25 µM) as shown by DCFDA fluorescence. A) Changes in fluorescence (% of Control) in cardiomyocytes at different time points; B) representative images of cardiomyocytes at 24 h. All treatments are the same as in Figure 4. Data are expressed as mean ± S.E.M. of five different experiments. *Significantly different ($P \leq 0.05$) from Control and Vit C and # significantly different ($P \leq 0.05$) from the Dox group at the same time point.

2.3 Stress-induced pathways

Time course on effects (1 to 24h) of Dox with and without Vit C were studied for up to 24h for different stress-induced proteins (ERK, p53, JNK and p38) and these data are shown in figure 10 A and B. After Dox treatment, ERK phosphorylation was found to be high at all time points, this activation reached a peak at 6h, and started to decrease after that time point for up to 24h. At all time points, this ERK activity was significantly ($P \leq 0.05$) higher than Control (Figure 10 A); JNK phosphorylation was detected within the first hour of Dox exposure and this activation was gradually increased from 6 to 24 h; p38 and p53 phosphorylations became evident within the first 3 hours, being significantly more pronounced at 6 to 24h (Figure 10 A). These Dox-induced patterns were significantly changed when cardiomyocytes were pre-treated for 1h with Vit C (Figure 10 B). ERK activation delayed in time and was significantly less elevated at 6h. Activation of the other signaling proteins p38, JNK and p53 due to Dox was significantly blunted by Vit C adjuvant treatment. Control values were set as 100% and no differences were seen in Control and Vit C groups (Figure 10 B).

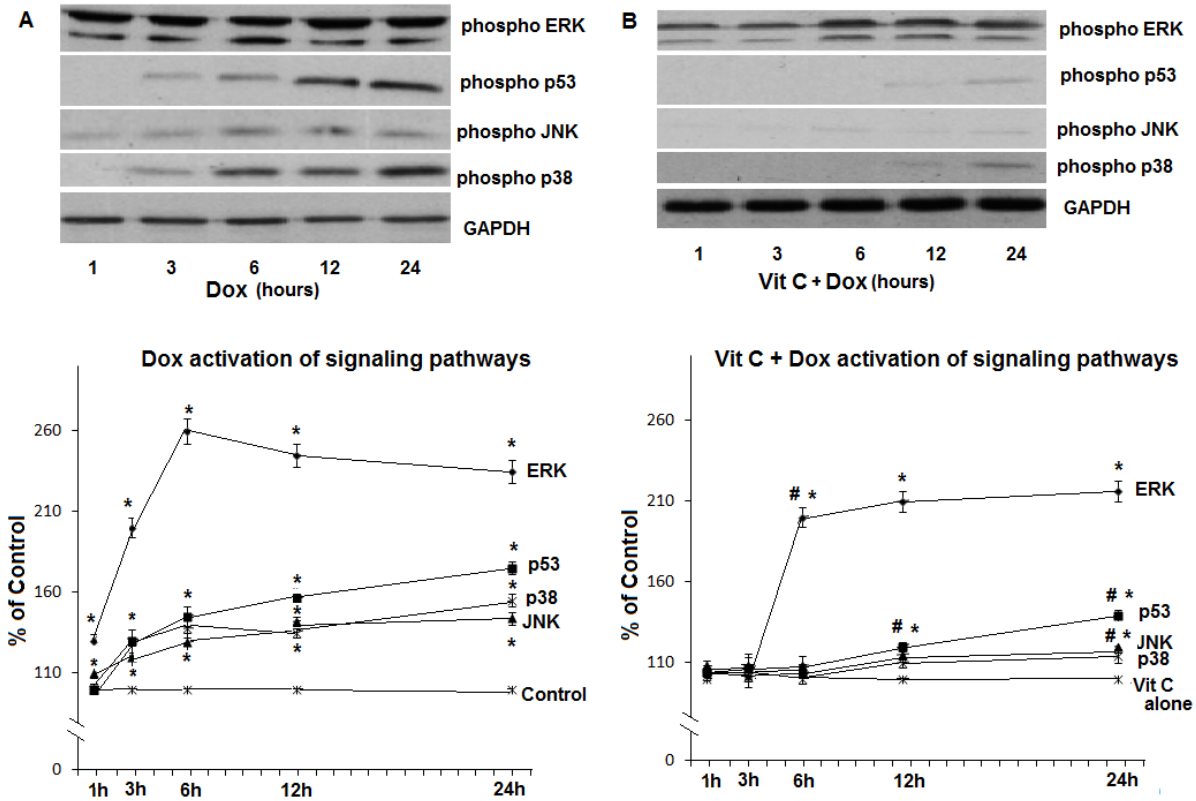

Figure 10 A and B. Time course on the effects of Vit C on Dox-induced changes in stress-induced proteins (ERK, p53, JNK and p38) in cardiomyocytes. A) Changes due to Dox alone; and B) Changes in Vit C + Dox group. Upper panels are Westen blot representations of phosphorylated proteins and lower panels are densitometric analysis of the activation of these phosphorylated proteins. Activation of the analyzed signaling pathways were compared against Control or Vit C alone, set as 100%. All treatments are the same as in Figure 4. Data are expressed as mean ± S.E.M. of five different experiments. *Significantly different ($P < 0.05$) from Control or Vit C and # significantly different ($P < 0.05$) from the Dox group at the same time point.

2.4 Apoptotic markers

The time-course effects (1 to 24h) of Dox and the influence of Vit C on apoptotic markers were studied using Western blot where the ratio of pro-apoptotic protein Bax over the anti-apoptotic protein Bcl-xL was quantified along with cleaved caspase-3, a product and indicator of caspase-3 activation and these data are shown in Figure 11 A and B. Although not all statistical significance differences are presented in symbols in the figure, study of Western Blot indicated a time-dependent rise in the pro-apoptotic protein Bax and decline in anti-apoptotic protein Bcl-xL (Figure 11 A). At 12 and 24h of Dox exposure, the increase in Bax expression and decrease in Bcl-xL expression resulting in an increase of the Bax/Bcl-xL ratio was significant ($P \leq 0.05$) as compared with the Control (Figure 11 A). Pre-treatment for 1h with Vit C significantly ($P \leq 0.05$) inhibited this Dox-induced rise in Bax/Bcl-xL ratio (Figure 11 B). Cleavage of caspase-3 due to Dox was noticed after 12 and 24h in culture (Figure 11 A). When Vit C was presented prior to Dox treatment, the effect on caspase-3 activation was completely abolished (Figure 11 B). Control values were set as 100% and no differences were seen in Control and Vit C groups.

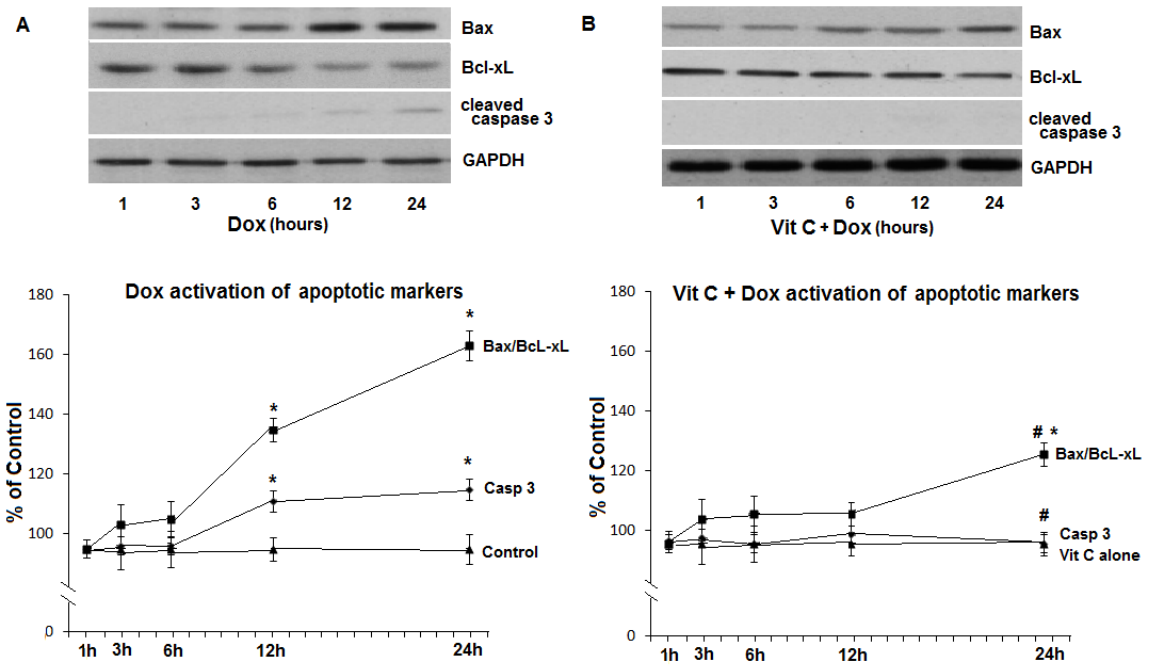

Figure 11 A and B. Time course on the effects of Vit C on Dox-induced changes in apoptotic markers (Bax/Bcl-xl ratio and Caspase 3 cleavage) in cardiomyocytes. A) Changes due to Dox alone; and B) Changes in Vit C + Dox group. Upper panels are Westen blot representations of proteins and lower panels are densitometric analysis of these proteins. Activation of the analyzed proteins were compared against Control or Vit C alone, set as 100%. All treatments are the same as in Figure 4. Data are expressed as mean ± S.E.M. of five different experiments. *Significantly different ($P < 0.05$) from Control or Vit C and # significantly different ($P < 0.05$) from the Dox group at the same time point.

3. Selection of doses of Dox and Vit C for study

Based on the dose-response and time course studies, for all the subsequent experiments, Dox was used at 10 µM concentration; and for Vit C, the concentration of 25 µM was selected. The duration for Dox exposure was 24h. For antioxidant effects, Vit C was added to the culture media 1h prior to addition of Dox.

3.1 Lipid hydroperoxides and antioxidant capacity

In Figure 12 A and B, oxidative stress was monitored in terms of lipid hydroperoxides along with the study of total antioxidant capacity in cell lysates. The production of lipid hydroperoxides was increased by about 50% in Dox-exposed cardiomyocytes which was only partially reduced ($P \leq 0.05$) in Vit C + Dox group. Lipid hydroperoxide levels were not different between Control and Vit C-treated cells (Figure 12 A).

The overall antioxidant capacity of the cells demonstrated in Figure 8 B takes into account the enzymatic antioxidant systems as well as the non-enzymatic defense present in the cell i.e. all the systems that detoxify reactive oxygen species (ROS). Dox-treatment led to 25% decrease ($P \leq 0.05$) in the total antioxidant capacity of cells (Figure 12 B). Vit C alone did show some increase but the change was not significant. Dox-induced decrease was significantly ($P \leq 0.05$) mitigated in the presence of Vit C.

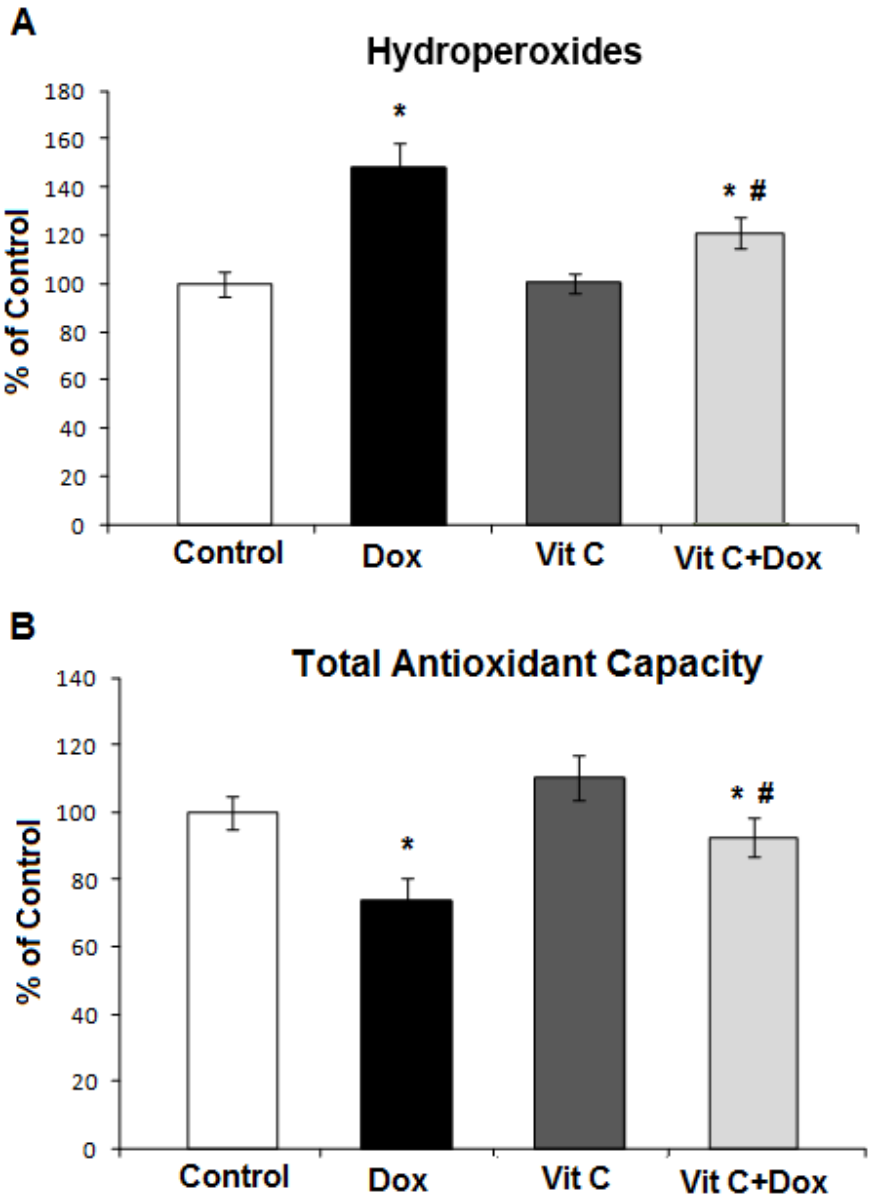

Figure 12 A and B. Effects of different treatments on the oxidative stress in cardiomyocytes. A) Hydroperoxides; and B) Total antioxidant capacity measured in cell lysates. Cardiomyocytes were treated with Vit C (25 µM) 1 hour prior to the addition of Dox (10 µM) for 24h. Data are expressed as mean ± S.E.M. of five different experiments. *Significantly different ($P < 0.05$) from Control and # significantly different ($P < 0.05$) from the Dox group.

3.2 Antioxidant enzymes

In order to further investigate the effects of Dox with and without Vit C on the endogenous antioxidant enzymes, protein expression of the major enzymes: glutathione peroxidase (GSH-Px), catalase, cytosolic Cu/Zn superoxide dismutase (SOD), and heme oxygenase were studied using Western blot analysis (Figure 13 A-D). Dox significantly decreased protein levels of GSH-Px by 15%, Cu/Zn SOD by 50%, and catalase by 40%. Vit C pre-treatment was able to completely prevent Dox-induced decrease in GSH-Px, and partially prevented the decrease seen in Cu/Zn SOD but had no effect on catalase levels. There was no difference found in heme oxygenase levels among all the studied groups.

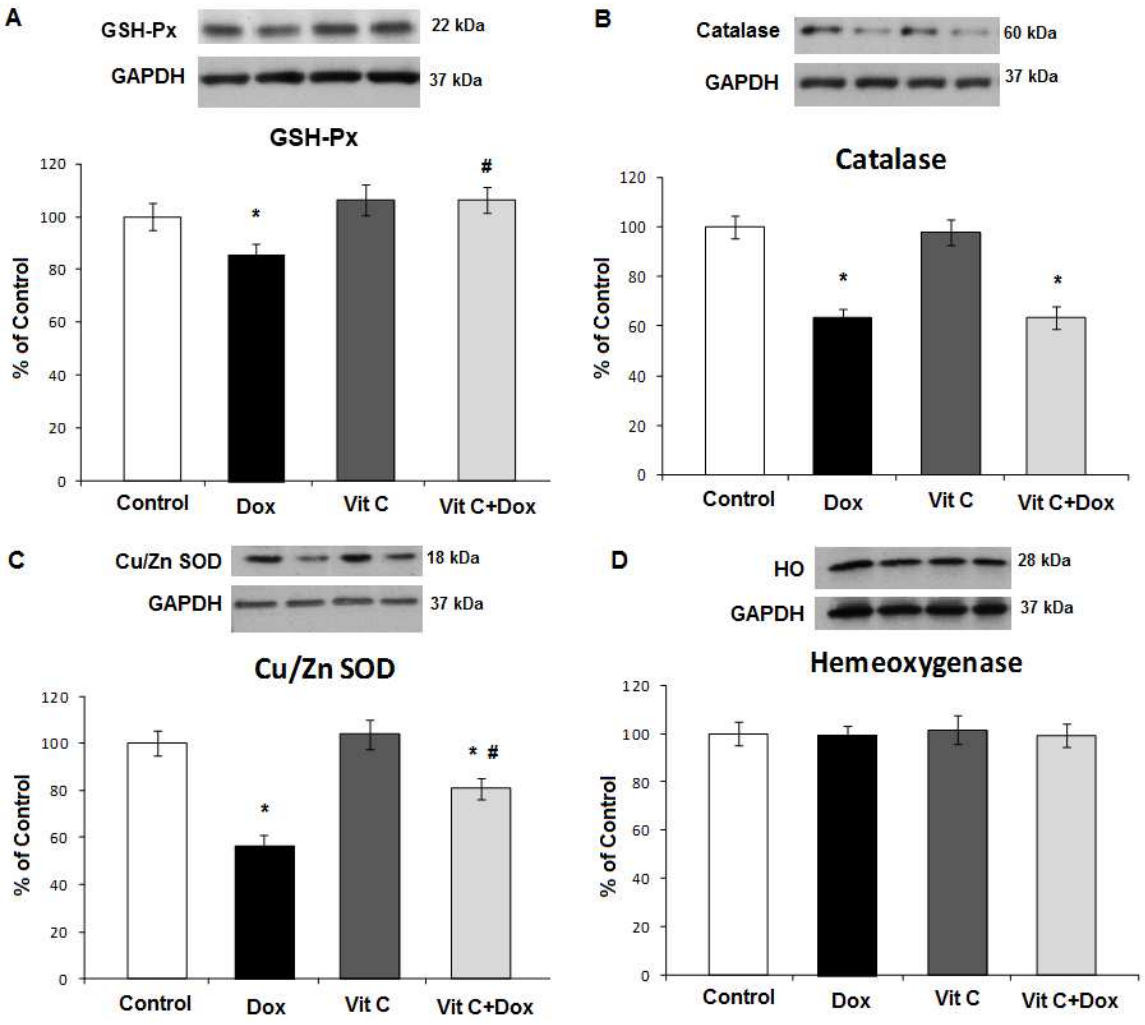

Figure 13 A, B, C and D. Effects of different treatments on the GSH-Px (A), catalase (B) Cu/Zn SOD (C) and Heme Oxygenase (D) protein expression in cardiomyocytes. In each figure, upper part is the Western blot and the lower part is densitometric analysis. All treatments are the same as in Figure 8. Data are expressed as mean ± S.E.M. of five different experiments. *Significantly different ($P < 0.05$) from Control and # significantly different ($P < 0.05$) from the Dox group.

3.3 Creatine kinase release

Membrane injury with the leakage of cell content leading to cell loss is a characteristic of Dox-induced cardiotoxicity. In this study, membrane injury was evaluated by the measurement of creatine kinase (CK) release from cardiomyocytes in the culture medium after treatment with Dox with and without Vit C (Figure 14). Dox-treatment led to an increase in cellular damage shown by a significant increase ($P \leq 0.05$) in CK release. Vit C alone did not show any significant effect but Dox-induced increase in CK release was significantly ($P \leq 0.05$) decreased in the presence of Vit C.

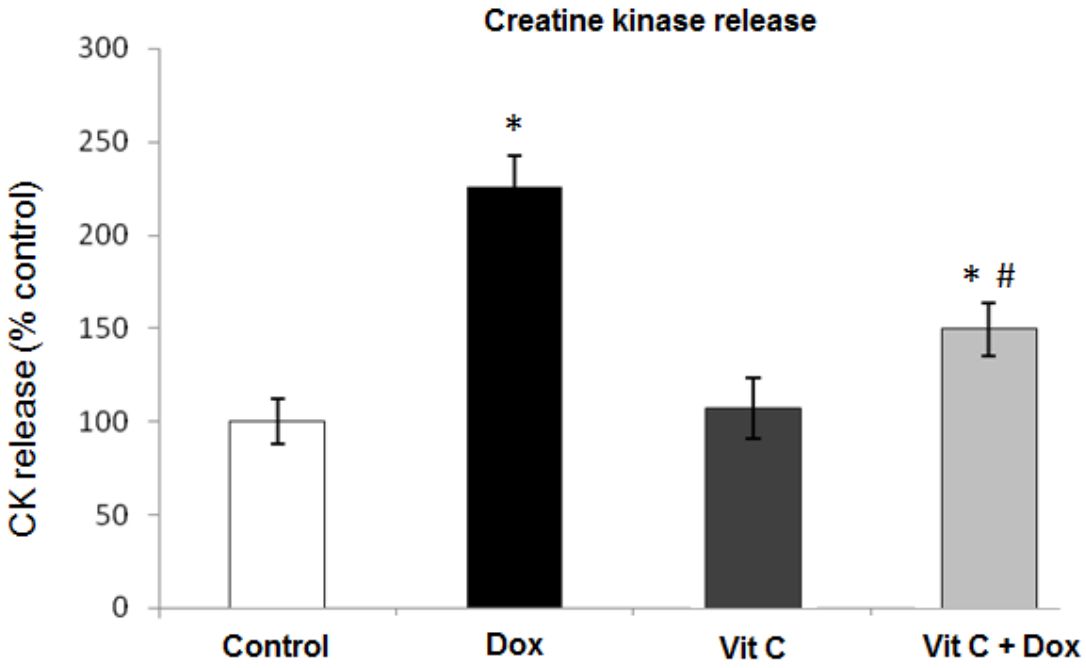

Figure 14. Cardiomyocyte injury as indicated by creatine kinase (CK) release in the culture medium. All treatments are the same as in Figure 8. Data are expressed as mean ± S.E.M. of five different experiments. *Significantly different ($P < 0.05$) from Control and # significantly different ($P < 0.05$) from the Dox group.

3.4 Apoptosis

Dox-induced apoptosis and its modulation by Vit C was studied by staining the cardiomyocytes with Annexin-PI (Figure 15 A and B) and Hoescht 33258 (Figure 16 A and B) as well as using pro-apoptotic markers (Figure 17 A and B).

3.4.1 Annexin staining

A common feature of apoptosis is the translocation of phosphatidylserine (PS) from the inner part of the plasma membrane to the outer layer, and this change can be detected by Annexin staining due to its high affinity for PS. Our study showed that, in the Control group, the majority of cells were rod-shaped, binucleated, and the nuclei had normal appearance. Exposure to Dox caused a 6 fold increase ($P \leq 0.05$) in Annexin staining in cells that excluded PI dye (Figure 15 A). Treatment with Vit C completely prevented this change caused by Dox (Figure 15 B).

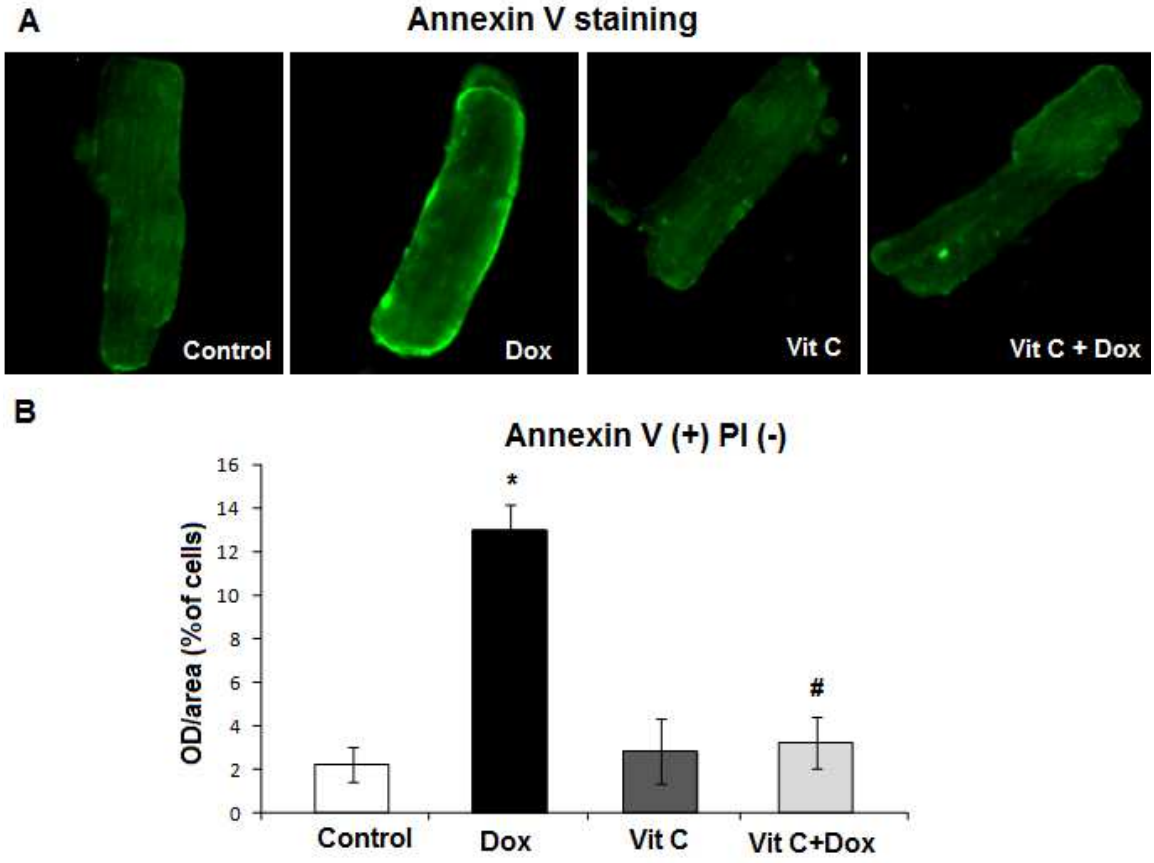

Figure 15 A and B. Apoptosis marker as seen by Annexin-V-Fluos staining. A) Representative cardiomyocytes after different treatments. Dox treatment resulted in the membrane staining positive for Annexin and excluding propidium iodide (PI) staining; and B) Percentage of cardiomyocytes stained positive for Annexin and negative for PI. All treatments are the same as in Figure 8. Data are expressed as mean ± S.E.M. of five different experiments. *Significantly different ($P \leq 0.05$) from Control. # significantly different ($P < 0.05$) from the Dox group.

3.4.2 DNA fragmentation

Hoescht 33258 staining was used to detect nuclear fragmentation and these data are shown in Figure 16 A and B. In the Control group, the nuclei had normal appearance. Exposure to Dox resulted in nuclear fragmentation (arrows) and the number of apoptotic cells significantly increased. Treatment with Vit C was able to significantly ($P \leq 0.05$) decrease this change caused by Dox (Fig. 16 A and B).

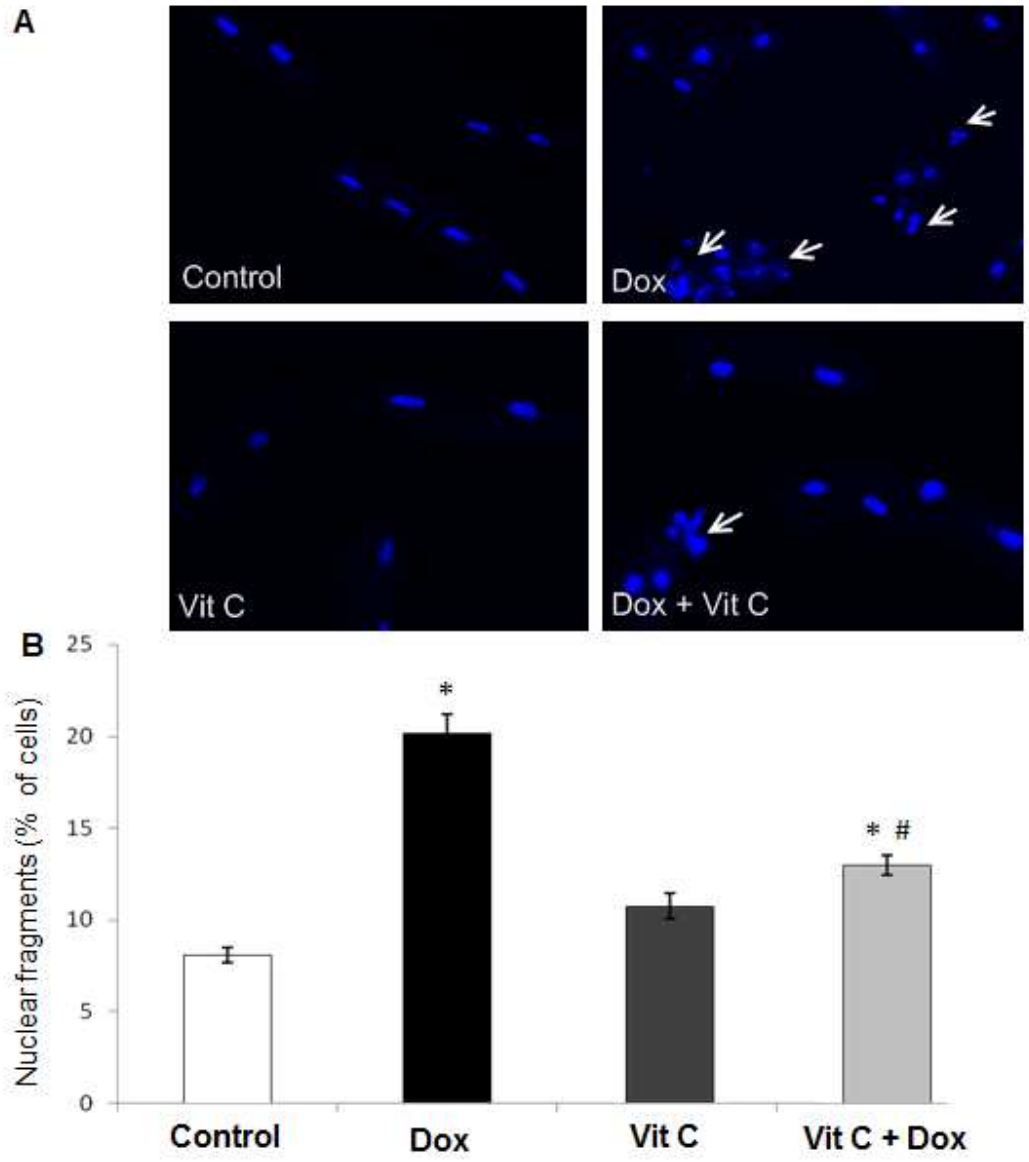

Figure 16 A and B. Nuclear fragmentation (arrows) as seen by Hoechst 33258 staining. A) Representative images of cardiomycytes; and B) Percentage of the nuclear fragments. All treatments are the same as in Figure 8. Data are expressed as mean ± S.E.M. of five different experiments. *Significantly different ($P < 0.05$) from Control and # significantly different ($P < 0.05$) from the Dox group.

3.4.3 Cytochrome C and Bax in mitochondria and cytosol

Pro-apoptotic protein Bax and Cytochrome C release were also analysed (Figure 17 A and B). Mitochondrial permeability change results in Cytochrome C release from mitochondria to the cytosol and activates downstream apoptotic signalling events. Dox caused a significant increase in cytosolic Cytochrome C and decrease in its mitochondrial content. This shift in Cytochrome C distribution was prevented by Vit C (Figure 17 A). Dox-induced overexpression of Bax was seen both in the mitochondria and cytosol, which was mitigated by Vit C treatment (Figure 17 B).

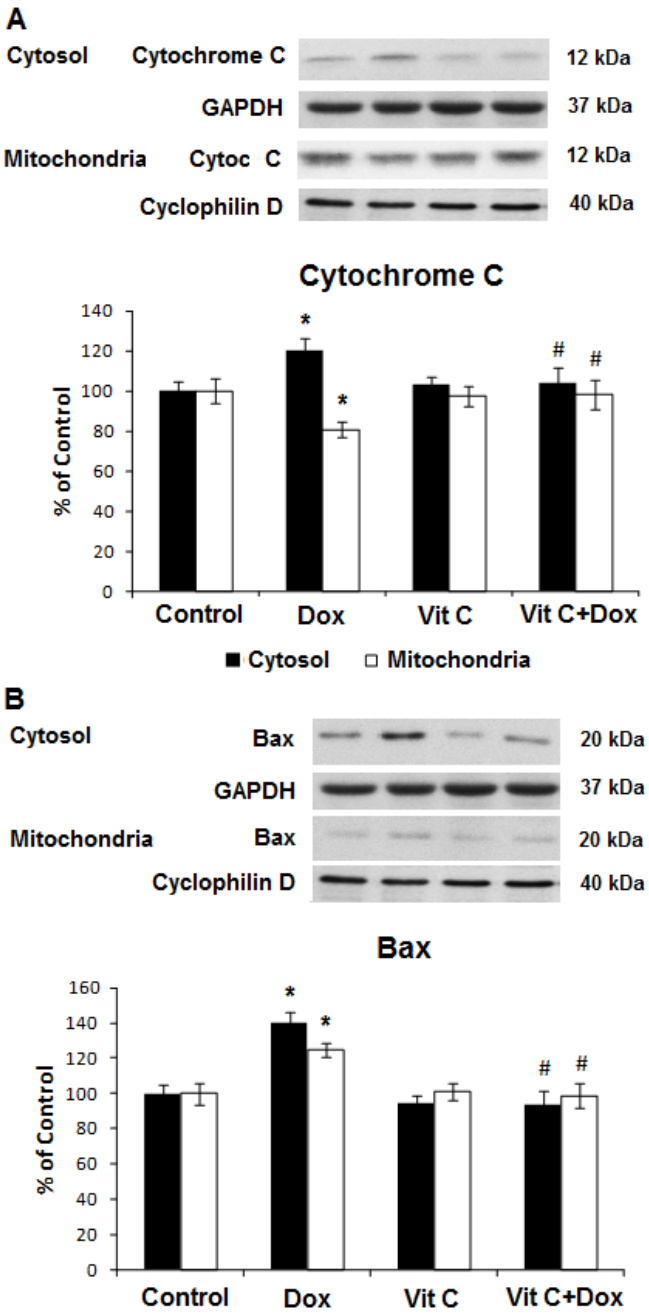

Figure 17 A and B. Effects of different treatments on Cytochrome C release from mitochondria to cytosol (A) representing its translocation and the Bax expression in cytosol and mitochondria (B) in cardiomyocytes. In both figures, upper part is the Western blots and the lower part shows densitometric analysis. All treatments are the same as in Figure 8. Data are expressed as mean ± S.E.M. of five different experiments. *Significantly different ($P < 0.05$) from Control and # significantly different ($P < 0.05$) from the Dox group.

4. Vit C transporters

In order to analyze Vit C transporter, we studied SVCT-2 due to its presence in the myocardium and its property of being the stereoselective high-affinity transport for Vit C. GLUT4 was chosen in order to understand whether Vit C could be entering the cell through its oxidized form DHA, besides this GLUT isoform is expressed exclusively in cardiac, skeletal, and adipose tissue (Fukumoto et al. 1989).

4.1 SVCT-2 and GLUT 4 localization

We studied SVCT-2 and GLUT4 localization by immunofluorescence. SVCT-2 was prominently labelled on the cell surface as well as in the vesicle compartments (Figure 18 A). Quantification of fluorescence intensity showed a significant decrease (20%) of SVCT-2 immunoreactivity after Dox treatment, which was partially reversed by co-treatment with Vit C (Figure 18 B). In the absence of insulin, GLUT4 was located in the intracellular compartments, and the intensity of immunofluorescence was low in all groups and the reaction was no different from its control shown in Figure 19 A. However, in the presence of insulin, GLUT4 was translocated to the cardiomyocyte surface and there was very prominent GLUT4-specific cell surface labelling. Cardiomyocytes treated with Dox expressed small but significantly higher GLUT4 immunofluorescence intensity in the presence of insulin than in all other groups (Figure 19 B).

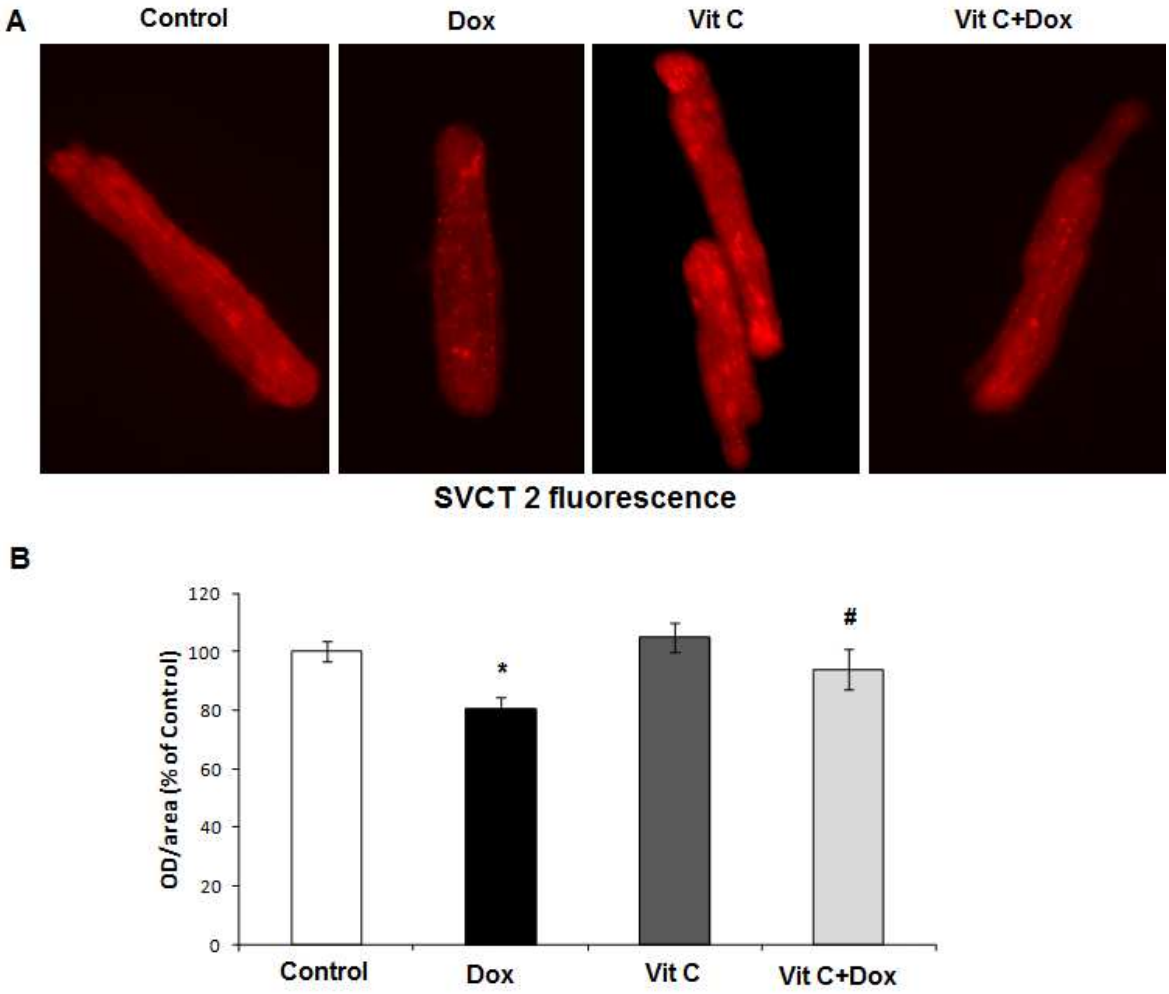

Figure 18 A and B. Effects of different treatments on the study of Sodium-dependent Vit C transporter (SVCT-2). A) Representative SVCT2 fluorescent microscopic images of cardiomyocytes; and B) Fluorescence intensity (% of Control). All treatments are the same as in Figure 8. Data are expressed as mean ± S.E.M. of five different experiments. *Significantly different ($P < 0.05$) from Control and # significantly different ($P < 0.05$) from the Dox group.

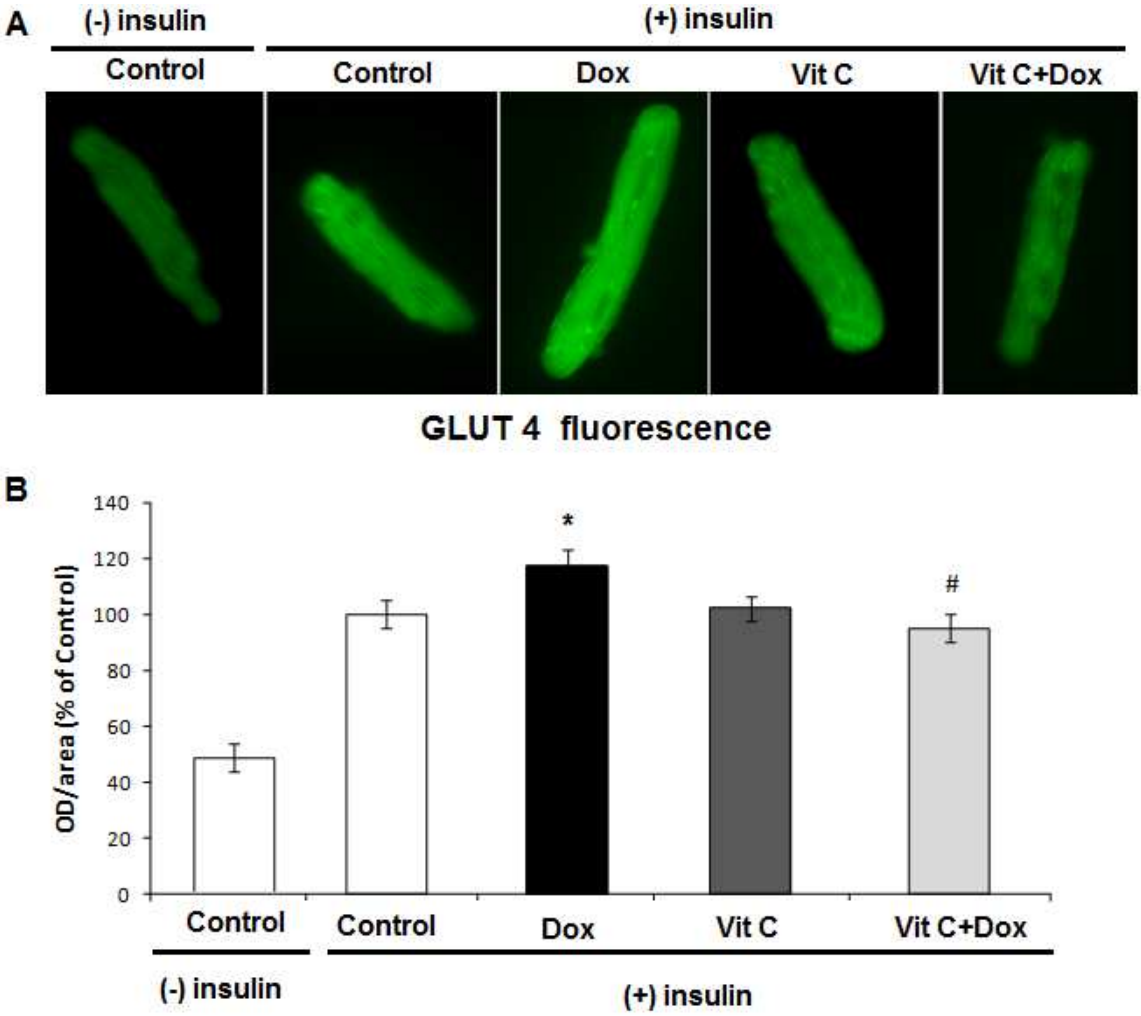

Figure 19 A and B. Effects of different treatments on the study of glucose transporter 4 (GLUT4) in the presence or absence of insulin. A) Representative GLUT4 fluorescent microscopic images cardiomyocytes; and B) Fluorescence intensity (% of insulin control). All treatments are the same as in Figure 8. Data are expressed as mean ± S.E.M. of five different experiments. *Significantly different ($P < 0.05$) from Control and # significantly different ($P < 0.05$) from the Dox group.

4.2 SVCT-2 and GLUT4 expression

To assess SVCT-2 and GLUT4 protein expression after respective treatments, western blotting of cell lysates was performed (Figure 20 A and B). In the Dox-treated group, SVCT-2 was downregulated by 22%, but Vit C was able to blunt the change caused by Dox. There was no difference between Control and Vit C alone groups (Figure 20 A). In accordance with immunofluorescence results, GLUT4 protein expression was slightly increased in the Dox group when compared to the other groups (Figure 20 B).

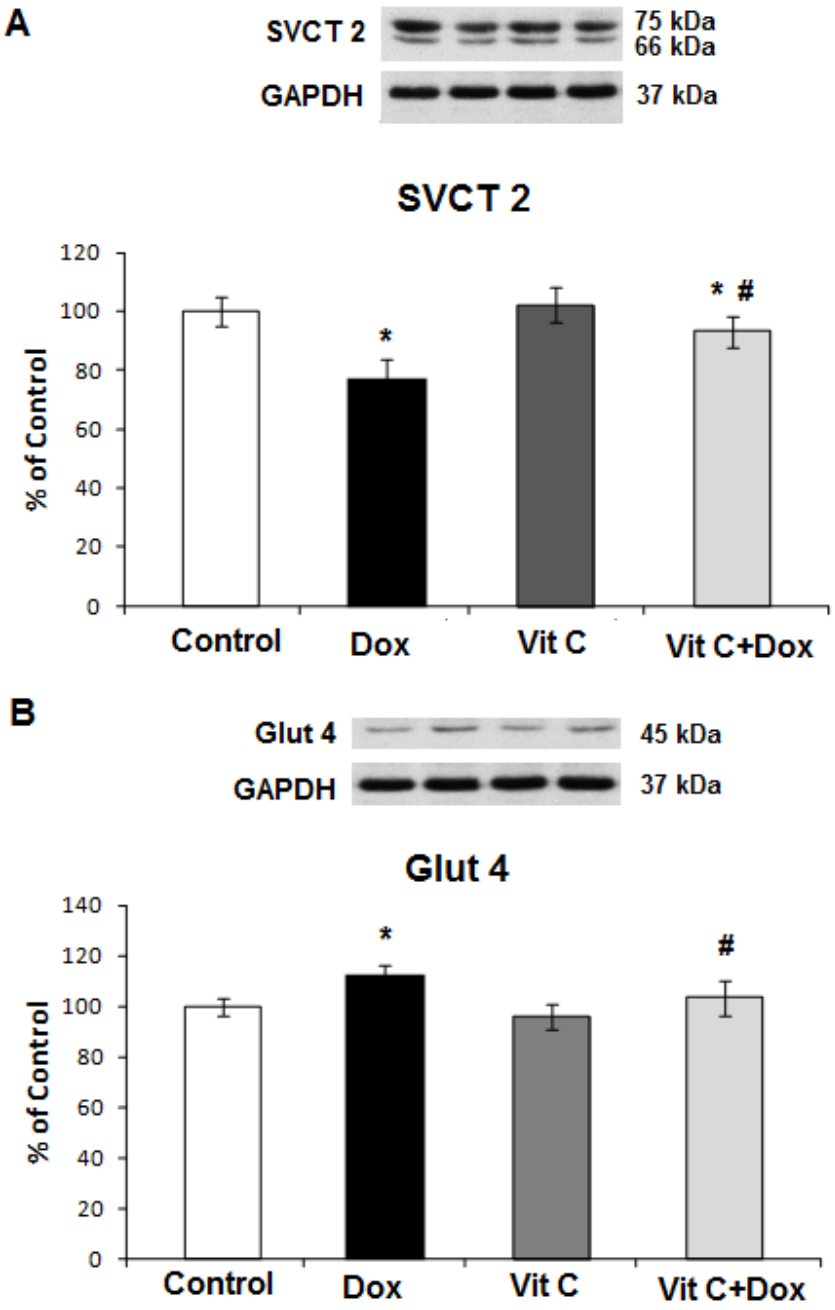

Figure 20 A and B. Effects of different treatments on the SVCT2 (A) and GLUT4 (B) transporters expression in cardiomyocytes. In both figures, upper part is the Western blots and the lower part shows densitometric analysis. All treatments are the same as in Figure 8. Data are expressed as mean ± S.E.M. of five different experiments. *Significantly different ($P < 0.05$) from Control and # significantly different ($P < 0.05$) from the Dox group.

5. Antioxidant effects of Vit C on Dox-induced changes

In order to understand whether Vit C protection against Dox-induced changes in stress-related signaling pathways, autophagy and apoptosis could be attributed mostly to its antioxidant properties, a well-known water soluble antioxidant, N-acetylcyteine (NAC), was used as positive control at the selected working dose of 50 µM (as described earlier).

5.1 Stress-induced pathways

The activation of p38, JNK and ERK MAPKs as well as p53 was expressed as the ratio of the phosphorylated to total protein. These results are presented in Figure 21 A-D. Dox-induced significant increase in p38, JNK and p53 activations was significantly reduced when either antioxidants, Vit C or NAC was added 1 h prior to Dox treatment (Figure 21 A, B and D). ERK activation was significantly higher than Control group in response to Dox treatments with and without either antioxidant treatment (Figure 21 C).

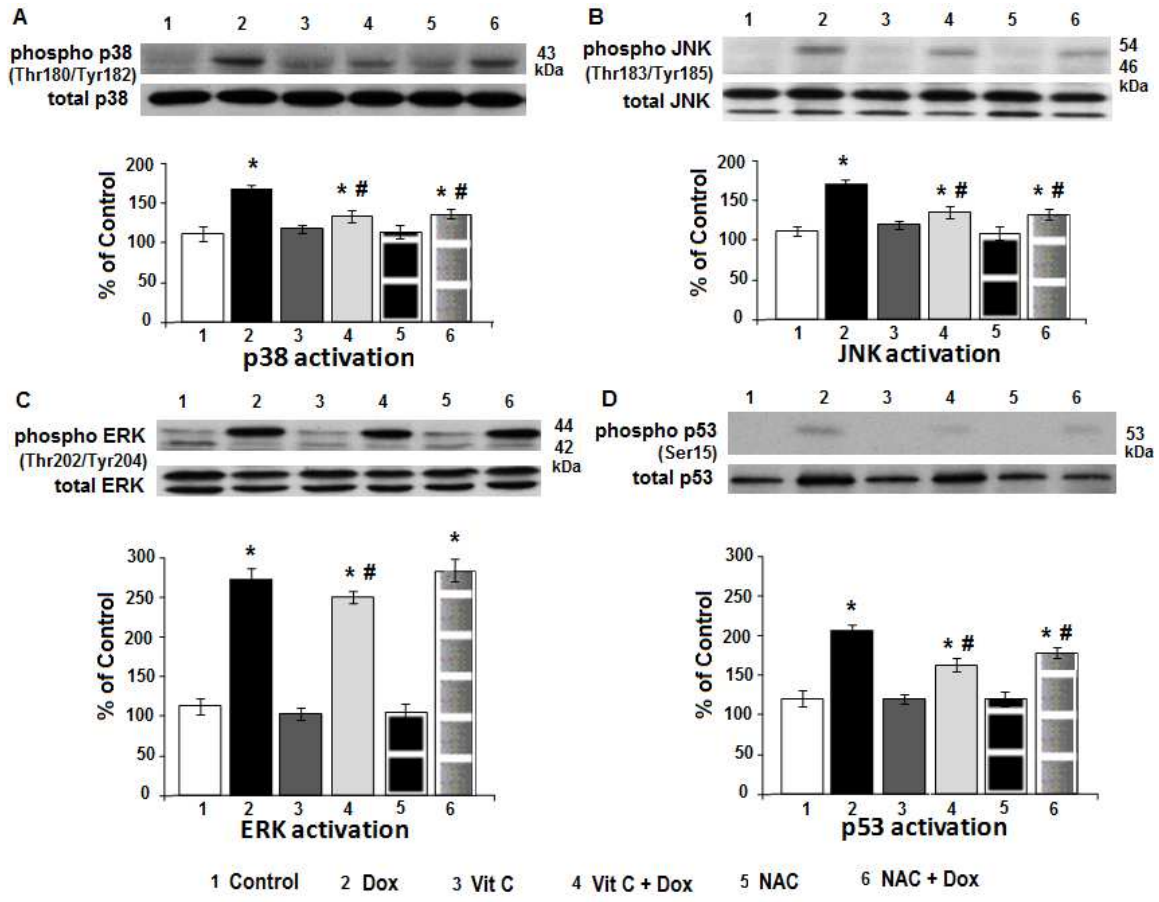

Figure 21 A, B, C and D. The effects of different treatments on stress-induced signaling pathways p38 (A), JNK (B), ERK (C), and p53 (D) in cardiomyocytes. In all figures, upper part is the Western blot analysis and the lower part shows densitometric analysis (% of Control). Cardiomyocytes were treated with Vit C (25 µM) or NAC (50 µM) 1 hour prior to the addition of Dox (10 µM) for 24h. Data are expressed as mean ± S.E.M. of five different experiments. *Significantly different ($P < 0.05$) from Control and # significantly different ($P < 0.05$) from the Dox group.

5.2 Markers of apoptosis and autophagy

In order to further investigate apoptosis and autophagy, more evidence for the confirmation of caspase-3 activation has been provided by monitoring the cleavage of its substrate, PARP along with Bax/Bcl-xL ratio. Dox-induced cleavage of caspase-3 and PARP was abolished by antioxidant treatments. The significant increase in Bax/Bcl-xL ratio caused by Dox was alleviated when either Vit C or NAC was added as adjuvant treatment (Figure 22 A-D).

When autophagy is induced, microtubule-associated protein, light chain 3 (LC3), is processed from LC3-I (18 kDa) to LC3-II (16 kDa) and incorporated into autophagic vacuoles. The Western blot analysis showed an increase in the formation of LC3-II after Dox treatment, suggesting that autophagy activation is supporting apoptosis, and this change was blunted by pre-treatment with Vit C or NAC. Control values were set as 100% and no differences were seen in Control and Vit C groups.

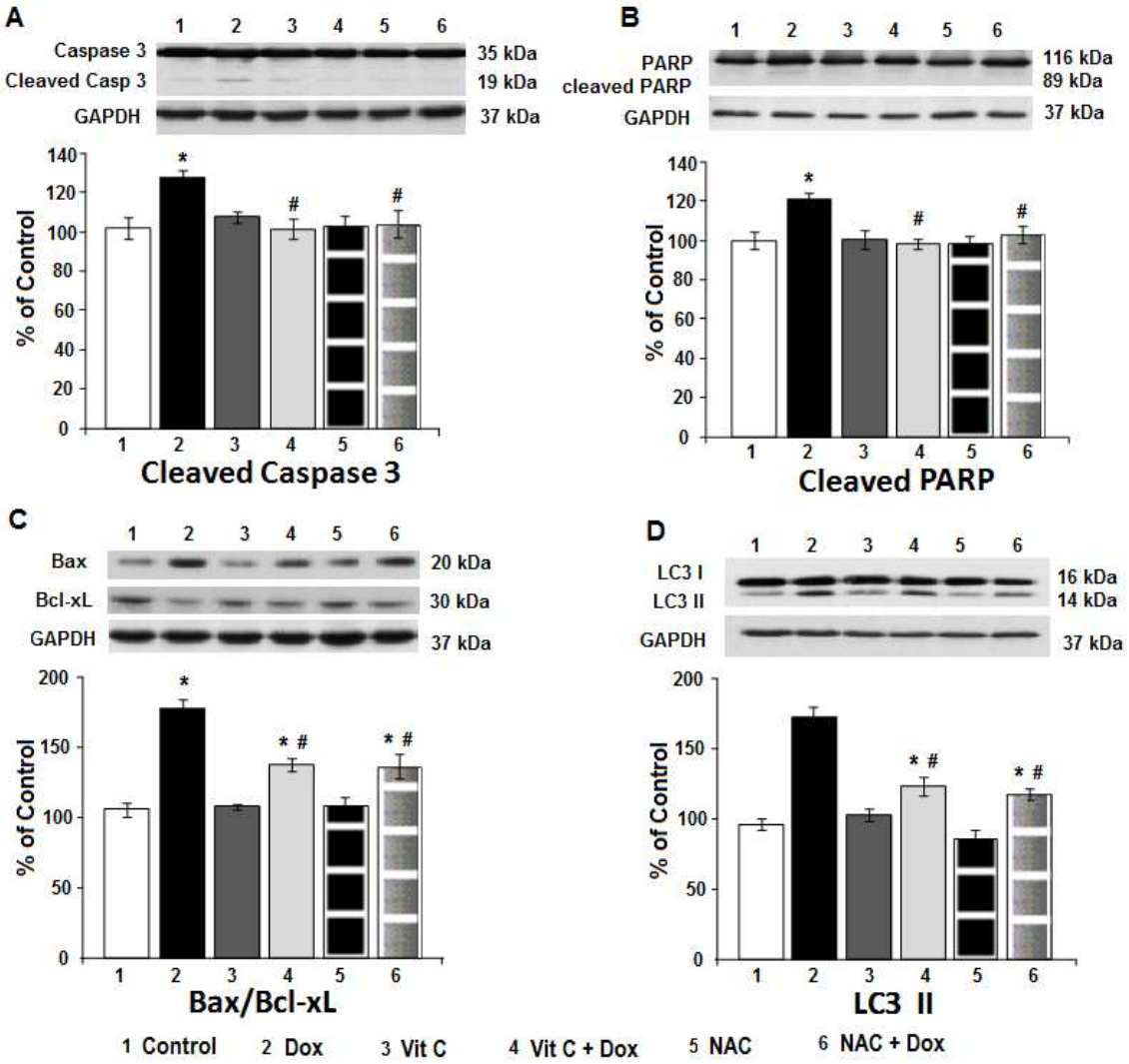

Figure 22 A, B, C and D. The effects of different treatments on apoptotic markers Caspase 3 cleavage (A), PARP cleavage (B), Bax/Bcl-xL ratio (C), and autophagic marker LC3 II (D) in cardiomyocytes. In all figures, upper part is the Western blot analysis and the lower part shows densitometric analysis (% of Control). All treatments are the same as in Figure 17. Data are expressed as mean ± S.E.M. of five different experiments. *Significantly different ($P < 0.05$) from Control and # significantly different ($P < 0.05$) from the Dox group.

6. Role of stress-induced pathways in Dox-induced changes

The role of different signaling proteins in Dox-induced changes was further analyzed by using well known pharmacological inhibitors of some of these intermediary proteins. In order to understand the importance of MAPKs and p53 activation, cells were cultured with SB203580 (pharmacological inhibitor of p38 that also inhibits JNK), PD98059 (pharmacological inhibitor of ERK) and pifitrin-α (pharmacological inhibitor of p53) 1h prior to addition of Dox for 24h. The concentration for each of these inhibitors was 25 µM, selection based on pilot studies and previous work from our laboratory (Dhingra et al 2007).

6.1 Viability and Reactive oxygen species

Treatment with p38/JNK and p53 inhibitors abrogated Dox-induced decrease in cardiomyocyte viability and these data are shown in Table 3. p38/JNK and p53 inhibitors also blunted the Dox-induced increase in the ROS production (Table 3). On the other hand, inhibition of ERK did not influence Dox-induced changes in either cell viability or ROS production (Table 3).

Table 3. Effects of different inhibitors on Dox-induced changes in viability and reactive oxygen species (ROS) production in cardiomyocytes.

GROUPS	Viability	ROS production
Control	91.5 ± 3.2	100 ± 7.8
Dox (10μM)	71 ± 2.1*	198 ± 10.6*
p38 inhib (25μM)	90.2 ± 3.1	105 ± 8.9
Dox (10μM) + p38 inhib (25μM)	80 ± 3.5*#	175 ± 7.5*#
ERK inhib (25μM)	91.2 ± 3.3	102 ± 6.9
Dox (10μM) + ERK inhib (25μM)	75 ± 3.5*	195 ± 7.5*
p53 inhib (25μM)	90.8 ± 3.5	103 ± 9.8
Dox (10μM) + p53 inhib (25μM)	81.2 ± 3.1*#	170 ± 8.8*#

Cardiomyocyte viability and ROS production were analyzed as described in Figures 2 and 3. Inhibitors were added 1h prior to 24h incubation with Dox. Data are expressed as mean ± S.E.M. of five different experiments. *Significantly different ($P < 0.05$) from Control and # significantly different ($P < 0.05$) from the Dox group.

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6.2 Effects of MAP kinase inhibition on apoptotic markers

We further investigated the role of p38/JNK and ERK inhibition on specific parameters of apoptosis. Inhibition of p38/JNK with SB203580 was able to prevent Dox-induced cleavage of caspase-3 and PARP as well as blunted the increase in the pro-apoptosis index Bax/Bcl-xL ratio (Figure 23 A-C). Inhibition of ERK with PD98059 did not interfere with all these apoptotic markers suggesting that Dox-induced cardiomyocyte apoptosis is more likely p38/JNK-dependent and ERK is not fully involved in this Dox-induced pathway.

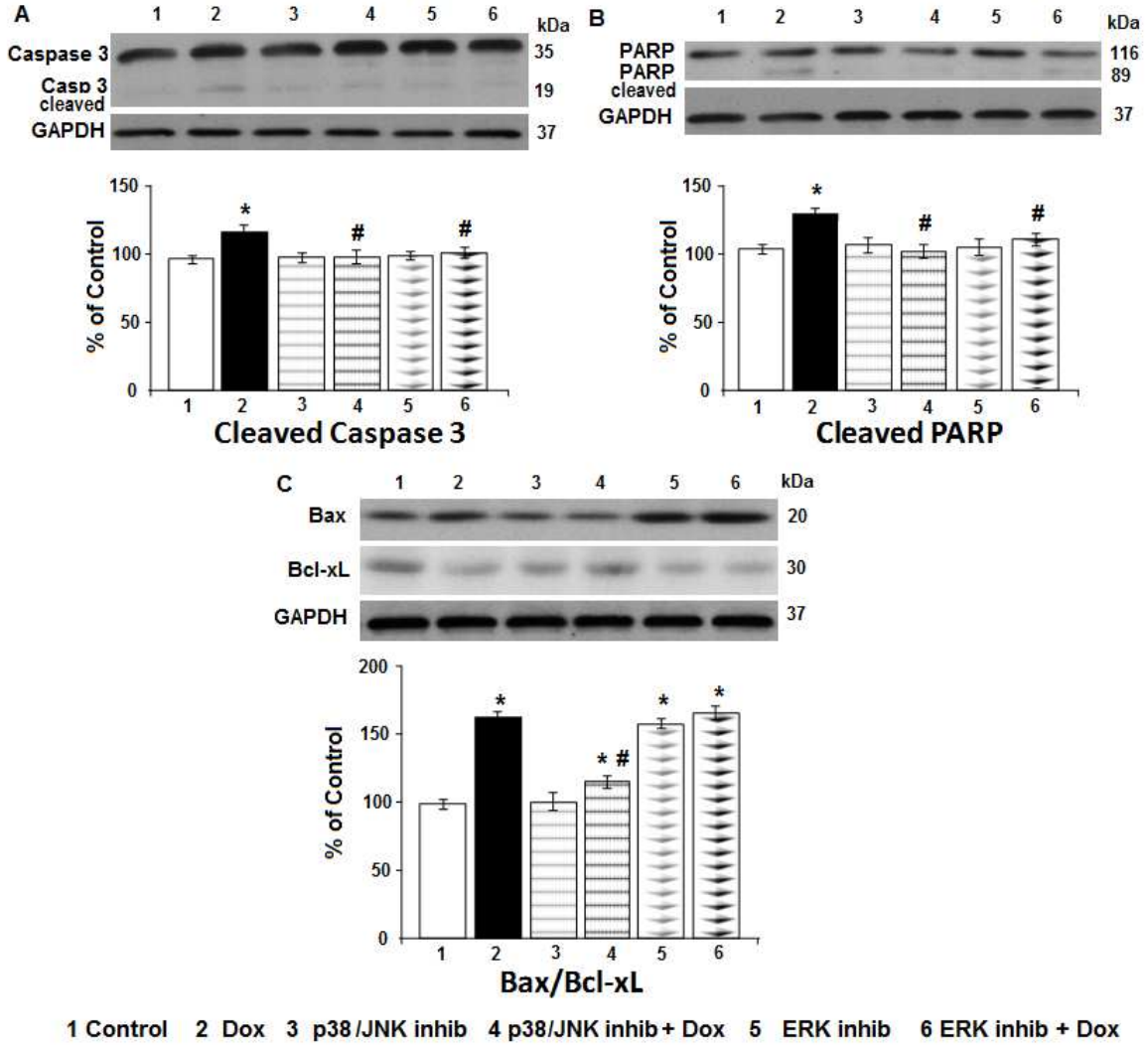

Figure 23 A, B and C. The effects of p38/JNK and ERK inhibition on apoptotic markers Caspase 3 cleavage (A), PARP cleavage (B), and Bax/Bcl-xL ratio (C) in cardiomyocytes. In all figures, upper part is the Western blot analysis and the lower part shows densitometric analysis (percentage of Control). Cardiomyocytes were treated with 25 µM of SB203580 (p38 inhib) or 25 µM of PD98059 (ERK inhib) 1 hour prior to the addition of Dox (10 µM) for 24h. Data are expressed as mean ± S.E.M. of five different experiments. *Significantly different ($P < 0.05$) from Control and # significantly different ($P < 0.05$) from the Dox group.

6.3 Effects of p53 inhibition on apoptotic markers

Figure 24 A-C shows the role of p53 inhibition on apoptosis. Dox-induced cleaved caspase 3 and PARP as well as Bax/Bcl-xL ratio was decreased by pifitrin-α indicating that Dox-induced apoptosis is also influenced by p53 activation. Control values were set as 100% and no differences were seen in Control and Vit C groups.

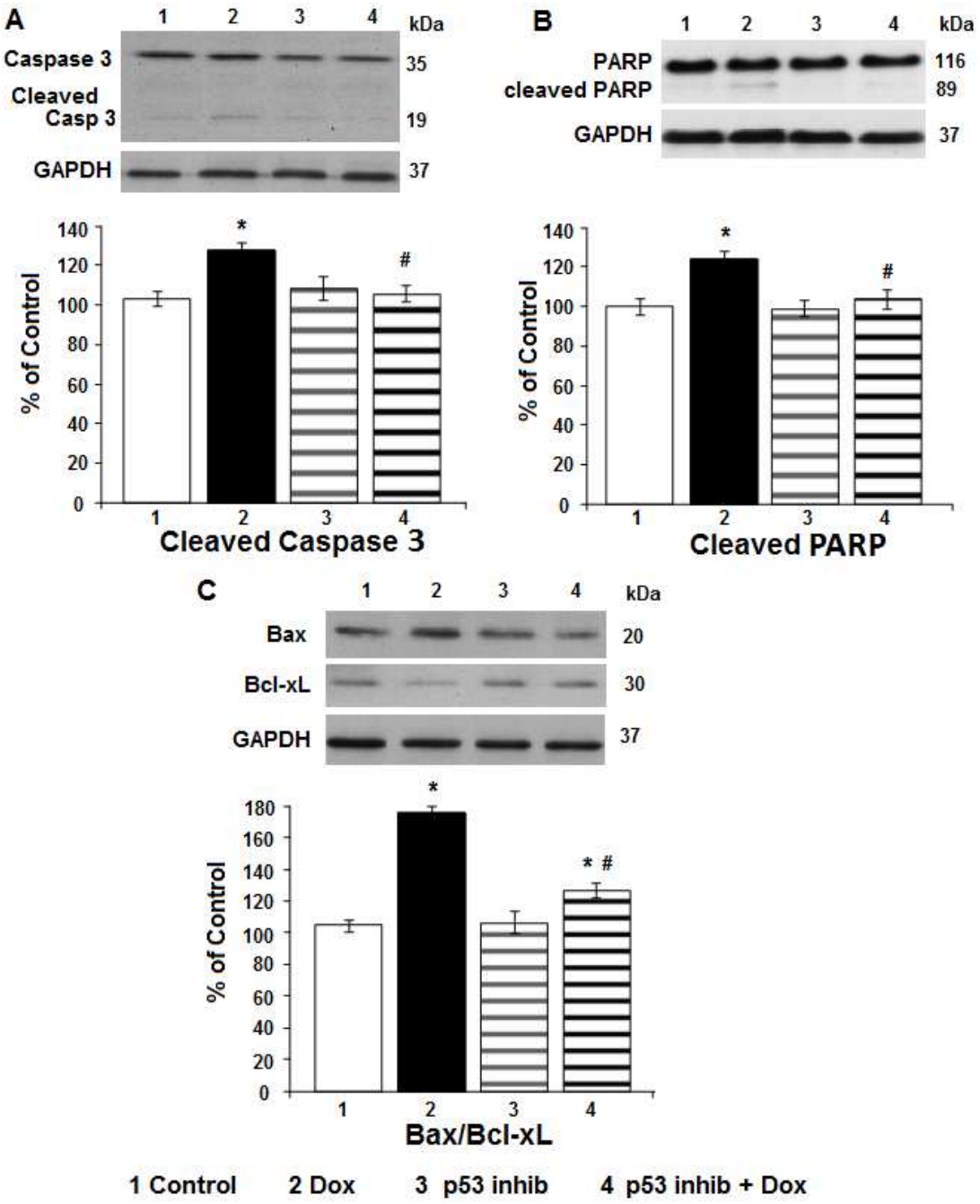

Figure 24 A, B and C. The effects of p53 inhibition on apoptotic markers Caspase 3 cleavage (A), PARP cleavage (B), and Bax/Bcl-xL ratio (C) in cardiomyocytes. In all figures, upper part is the Western blot analysis and the lower part shows densitometric analysis (percentage of Control). Cardiomyocytes were treated with 25 µM of pifithrin-α (p53 inhib) 1 hour prior to the addition of Dox (10 µM) for 24h. Data are expressed as mean ± S.E.M. of five different experiments. *Significantly different ($P \leq 0.05$) from Control and # significantly different ($P < 0.05$) from the Dox group.

7. Sca-1 positive cells

The Sca-1 positive population that can be isolated from the heart represents a small fraction of the total number of cardiomyocyte and non-yocyte cells (Smits et al, 2009). Using the magnetic cell sorting system (MACS) for cell isolation, the number of Sca-1 positive cells isolated from each adult rat heart in the present study ranged from 10,000 to 13,000 cells. For the bone marrow (BM), both femurs and tibias were used and the number of isolated Sca-1 positive cells ranged between 100,000 to 120,000. The purity of the isolation from both tissues was around 89%. In the FACS analysis, Sca-1 positive cells bound to the Sca-1 magnetic beads and were retained in the magnetic column, while the cardiac negative cells passed freely through the MACS column (Figure 25 A). The circled positive fraction shown in Figure 25 A was further analyzed under fluorescence microscopy which revealed the membrane bound Sca-1 antibody (Figure 25 B). Similar results were obtained after bone marrow Sca-1 positive cell isolation (data not shown).

Due to the very low number of Sca-1 positive cells isolated from the heart, very sensitive techniques designed to precisely detect changes using small number of cells were selected. For this purpose, luciferase technology was used to detect ATP levels and AK release. These two techniques are able to detect changes using only 100 cells. DCFDA fluorescence was used to detect ROS production and MTT assay was chosen to assess cell viability. In order to compare the results obtained for cardiomyocytes, and cardiac as well as bone marrow Sca-1 positive

cells, same number of cells and culture conditions were established for each parameter analyzed.

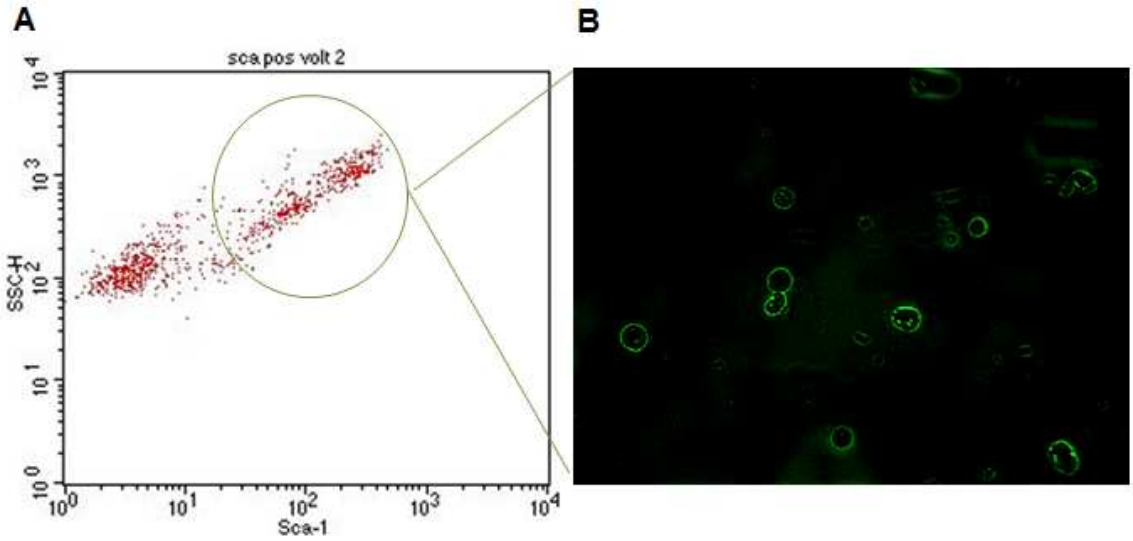

Figure 25 A and B. Cardiac derived Sca-1 positive cells. A) FACS analysis showing Sca-1 positive (circle) cells. B) Representative cells showing Sca-1 positive fluorescent staning on the cell surface.

7.1 Effects of Dox and Vit C in cardiac and bone-marrow derived Sca-1 positive cells as compared to cardiomyocytes

7.1 .1 Viability

Cardiomyocytes and Sca-1 positive cells (cardiac and bone marrow - BM) were treated with increasing concentrations of Dox (0.01 to 20 µM). The cardiomyocyte viability was decreased in a dose-dependent manner, with significant decreases observed with doses of Dox of 0.1-20 µM (Figure 26 A). Sca-1 positive cells isolated from the heart showed also a dose-dependent decrease in viability, but this decrease was more pronounced ($P \leq 0.05$) than seen in cardiomyocytes. BM Sca-1 positive cells followed the same pattern as cardiac Sca-1 positive cells (Figure 26 A). Physiological doses of Vit C (25 and 50 µM) were used alone (data not shown) or in combination with supraclinical dose of Dox (10 µM) in order to analyze its beneficial effect (Figure 26 B). Vit C co-treatment with 25 or 50 µM was able to partially blunt to the same extent this decrease in viability caused by Dox in cardiomyocytes. Vit C pre-treatment followed by Dox exposure showed beneficial effects in improving the cell viability. The addition of 50 µM of Vit C with Dox treatment was significantly ($P \leq 0.05$) more beneficial to Sca-1 positive cells from both tissues, reaching the same partial recovery in viability seen in cardiomyocytes. 100 µM of Vit C in the presence of Dox did not show any additional benefit (data not shown).

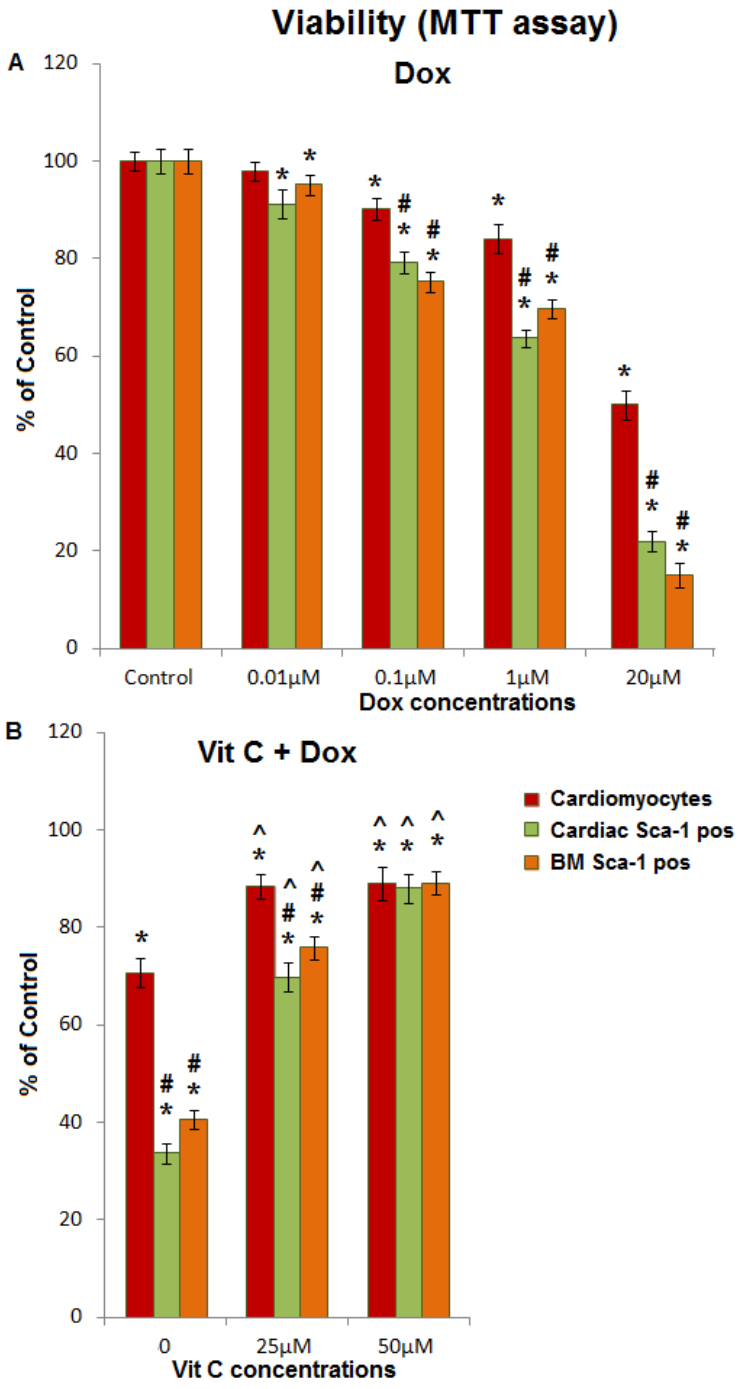

Figure 26 A and B. A) Effects of different concentrations of Dox, 0.01-20 µM on cardiomyocytes and Sca-1 positive cell (derived from cardiac tissue and bone marrow) viability using MTT assay. B) Effects of Vit C (0, 25 and 50 µM) on Dox (10 µM) induced changes in viability. Data expressed as mean percentage ± S.E. of five different experiments. Significantly different (P ≤ 0.05) * from their respective control group # from cardiomyocytes at the same dose ^ from Dox 10µM/Vit C 0.

7.1.2 ATP production

For the next set of experiments, we excluded the 20 µM dose of Dox due to the reduced cell viability and lack of enough number of surviving cells at 24 hours for further studies.

The ATP levels found in different groups of cells at different doses of Dox and Vit C + Dox are shown in Figure 27 A an B. ATP levels were reduced in all cell types in a dose-dependent manner (Figure 27 A). However, cardiac and BM Sca-1 positive cells were more sensitive than cardiomyocytes to this decline in ATP, with a significant ($P \leq 0.05$) depletion found with the dose of Dox as low as 0.01 µM. Vit C pre-treatment was shown to be beneficial by blunting the effect of 10 µM Dox on the ATP levels (Figure 27 B). At 50 µM of Vit C, the protection against Dox-induced depletion in ATP levels on Sca-1 positive cells was the same extent seen in cardiomyocytes.

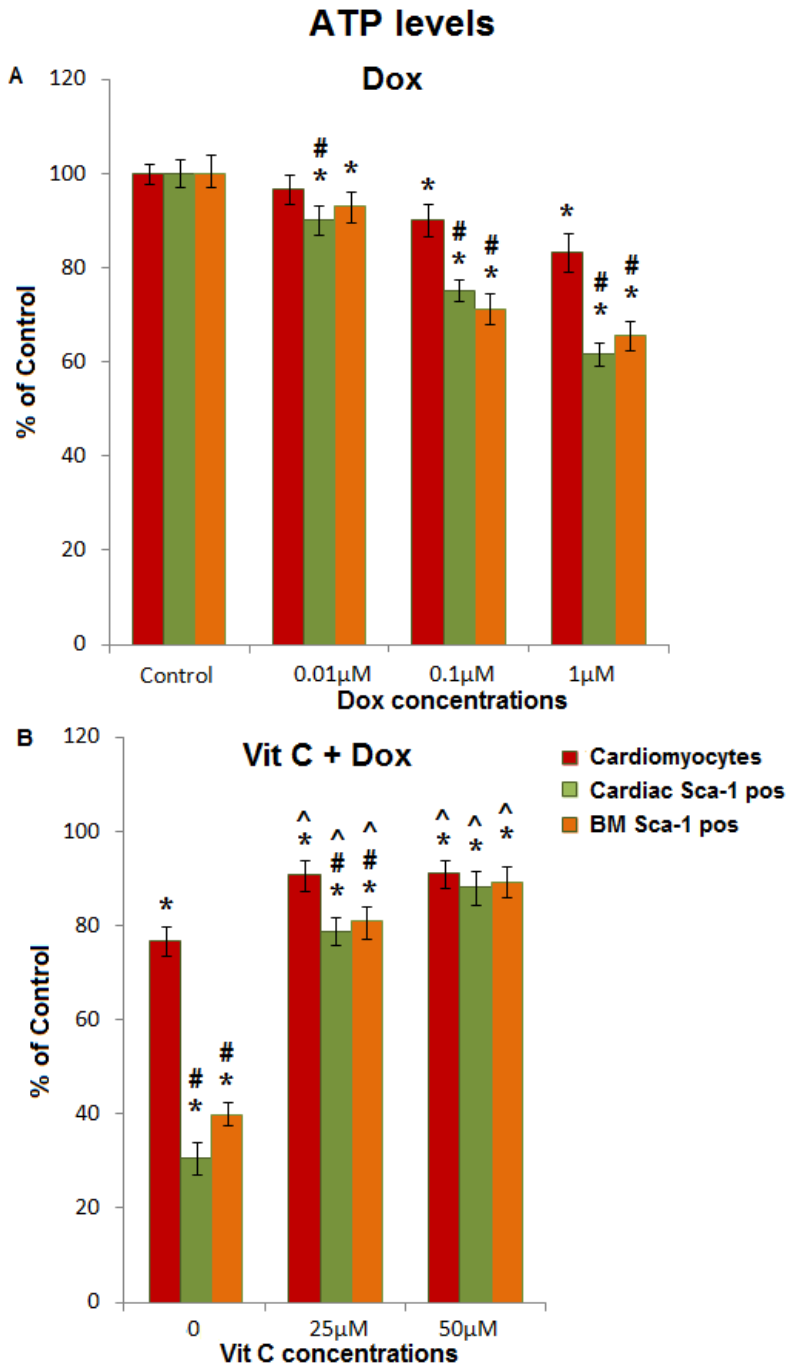

Figure 27 A and B. A) Effects of different concentrations of Dox, 0.01-1 µM on cardiomyocytes and Sca-1 positive cell (derived from cardiac tissue and bone marrow) Adenosine Triphosphate (ATP) levels detected by Vialight assay. B) Effects of Vit C (0, 25 and 50 µM) on Dox (10 µM) induced changes in ATP levels. Data expressed as mean percentage ± S.E. of five different experiments. Significantly different (P ≤ 0.05) * from their respective control group # from cardiomyocytes at the same dose ^ from Dox 10µM/Vit C 0.

7.1.3 Adenylate kinase release

Membrane leakage was assessed by measuring adenylate kinase (AK) release in the culture medium. Cardiomyocytes showed membrane leakage at doses of 0.1-10 µM of Dox, being significantly ($P \leq 0.05$) more pronounced with the increase in the dose (Figure 28 A). Cardiac and BM Sca-1 expressing cells showed significantly elevated cell injury with doses as low of 0.01 µM and higher (Figure 28 A). Vit C pre-treatment prior to addition of Dox offered a significant protection at concentrations of 25 and 50 µM. 50 µM of Vit C showed a further positive effect on Sca-1 positive cells (Figure 28 B).

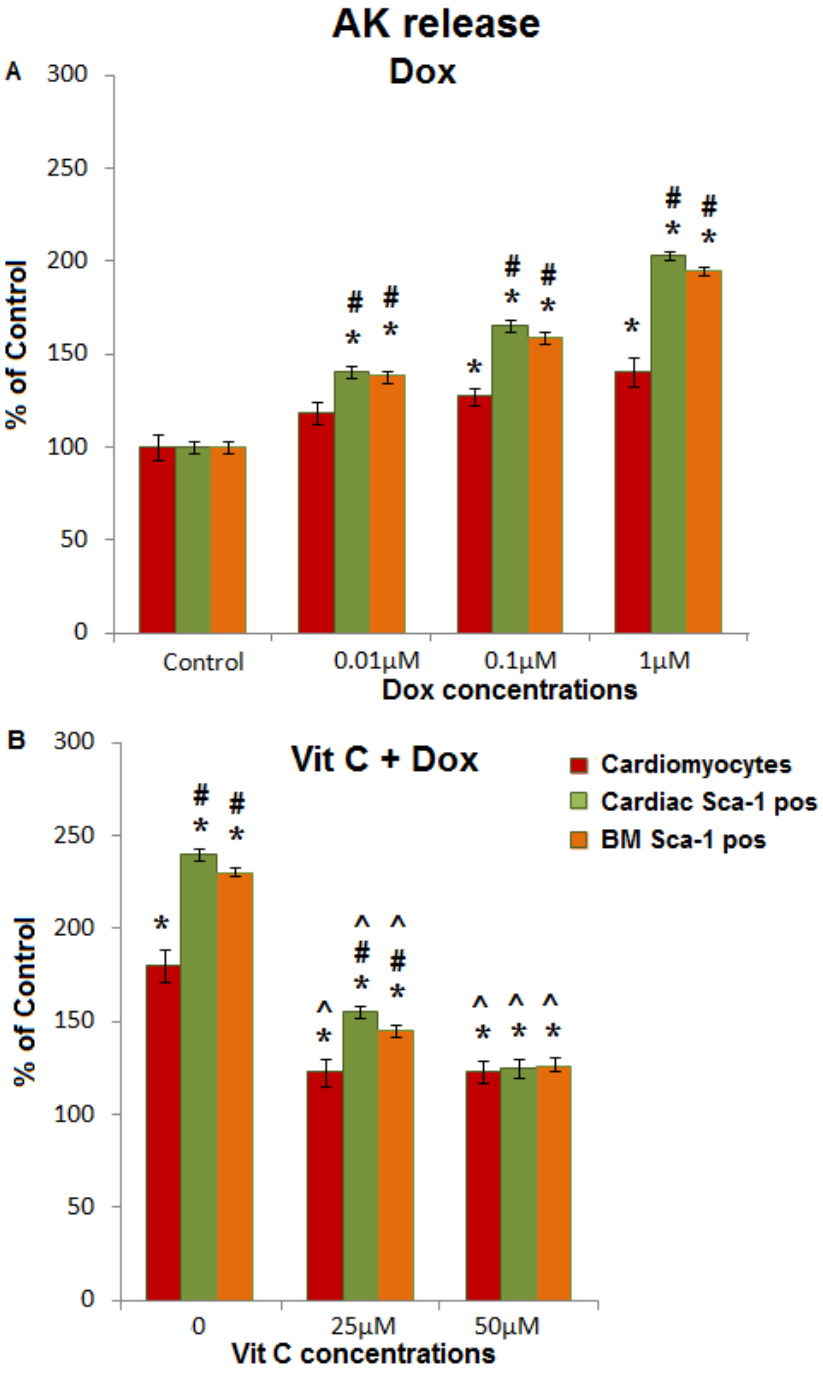

Figure 28 A and B. A) Effects of different concentrations of Dox, 0.01-1 µM on cardiomyocytes and Sca-1 positive cell (derived from cardiac tissue and bone marrow) Adenylate kinase (AK) released in the culture media detected by Toxilight assay. B) Effects of Vit C (0, 25 and 50 µM) on Dox (10 µM) induced changes in AK release. Data expressed as mean percentage ± S.E. of five different experiments. Significantly different (P ≤ 0.05) * from their respective control group # from cardiomyocytes at the same dose ^ from Dox 10µM/Vit C 0.

7.1.4 ROS levels

Direct ROS production was analyzed using DCFDA fluorescence in all cell groups and these results are presented in Figure 29 A and B. Dox-induced ROS production in cardiomyocytes was apparent at 0.1 µM and higher concentrations. However, in Sca-1 positive cells, Dox-induced ROS production was seen at the lowest concentration (0.01 µM) used. For the Sca-1 positive cardiac as well as BM cells, there was a more pronounced increase in ROS levels when compared to cardiomyocytes. Effects of Vit C (25 and 50 µM) were studied in the different cell types exposed to 10 µM of Dox. Vit C (25 and 50 µM) was effective in alleviating the ROS production caused by Dox, with the higher concentration showing slightly better effect in Sca-1 positive cells.

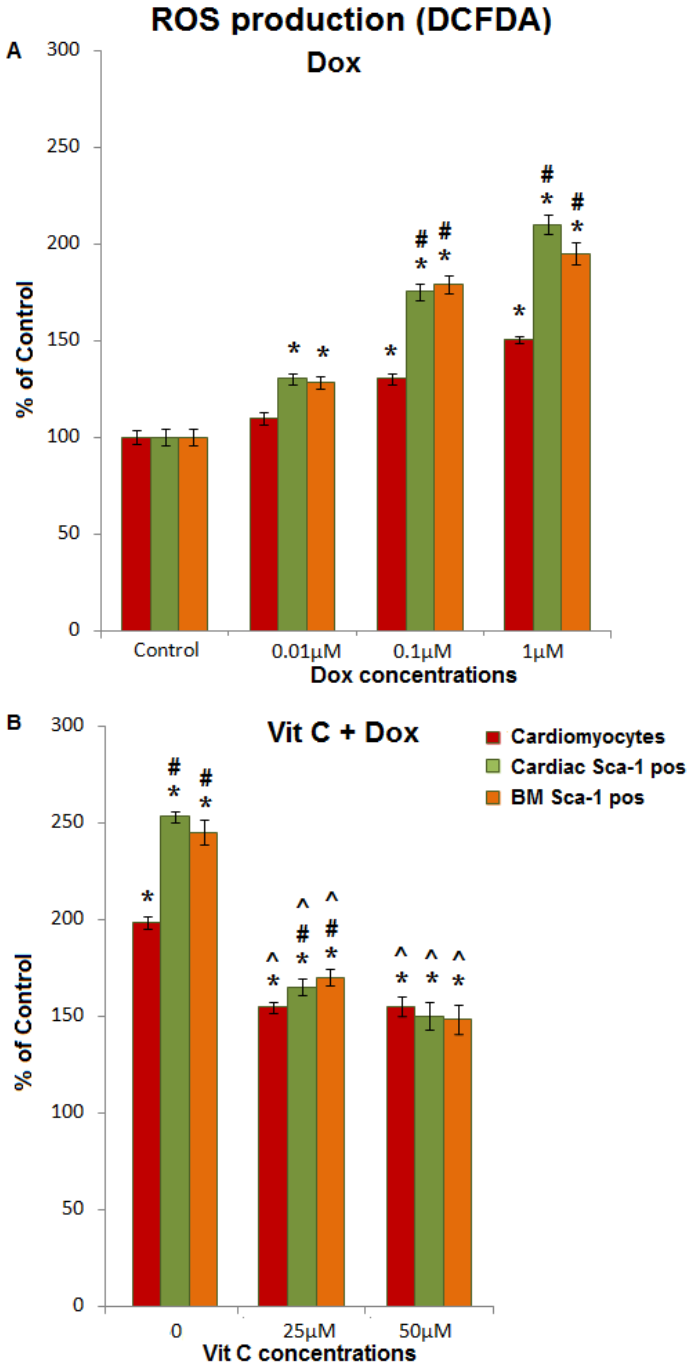

Figure 29 A and B. A) Effects of different concentrations of Dox, 0.01-1 µM on cardiomyocytes and Sca-1 positive cell (derived from cardiac tissue and bone marrow) ROS production detected by DCFDA fluorescence. B) Effects of Vit C (0, 25 and 50 µM) on Dox (10 µM) induced changes in ROS production. Data expressed as mean percentage ± S.E. of five different experiments. Significantly different (P ≤ 0.05) * from their respective control group # from cardiomyocytes at the same dose ^ from Dox 10µM/Vit C 0.

VI. Discussion

Dox-induced cardiomyopathy still remains an important clinical problem (Yeh et al. 2004; Singal and Iliskovic 1998; Ludke et al. 2009). Research on the underlying mechanisms of this unique cardiomyopathy is crucial to develop therapeutic strategies and prevent premature cardiac cell loss in patients subjected to anthracycline treatment. The present approach has unique strength in that it utilized adult cardiomyocytes as the cell model that is much more relevant to the disease state being investigated but not commonly utilized previously. Additionally, the utilization of Sca-1 positive cells isolated from heart tissue as well as bone marrow enhanced the impact of our experimental findings.

Among different mechanisms for Dox-induced cardiotoxicity, occurrence of oxidative stress has gained significant support (Singal et al. 1997; Kumar et al. 2001; Mizutani et al. 2005). Despite such an understanding of the subcellular basis of Dox-induced cardiomyopathy (Singal and Iliskovic 1998; Minotti et al. 2004), we still do not have an ideal cardioprotective agent to prevent this side effect (Ludke et al. 2009). Different antioxidants have been tried to reduce Dox-induced oxidative stress as well as its effects but only with a limited success (Singal and Iliskovic 1998; Quiles et al. 2002; Injac and Strukelj 2008). My study focused on the role of Vit C, a water soluble antioxidant, in Dox-induced oxidative stress in cardiomyocytes, with the ultimate goal of reducing oxidative stress with a nutritional approach. The study on isolated adult cardiomyocytes shows a Dox-induced increase in reactive oxygen species (ROS), oxidative stress, and disturbance in

downstream signaling leading to cell injury and culminating in cell loss. Most of these changes are prevented by the use of Vit C.

The cardiomyocyte loss contributes to an impaired cardiac function, and in addition, progenitor cells such as Sca-1 expressing cells when depleted due to Dox exposure also compromise the cardiac repair system. In fact, the Dox-induced cardiomyopathy may also be viewed as a "stem cell disease" (De Angelis et al. 2010, Huang et al. 2010) in which. In this study, the data showed a net loss of Sca-1 positive cells exposed to Dox. Thus, it is possible that Dox-induced loss of cardiomyocytes together with early damage to cardiac or circulating stem cells due to the drug treatment may result in permanent cardiotoxicity. In particular, this study showed that Dox-induced injury to these stem cells could also be associated to the increase in ROS production. In addition, antioxidant Vit C offered protection against Dox-induced cardiomyocyte cell loss as well as Dox-induced "stem cell disease". These findings may contribute to the design of novel therapeutic approaches to prevent the development of Dox-induced heart failure.

Dox-induced oxidative stress and cell injury/loss

Our detailed *in vitro* study of Dox-induced effects showed that cardiomyocyte injury occured in a dose-dependent manner. Dox in clinical doses (0.1 – 1µM) decreased cell viability and at supraclinical doses (10 – 20 µM) caused a significantly higher cell loss. The subclinical dose (0.01 µM) was without any effect on the depletion of cardiomyocytes. These *in vitro* data using 0.01 to 20 µM of Dox, support the well known clinical findings that both early- and late-onset

cardiotoxicity are characterized by a dose-dependent progressive heart failure (Swain et al. 2003; Pinder et al. 2007; van Dalen et al. 2008). Clinically, lower doses were also found to be associated with cardiac abnormalities only when other risk factors such as age, previous irradiation or pre-existing heart disease were present (Von Hoff et al. 1979; Singal and Iliskovic 1998; Lipshultz et al. 2005; Ludke et al. 2009).

An increase in creatine kinase and adenylate kinase release in the culture medium of treated cells found in this study suggests that Dox has altered cardiomyocyte permeability, increased cell injury and membrane leakage. This effect may be due to solubility of Dox in the membrane and its reported direct effects on the sarcolemma (Singal and Panagia 1984; Singal et al. 1997). Our findings also demonstrated that Dox progressively increased the ROS production with increasing doses. There is enough evidence that production of ROS, as a by-product of Dox metabolism, and promotion of oxidative stress play a role in Dox-induced cardiotoxicity due to the unique vulnerability of the heart (Singal and Iliskovic, 1998, Ludke et al. 2009). Cardiomyocytes depend on oxidative phosphorylation to execute their contractile function, where antioxidant reserve may already be tightly balanced with the ROS production under physiological circumstances (Doroshow et al. 1980; Ludke et al. 2009). Upon an insult such as Dox-induced oxidative stress in the heart, the cardiomyocyte becomes overwhelmed and more prone to oxidative damage. Since Dox-induced cardiomyopathy and heart failure is considered progressive, myocardial damage may start with the first dose of the drug and each administration can constitute additive or sequential damage with higher production of ROS (Elliott 2006).

The dose-dependent decrease in cardiomyocyte viability shown in the present study was also associated with a decrease in ATP production which was significantly accentuated with higher doses of Dox. In this regard, the MTT assay used as a measure of cell viability as well as the direct analysis of ATP levels, may also suggest mitochondrial impairment, since MTT assay assesses the reactions occurring in mitochondrial reductase enzymes of living cells where these organelles are mainly responsible for ATP synthesis. Mitochondria have already been suggested to play an important role in cell death due to Dox (Zhou et al. 2001; Lebrecht and Walker 2007; Wallace 2007).

Dox not only affected cardiomyocytes in a dose-dependent manner, but more prominently affected Sca-1 positive cells either derived from heart or bone marrow at the lowest subclinical dose of Dox. In these cells, Dox-induced a dose dependent decrease in cell viability as well as ATP levels, and increased both cell leakage and ROS production. Cardiac progenitor cells with cardiogenic potential have been recognized as a subset source of cells within the adult heart (Oh et al. 2003; Beltrami et al. 2003; Martin et al. 2004; Laugwitz et al. 2005; Rosenblatt-Velin et al. 2005; Moretti et al. 2006; Cai et al. 2008). Resident cardiac stem cells are a newer concept in heart physiology and still in debate. Although, their role in heart structure and function is poorly understood, different subsets of cells have been shown to be committed to form cells of cardiac lineage in support of tissue repair such as cardiomyocytes, smooth muscle cells, and endothelial cells under ideal conditions (Oh et al. 2003; Beltrami et al. 2003; Urbanek et al. 2005; Linke et al. 2005). Thus the depletion in circulating or cardiac Sca-1 positive cells even at the subclinical dose of Dox (0.01 μM) may compromise this repair system of the

heart. Dox-induced acute changes in Sca-1 positive cells could be potentially associated with the late onset cardiomyopathy. Although our findings showed that Sca-1 positive cells isolated from heart or bone marrow are more sensitive than cardiomyocytes to Dox-induced injury, the mechanisms for these findings are not understood at this point. Huang et al. (2010) concluded that the decreased number and accelerated senescence of cardiac progenitor cells in young “juvenile” mice exposed to clinical concentrations of Dox may later in life contribute to the Dox-induced heart dysfunction. Future research is needed to validate whether the premature cardiac progenitor cell loss seen in their study and supported by ours does happen in patients subjected to anthracycline treatment and may play a role in the late-onset cardiomyopathy. In addition, it can be hypothesized that the high ROS levels produced in Sca-1 positive cells after exposure to Dox may compromise the ability of cardiac or bone marrow stem cells to divide, differentiate and contribute to cardiac regeneration.

Dox-induced activation of stress-induced signaling pathways

In order to understand the temporal changes leading to cardiomyocyte loss, we studied the time-course changes due to Dox. At the supraclinical dose of Dox (10 µM), in these time-course studies there was an increase in ROS, followed by activation of the signaling messengers such as mitogen activated protein kinases (MAPK) and p53. This was later associated with an increase in apoptotic markers shown by increase in Bax/Bcl-xL ratio and caspase-3 activation, impairing cardiomyocyte viability. It was interesting to note that cardiomyocyte viability was

significantly reduced at 12 and 24 hours after Dox treatment when ROS production was significantly elevated above the 50% mark from Control.

In the present study, ERK MAPK was found to be high at all time points after Dox exposure, but with a peak at 6 hours and then declined. In addition, by using a pharmacological inhibitor for this pathway, we did not find any association with cell loss, suggesting that this elevation in ERK might play a different role. In Dox-induced cardiotoxicity, previous work from our laboratory has demonstrated that ERKs, indeed, are upregulated as an early adaptive and/or protective response (Lou et al. 2005). This change was transient and was followed by downregulation during the heart failure stage (Lou et al. 2005). Conversely, we have shown that phosphorylation of p38 and JNK MAPKs presented a gradual and persistent increase for up to 24 hours. Our findings are supported by previous studies where it has been reported that these MAPKs increase early and persist until the heart failure stage, suggesting that these pathways may play a dominant role in the progression of anthracycline-induced cardiomyopathy and heart failure (Lou et al. 2005; Lou et al. 2006). Conflicting results showing different patterns of MAPK activations have also been reported in response to Dox treatment (Spallarossa et al. 2006; Tokarska-Schlattner et al. 2010). The functional complexity and ambiguity of results could be a consequence of the temporal profiles of activation, feedback networks, intracellular compartmentalization, and signal complexity after different doses of Dox.In agreement with our findings, more recent studies have shown that oxidative stress caused by Dox activates signaling pathways and this will culminate in apoptotic cell death contributing to cardiomyopathy (Venkatesan et al. 2010; Ghosh et al. 2011). In this regard, it has already been suggested that ROS

activates MAPK family proteins (Lou et al 2005; Lou et al. 2006). Among them, p38 and JNK MAPKs may play critical roles in Dox-induced cell death pathways (Balachandar et al. 2010). In the present study, pharmacological inhibition of p38/JNK MAPKs abolished the increase in apoptotic markers Bax/Bcl-xL ratio, caspase-3 and PARP cleavage supporting the role of these kinases in Dox-induced apoptosis. Both p38 MAPK and JNK have been shown to induce Bax activation in human hepatoma HepG2 and porcine kidney LLC-PK1 cells exposed to various cell death agonists (Kim, Ryu and Song, 2006) and in cardiomyocytes exposed to Dox (Venkatesan et al. 2010). Furthermore, apoptotic cell death is usually mediated by a complex interplay of a number of pro-apoptotic and anti-apoptotic proteins, with factors such as Bcl-xL controlling the balance to guarantee resistance to apoptosis (Reeve et al. 2007). When the balance has overcome the favouring of apoptosis, formation of apoptosomes with subsequent activation of caspase will take place, with the active form of caspase-3 cleaving its substrate, PARP (Li et al. 1997; Liu et al. 2008). In support of our findings, studies on neutrophils further showed that MAPK inhibition can prevent Bax from heterodimerizing with Bcl-xL and other antiapoptotic factors (Gardai et al. 2004). Since cellular apoptosis can be at least partially responsible for the pathogenesis of Dox-induced cardiotoxicity (Reeve et al. 2007), *in vivo* studies have been conducted employing anti-apoptotic strategies to manage this complication with certain success (Kalay et al. 2006; Liu et al. 2008).

The stress-responsive tumour suppressor protein p53 plays a critical role in regulating either cell survival or death depending on the nature of cells and type of stress involved (Nithipongvanitch et al. 2007; Venkatesan et al. 2010). In Dox-

induced cardiotoxicity, high doses of Dox were shown to induce p53 activation in cardiomyocytes which was closely associated with apoptosis (Spallarossa et al. 2009, Yoshida et al. 2009). In the present study, phosphorylated p53 started to increase at 3 hours after Dox treatment, subsequent to the increase seen in ROS. In this regard, Yoshida and colleagues (2009) performed a study in neonatal rat cardiomyocytes with a 1 μM Dox dose and suggested that p53 activation is via oxidative DNA damage and it was also associated with apoptosis. In their study, pitavastatin attenuated Dox-induced cardiotoxicity through antioxidant effects. In our study, treatment with antioxidant Vit C and pifitrin-α, the pharmacological inhibitor of p53, abrogated Dox-mediated cardiomyocyte decrease in cell viability and increase in apoptotic markers. These findings indicate that the supraclinical dose of Dox is a potent inducer of p53 activation, and stimulates cardiomyocyte death in a p53-dependent manner.

In our study, Dox treatment to cardiomyocytes not only increased the direct ROS production as analyzed by DCFDA fluorescence, but also increased lipid hydroperoxides, and decreased the total antioxidant capacity of the cells with reduction of important antioxidant enzymes expression such as GSH-Px, Cu/Zn SOD and catalase. Dox-induced cardiotoxicity was already shown to disturb antioxidant defense systems as well as repair pathways (Swift et al. 2007; Kang 2007). Dox has also been reported to decrease the activity of the well known antioxidant enzymes GSH-Px, Cu/Zn SOD and catalase (Lim et al. 2004; Aries et al. 2004; Timolati et al. 2006). Although in the present study, at the dose and time of Dox exposure to cardiomyocytes, hemeoxygenase protein expression did not show any difference among different groups, a decrease in HO-1 has shown to

play a role in Dox-induced cardiotoxicity (Suliman et al. 2007; Bernuzzi et al. 2009). All of these findings support the increase in oxidative stress caused by an increase in free radical production and a decrease in diverse antioxidant enzymes and reserve sustaining the cardiac damage caused by Dox. Cardiomyocyte viability was significantly reduced at 12 and 24 hours after Dox treatment when ROS production was significantly elevated above the 50% mark from Control. This correlation suggests that there is a threshold for ROS to play its detrimental role.

As mentioned ealier, a link between increased oxidative stress induced by Dox and apoptotic cell death contributing to the cardiac dysfunction has gained substantial support (Mizutani et al. 2005; Danz et al. 2009). In our study, after Dox treatment for 24 hours, about 20% of the cells were dead as determined by trypan blue staining. Apoptotic cell death was found in 10-12% of cardiomyocytes since 12% of cells undergoing nuclear fragmentation and 10% presented annexin staining with PI exclusion representing the actual apoptotic cell death. In support of apoptosis, we also found an increase in pro-apoptotic protein Bax in cytosolic as well as in mitochondrial fractions which could be associated with the release of Cytochrome C from mitochondria to cytosol. Cytochrome C release has already been related to downstream pro-caspase processing and apoptosis (Reeve et al. 2007). These factors are considered to act as intermediates in the intrinsic apoptotic cell death pathway (Li et al. 1997; Gross et al. 1998) which may suggest that the mitochondrial pathway is contributing to Dox-induced cell death in our study.

Considerable data indicate that cardiomyocyte death through apoptosis, necrosis, and other forms where autophagy might take place as a primary

contributor to the progression of Dox-induced cardiomyopathy (Lebrecht and Walker, 2007). In fact, an increase in the autophagic marker LC3 II which is currently used as an indicator of the number of autophagossomes (Itakura and Mizushima 2010) after Dox exposure was seen in the present study. In this regard, autophagy in support of cell death was also very recently reported in Dox-induced cardiotoxicity (Lu et al. 2009; Kobayashi et al. 2010). The different types of cell death may result from the activation of various pathways that include oxidative stress, mitochondrial damage, DNA damage, and induction of pro-apoptotic proteins and cell membrane injury (Zhou et al. 2001; Lebrecht and Walker, 2007; Wallace 2007). We have shown that high dose of Dox induces cardiotoxicity that is mediated by a progressive increase in ROS levels, followed by activation of stress-induced pathways p38 and JNK MAPKs and p53 leading to apoptosis. In addition, since our results showed more cardiomyocytes dying (20%) compared to 10-12% undergoing actual apoptosis, it can be suggested that necrosis might also be taking place and all types of cell death contributed to the cardiomyocytes loss seen after a supraclinical dose of Dox.

Vit C protection against Dox-induced changes

Vit C is considered to be one of the most potent and least toxic antioxidants for humans (Sauberlich 1994). Since Vit C is readily soluble in water, it can serve as an antioxidant in the water phase inactivating biologically relevant radicals and other oxidants. Furthermore, Vit C is also well known to regenerate vitamin E (Vit E) from Vit E α-tocopheryl which serves as antioxidant in the lipid phase, in the

membranes (Amorati et al. 2002). Although earlier studies on animals have shown that Vit C reduced cardiotoxicity and prolonged life after Dox exposure (Fujita et al. 1982; Shimpo et al. 1991; Wold et al. 2005; Santos et al. 2007), there is no information about subcellular basis of this beneficial effect. Thus, our *in vitro* studies provide a better understanding on the molecular mechanisms of antioxidant therapy against Dox-induced changes.

Our data showed that the presence of Vit C (25, 50 and 100 µM) prior to the addition of Dox to cardiomyocytes was able to minimize oxidative stress and prevent the decrease in cell viability due to the drug toxicity. The most optimal beneficial effects of Vit C in cardiomyocytes were seen with a dose as low as 25 µM of the vitamin. On the other hand in Sca-1 positive cells, a higher dose of Vit C (50 µM) was required to see an optimal protection. This may be due to a higher sensitivity of Sca-1 positive cells to Dox-induced changes. We provide further evidence that oxidative stress plays a key role in Dox-induced cardiac injury which is mitigated by antioxidants. In addition, we have shown for the first time that Vit C mitigates Dox-induced oxidative damage not only in cardiomyocytes but also in cardiac and bone marrow stem cells.

As the Vit C exchange among different tissues and plasma is very well regulated (Sauberlich 1994), we studied the sodium-dependent Vit C transporter-2 (SVCT-2) in cardiomyocytes. It is also known that SVCT-2 in mammalian cells is the major transport system of ascorbate (Rajan et al. 1999). The present study provided evidence for the first time that Dox treatment downregulates SVCT-2 protein expression on the cardiomyocyte cell surface. Previous studies in different

cell types have also suggested that in addition to its membrane localization, SVCTs can also be found in intracellular compartments (Subramanian et al. 2004; Boyer et al. 2005). A specific reduction in SVCT-2 in cardiomyocytes implies that the entry of Vit C may be affected *in vivo* with Dox exposure impairing its intracellular distribution. Thus, the downregulation of SVCT-2 along with the associated decrease in the overall antioxidant capacity may also have accentuated Dox-induced cardiomyocyte damage. In this regard, we also noticed that SVCT-2 was present in the sarcolemma as well as vesicle compartments within the cardiomyocyte. The reduction in SVCT-2 seen in the present study may suggest that Dox could potentially influence the flux of other transporter-dependent exogenous defense molecules as well as influence their intracellular distribution. Since Vit C was able to blunt Dox-induced decrease in SVCT-2 as well as partially rescue the cardiomyocytes from Dox-induced injury, the importance of antioxidant co-treatment with Vit C is highlighted.

Upon insulin stimulation, GLUT4 is translocated to the plasma membrane where it facilitates the uptake of glucose (Pessin et al. 1999) and due to similarity in their structures it has already been shown to support the influx of oxidized Vit C (DHA) (Vera et al. 1994). We noticed a small but significant increase in GLUT4 after Dox treatment and no such Dox-induced increase was found when Vit C was present. Thus, it is likely that there is no increase in transport of oxidized Vit C when Dox and Vit C were present in combination. Furthermore, the entry of DHA into cells can be competitively inhibited by glucose (Vera et al 1994). In this regard, the lipid peroxidation caused by Dox has been reported to evoke an adaptive response resulting in the upregulation of glucose influx, most likely due to a shift in

the metabolic regulation to restore cellular energy (Hrelia et al. 2002; Strigun et al. 2012). Thus an increase in lipid peroxidation may have influenced Dox-induced change in metabolic regulation by favoring glucose transport through GLUT4 rather than oxidized Vit C.

In this study, Vit C (25 µM) delayed and reduced Dox-induced activation of stress-induced pathways, ROS production and the rise in apoptotic markers, partially rescuing cardiomyocyte loss. Our findings showed that Vit C was not only able to prevent apoptosis but also autophagy. Despite Vit C's major function as a water-soluble antioxidant, this vitamin also functions as a cofactor for enzymes involved in the biosynthetic reactions. Since N-Acetyl Cysteine (NAC), another water-soluble antioxidant also showed protection against Dox-induced changes in cardiomyocytes, it is likely that the benefitial effects of Vit C are due to its antioxidant properties. Furthermore, pre-treating the cells with Vit C, specifically prevented GSH-Px decrease caused by Dox and partially prevented the decrease in SOD, but had no effect on rescuing catalase. These data suggest that there is some selectivity in the beneficial effects of Vit C on antioxidant enzymes. Some other antioxidants such as probucol, calceolarioside and ginkgo biloba extract have already been shown to increase or preserve the endogenous antioxidant enzymes which are decreased by Dox (Li T and Singal 2000; Kim et al. 2006; Liu et al. 2009; Zhu et al. 2009; Asensio-Lopez et al. 2011) suggesting that antioxidant therapy may be a reliable cardioprotective strategy.

As described earlier, in order to achieve physiological blood concentrations of 60–100 µM, daily oral doses of around 400 mg of Vit C are nedeed (Padayatty et al. 2004). As, in our study, Vit C was delivered in its active form directly to cells,

titration of an appropriate oral dose to achieve target blood levels of Vit C appears to be of paramount importance. In order to sustain higher physiological concentrations of this vitamin in the circulating blood, it appears that a frequent oral intake of physiological dose may be one alternative (Verrax and Calderon 2009). Furthermore, cancer patients who generally present lower concentrations of ascorbic acid in plasma (Goncalves et al. 2005) could have additional benefits from Vit C supplementation. Thus, we provide a stronger basis for the design of further animal and clinical studies on Vit C as adjuvant therapy in Dox-induced cardiomyopathy.

VII. Conclusions

In conclusion, our findings have provided additional understanding on the mechanisms involved in Dox-induced cardiomyopathy (Figure 30). Dox causes an increase in reactive oxygen species (ROS), lipid peroxidation and a decrease in antioxidant defence in cardiomyocytes leading to detrimental oxidative stress. Dox-induced decrease in Sodium-dependent Vit C transporter 2 (SVCT-2) may also contribute to decrease Vit C entry and consequently enhance oxidative stress. Stress-induced pathways p38 and JNK MAPKs and p53 are activated after Dox-induced increase in ROS levels, and they may participate in a positive feedback leading to more ROS production and enhancement of apoptic-cell death. Subcellular changes associated with membrane injury, metabolic disturbances, activation of apoptosis and autophagy indicate that Dox influences multiple targets that may serve as mediators of this unique cardiomyopathy. Dox exposure not only damages cardiomyocytes, but more prominently also depletes Sca-1 expressing cells with a dose-dependent decrease in cell viability, reduction in the ATP levels and increase in ROS production and cell leakage. As antioxidant Vit C and/or NAC efficently decrease the ROS produced by Dox and partially rescue all Dox-induced changes to cardiomyocytes as well as Sca-1 positive cells, there is additional support that oxidative stress plays a major role in the progression of Dox-induced cardiomyopathy. Pharmacological inhibitors for p38/JNK and p53 also inhibit apoptosis and partially rescue cardiomyocyte loss; it is likely that modulation

of these pathways may also result in an alternative approach for cardioprotection against Dox-induced cardiomyocyte damage. Although the precise mechanism underlying Dox-induced toxicity to Sca-1 positive cells as well as their actual role in Dox-induced heart failure remains elusive, a lack of replacement cardiomyocytes would be expected to contribute in the delayed cardiomyopathy. Thus this study provides further evidence for cardioprotective strategies against Dox-induced oxidative stress leading to cardiomyopathy. However, pre-clinical studies and clinical trials designed to determine whether these in vitro findings could be extrapolated to the expected clinical outcomes are needed.

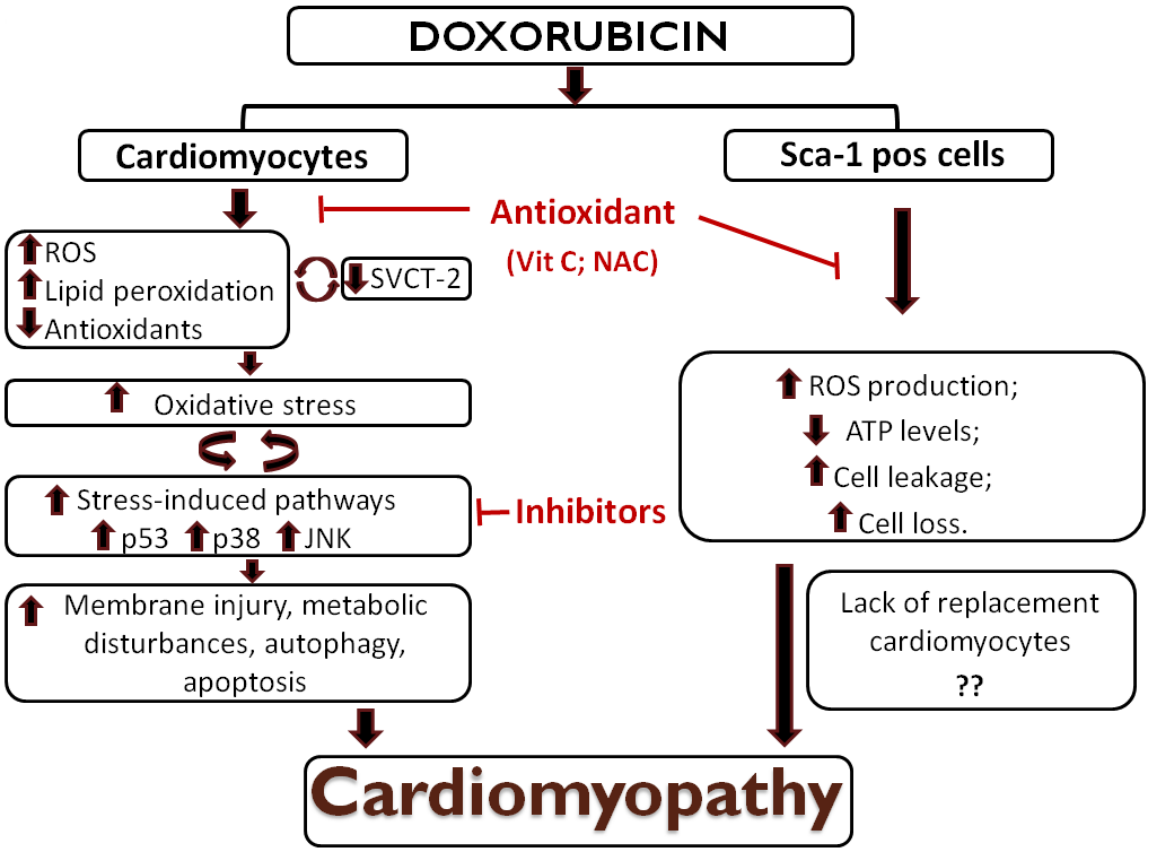

Figure 30. Schematic representation of Dox-induced subcellular changes leading to cardiomyopathy. Dox induces oxidative stress and downregulates Sodium-dependent Vit C transporter-2 (SVCT) causing damage to cardiomyocytes and Sca-1 positive cells. It involves the activation of p38 and JNK MAPKs, and p53 coupled with subcellular disturbances. Loss of cardiac progenitor cells (Sca-1 positive cells) thus lack of replacement cardiomyocytes may contribute in delayed Dox-induced cardiomyopathy. Vitamin C and/or N-AcetylCysteine (NAC) blunt most of Dox-induced changes, and improve cardiomyocyte and Sca-1 positive cell survival. Pharmacological inhibitors for p38/JNK and p53 also alleviate Dox-induced cardiomyocyte apoptosis.

VIII. References

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