Cardioprotective role of Vitamin C in the mitigation of oxidative/nitrosative stress in Doxorubicin-induced cardiotoxicity

By

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ABSTRACT

Doxorubicin (Dox) cardiotoxicity is a serious concern in its use for the treatment of cancers. Oxidative stress (OS) and nitrosative stress (NS) are suggested to be the main causes of this cardiotoxicity. We used rat cardiomyocytes as well as whole animals to characterize changes in Dox-induced apoptosis, inflammation, OS/NS, cardiac structure/function and Vitamin C (Vit C) transporter proteins and the mitigation of these effects by Vit C.

In cardiomyocytes, Dox (10 μM) caused an increase in both superoxide radical and Nitric oxide (NO), resulting in the generation of peroxynitrite, protein nitration and nitrosylation. Dox increased Nitric oxide synthase (NOS) activity via the upregulated protein expression of inducible NOS (iNOS) and the altered protein expression as well as activation of endothelial NOS (eNOS). Dox also reduced the stability of dimeric eNOS and increased ratio of monomeric/dimeric eNOS. Dox-induced increase in TNF-α and a reduction in IL-10 was also noted. These Dox-mediated alterations in cardiomyocytes were attenuated by Vit C (25 μM) pretreatment.

In the rat model of Dox-induced cardiotoxicity (cumulative dose, 15mg/kg), both systolic and diastolic functions were decreased and there was structural damage in hearts. These changes were associated with increased levels of myocardial reactive oxygen species; reduction in anti-oxidant enzyme activities (SOD, GPx and catalase); increased expression of apoptotic proteins (Bax, Bnip-3, Bak and Caspase-3) and inflammation. An increase in OS/NS was indicated by an increase in superoxide, protein carbonyl formation, lipid peroxidation, NO, NOS activity, protein nitrosylation and iNOS expression. Dox increased the levels of cardiac TNF-α, IL-1β and IL-6 while the expression of Vit C transporter proteins (SVCT-2 and Glut-4) was reduced. Vit C (50

mg/kg) prevented all these changes, improved Dox-mediated systolic and diastolic dysfunctions, prevented structural damage and improved animal survival.

These results suggest that Vit C provides cardioprotection by reducing OS/NS as well as inflammation via modulation of Dox-induced increase in the NO levels and NOS activity. The molecular details in this study provide a rationale for a prophylactic use of Vit C to reduce chemotherapy induced cardiotoxicity.

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LIST OF FIGURES

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Publication

1. G Akolkar, A.K Bagchi, P Ayyappan, D. S. Jassal, and P. K. Singal. Doxorubicin induced nitrosative stress is mitigated by vitamin C via the modulation of nitric oxide synthases. *American Journal of Physiology.Cell Physiology* 312: 418-427, 2017.

2. G Akolkar, D Dias, P Ayyappan, A. K Bagchi, D. S Jassal, V M Salemi, M C Irigoyen, K DeAngelis, P K. Singal. Attenuation of oxidative/nitrosative stress and inflammation by Vitamin C in Doxorubicin-induced cardiomyopathy; American Journal of physiology – heart and circulatory physiology (In press) 2017.

LIST OF ABBREVIATIONS

ABTS- 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
$A^{-\bullet}$ - ascorbate radical
AH^- - ascorbate
AMPK- adenosine monophosphate activated protein kinase
BH4- tetrahydrobiopterin
BSA- bovine serum Albumin
BW- body weight
Ca^{2+} - calcium
cGMP- cyclic guanosine monophosphate
CVD- cardiovascular diseases
Cyt C- cytochrome C
DAF-2DA - diaminofluorescein-2diacetate
DHA- dehydroascorbic acid
DHE – dihydroxyethidium
DISC- death inducible signaling complex
DKG- 2,3-diketogulonic acid
DMF- dimethyl formamide
DMSO dimethyl sulfoxide
Dox- doxorubicin
DTT- dithiothreitol
E/A- early/active filling
ECL – enhanced chemiluminescence
EF- ejection fraction
ELISA- enzyme linked immunosorbent assay
eNOS- endothelial nitric oxide synthase
FADD- Fas associated death domain
FBS- fetal bovine serum
Fe^{2+}- ferrous iron
Fe^{3+}- ferric iron
FS- fractional shortening
GAPDH- glyceraldehyde 3-phosphate dehydrogenase
GLUT-4- glucose transporter -4
GPx – Glutathione Peroxidase
GSH- reduced glutathione
GSSG- glutathione
HF- heart failure
HIF- hypoxia inducible factor
HPF- hydroxyphenyl fluorescein
HR- heart rate
HRP- horseradish peroxidase
H_2O_2- hydrogen peroxide
IL – interleukin
IMM- inner mitochondrial membrane

IRE- iron regulatory element
IRP- iron regulatory protein
iNOS- inducible nitric oxide synthase
IVRT- isovolumic relaxation time
KDa- kilo Dalton
LC3- light chain 3
LT- low temperature
LTCC- L type calcium channel
LV- left ventricular
LVEF- left ventricle ejection fraction
MMP- mitochondrial membrane permeability
MPTP- mitochondrial permeability transition pore
MTT - (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide)
MUGA- multigated acquisition scans
NADPH- nicotinamide adenine dinucleotide phosphate
nM- nano molar
NO- nitric oxide
NOS- nitric oxide synthase
NT- nitrotyrosine
O_2- molecular oxygen
OMM- outer mitochondrial membrane
PAGE - polyacrylamide gel electrophoresis
PARP- poly ADP ribose polymerase
PBS – phosphate buffered saline
PI- propidium iodide
PKB- protein kinase B
PMSF- phenylmethylsulfonyl fluoride
pM- pico molar
PSA- prostrate specific antigen
PVDF- polyvinylidene difluoride
RAS- renin angiotensin system
RIP- receptor induced protein
RNS- reactive nitrogen species
ROS- reactive oxygen species
RT- room temperature
RyR- ryanodine receptor
SDS - sodium dodecyl sulfate
Ser- serine
SERCA- sarcoendoplasmic reticulum calcium ATPase
sGC- soluble guanylate cyclase
SOD- superoxide dismutase
$O_2^{-\bullet}$ - superoxide anion
SVCT-2- sodium dependent vitamin C transporter
TBARS- thiobarbituric acid reactive substances
TBST - tris buffered saline with Tween-20
TCA- trichloroacetic acid

TfR- transferrin receptor
Thr- threonine
TNF-α- tumor necrosis factor- α
TOH- tocopherol
Top I - topoisomerase I
Top II- topoisomerase II
TRADD- TNFα receptor associated death domain
TRAP- total antioxidant capacity of plasma
Vit C- vitamin C
Vit E- vitamin E

I. INTRODUCTION

The number of cancer survivors has significantly increased world-wide as a result of earlier detection as well as better therapeutic management using a combination of radiation, chemotherapy and surgery (Albini el al, 2010; Truong et al, 2014; Zhang et al, 2016). However chemotherapy induced cardiotoxicity is a major concern in cancer therapeutic practice and remains the chief factor limiting optimal drug delivery to cancer patients (Zhang et al, 2009). Anthracyclines such as Doxorubicin (Dox) are very potent chemotherapy drugs used for treatment of a variety of cancers such as breast cancer, ovarian cancer, lymphoma, sarcoma and pediatric leukemia (Lefrak et al, 1973; Ludke et al, 2009; Singal and Iliskovic, 1998). Use of Dox is associated with the development of dose dependent, cumulative asymptomatic cardiotoxicity that presents even after many years of cessation of chemotherapy. Dox-induced cardiotoxicity may lead to cardiac dysfunction and cardiomyopathic changes resulting in severe heart failure and death (Wallace et al, 2003; Yeh et al, 2004). The prevalence of cardiovascular complications has increased in cancer survivors accounting for about 33% deaths arising from chemotherapy associated heart failure (Lefrak et al, 1973; Vejpongsa and Yeh, 2014; Zhang et al, 2016).

With this increasing number of cancer survivors, development of protective strategies and beneficial therapies to target against Dox-induced cardiotoxicity needs dire attention. Several approaches for better prevention, early monitoring and treatment approaches have been atempted for reduction of cardiotoxic side effects. Some of the approaches include development of less toxic derivatives, a better delivery of anthracyclines, identification of pre-existing risk factors, use of cardioprotective agents such as dexrazoxane, use of conventional heart failure therapy

drugs (Andreadou et al, 2007; Granados-Principal et al, 2010; Oktem et al, 2012; Vincent et al, 2013). However, there is still no consensus on the best approach or on a satisfactory clinically applicable preventive strategy to attenuate Dox-mediated cardiotoxicity. Thus Dox-induced cardiotoxicity presents a serious clinical problem affecting overall morbidity, quality of life and survival of cancer patients. Amongst the pleothra of agents investigated for cardioprotective effects, study of the benefical effects of vitamin C (Vit C) is of special interest and of clinical relevance owing to its reduced plasma levels in various pathological conditions including cancer (Mayland et al, 2005; Vita et al, 1998). This thesis focuses on the elucidation of mechanisms involved in Dox-induced cardiotoxicity as well as investigating the beneficial role of Vit C.

Although Dox-induced cardiotoxicity is associated with an alteration of several cellular events including increased generation of reactive oxygen species (ROS), lipid peroxidation, mitochondrial dysfunction, and calcium overload leading to cellular oxidative stress as well as activation of various cell death pathways such as apoptosis and necrosis, the exact mechanism underlying this cardiotoxicity is not still completely understood (Ludke et al, 2009; Shi et al, 2011; Zeglinski et al, 2011). There is increasing evidence for oxidative stress as a major mechanism involved in Dox-induced cardiotoxicity (Ludke et al, 2009; Shi et al, 2011; Singal and Iliskovic, 1998; Zeglinski et al, 2011; Zhang et al, 2009). In addition to oxidative stress, generation of nitrosative stress through increased production of potent reactive nitrogen species (RNS) such as peroxynitrite as a result of reaction of superoxide with nitric oxide (NO) (Akolkar et al, 2017; Mukhopadhyay et al, 2009; Rochette et al, 2015; Weinstein et al, 2000) also remains a likely possibile cause of this cardiotoxicity. Increased levels of cardiac NO were observed during the progression of Dox-induced cardiomyopathic changes (Sayed-Ahmed et al, 2001). This can be via the activation of inducible nitric oxide synthase (iNOS) under pathological

stimuli such as stress or inflammatory cytokines (Dimmeler and Zeiher, 1997; Grisham et al, 1999; Kroncke et al, 1997; Singh and Gupta, 2011). Increased peroxynitrite leads to nitration of many contractile and calcium handling proteins and may result in cardiac dysfunction (Mihm et al 2002; Weinstein et al, 2000). Prolonged oxidative/nitrosative stress also acts as a trigger for the development of acute and chronic inflammation (Salvemini et al, 2006) leading to heart failure (Ferdinandy et al, 2000). Additionally, Dox affects endothelial nitric oxide synthase (eNOS) causing twofold damage: i) promoting increased generation of superoxide anion and ii) reducing bioavailable NO which is crucial for maintaining cardiovascular function (Neilan et al, 2007). Furthermore these cellular changes can trigger death of cardiomyocytes; however, exact details of these processes in Dox-mediated cardiac dysfunction remain to be elucidated. As oxidative stress is suggested to be the basis of Dox-induced cardiotoxicity, a strategic use of antioxidant(s) to reduce oxidative/nitrosative stress can also be a viable strategy for the mitigation of pathological conditions in Dox-induced cardiotoxicity. This aspect is also the focus of the present thesis.

Clinical trials using Vit C or other antioxidants have so far provided inconclusive results. This inconsistency may be due to a number of factors such as the dose and duration of the treatment used, end points evaluated and comorbidities in the patient population involved. In this regard, the time of administration of Vit C is critical (Farbstein et al, 2010). Moreover, most studies have investigated these effects in already sick or at risk populations, where extensive damage may have already occurred. Oral supplementation of Vit C to healthy, well-nourished population having normal plasma Vit C does not provide any additional benefits to raise the plasma levels of Vit C (Levine et al, 1999). Thus supplementation of Vit C can be beneficial in patients with increased generation of ROS and compromised antioxidant defense such as cancer

patients. Several studies of cancer patients have shown increased production of ROS while reduction in antioxidant enzyme activities and plasma Vit C levels (Hoffman, 1985; Kasapovic et al, 2010; Weijl et al, 2004; Yeh et al, 2005). Hence supplementation of Vit C in cancer patients undergoing chemotherapy may be beneficial in reducing oxidative/nitrosative stress mediated cardiotoxicity and enhance patient's well-being (Riordan et al, 2005). While Vit C may be beneficial to normal cell, adverse effects of its antioxidant property on the anti-tumor efficacy of anticancer drugs have raised doubts for its use as a protective agent in Dox-induced cardiotoxicity. Nevertheless, studies have shown a dissociation of anti-tumor mechanism of Dox from oxidative stress. Furthermore, Vit C does not alter the efficacy of Dox and antioxidants have been reported to increase the survival of Dox-treated animals (Block et al, 2007; Simone et al, 2007; Siveski-Iliskovic et al, 1995). In fact high oxidative stress generated during chemotherapy can interfere with the cytotoxic effects of chemotherapy drugs (Conklin, 2009). High dose intravenous Vit C is gaining popularity as adjuvant chemotherapy agent to increase the efficacy of chemotherapy as well as provide cardioprotective effects (Drisko et al, 2003). However, the effect of Vit C on nitrosative stress in relation to cardiac function and structure in Dox mediated cardiotoxicity has not been described yet.

As a water soluble antioxidant, Vit C is transported into cells in its reduced as well as oxidized forms as ascorbic acid and dehydroascorbic acid (DHA) by sodium dependent transporter (SVCT) or glucose transporters (GLUT) respectively (Corti et al, 2010; Rivas et al, 2008). In isolated cardiomyocytes, Dox has shown to reduce protein expression of SVCT-2 and GLUT-4 (Ludke et al, 2012[b]). Hence it can be anticipated that the reported reduction in the cellular levels of Vit C in cancer patients may be as a result of downregulation of Vit C

transporters. However, study of Vit C transporters in the hearts of Dox treated animals is needed for a complete understanding of Dox-mediated toxicity.

The current work examined in detail the molecular processes involved in Dox-induced nitrosative stress in relation to its cardiotoxic effects in isolated cardiomyocytes as well as *in vivo* in rats. We characterized the effects of Dox on nitrosative stress in isolated cardiomyocytes and in rats via monitoring increased generation of peroxynitrite, alteration in NOS activity as well as protein expression, upregulation of iNOS and enhanced protein nitration/nitrosylation. We also observed Dox-mediated increase in the levels of inflammatory cytokines in Dox-treated cardiomyocytes. Dox also reduced the expression of cardiac Vit C transporter proteins. Using Vit C, we observed a beneficial effect in reducing Dox-mediated nitrosative stress and inflammatory cytokines, while upregulating the expression of Vit C transporter proteins in isolated cardiomyocyte and in rats. Furthermore, oxidative stress parametes were also studied to have a complete understanding of oxidative/nitrosative stress mechanisms. Dox mediated increase in ROS and reduction in antioxidant enzyme defense was reduced by Vit C.

Based on the results obtained, it is suggested that Dox-induced oxidative/nitrosative stress has a role in cardiac injury affecting cardiac structure-function and viability. Furthermore, Vit C attenuated Dox-mediated oxidative/nitrosative stress and provided protection by improved cardiac structure, function and hence survival of Dox-treated animals.

II. LITERATURE REVIEW

1. Doxorubicin

1.1 Background

Doxorubicin (Dox), also known as Adriamycin, is an anti-tumor drug that was isolated in early 1960 from a pigment producing fungus *Streptomyces peucetius* var. *caesius* (Minotti et al, 2004; Weiss, 1992; Young et al, 1981). Since late 1960s, Dox has been used in clinical practice as a frontline agent for the treatment of various cancers such as childhood cancers, acute leukemia, Hodgkin's and non-Hodgkin's lymphoma, solid tumors, soft tissue sarcoma and breast cancer (Lefrak, 1973; Singal and Iliskovic, 1998). It is administered either as a single agent or in combination with other chemotherapeutic drugs (Minotti et al, 2004; Lipshultz, 1999). Unfortunately, as with other chemotherapy drugs, administration of Dox is also accompanied by adverse drug reactions such as compromised immune system, reduction in white cell count, nausea, vomiting, alopecia and cardiac effects. Most of these side effects are temporary and are improved soon after the cessation of the drug treatment. However, cardiovascular side effects leading to heart failure are a serious concern (Lefrak et al, 1973; Singal and Iliskovic, 1998). As the number of cancer survivors have increased significantly because of early tumor detection and improved chemotherapy regime, this has also increased their vulnerability to the development of cardiomyopathy resulting in compromised quality of life, morbidity and mortality (Chung and Youn, 2016; Lipshultz et al, 2015). Chemotherapy mediated heart failure (HF) is the most prevailing cause for death in long term and childhood cancer survivors accounting for about 33% deaths in these patients (Vejpongsa and Yeh, 2014; Zhang et al, 2016). Despite the life threating cardiotoxicity manifested by Dox, it is still in clinical use and included in chemotherapy

regimens. Hence approaches for a reduction of Dox-mediated cardiotoxicity need to be undertaken.

1.2 Structure

Chemically, Dox is composed of aglyconic moiety consisting of tetracyclic rings and a sugar moiety. The tetracyclic rings contain quinone-hydroquinone groups in their ring B and C. Ring D has substitutions with methoxy group at C-4 position; a short side chain at C-9 and carbonyl group at C-13. Ring A has sugar moiety, daunosamine (3-amino-2,3,6-trideoxy-L-fucosyl), attached at C-7 by a glycosidic bond. (Figure 1) (Tacar et al, 2012). The chemical structure of Dox is very similar to another anthracycline, Daunorubicin, also isolated from *Streptomyces peucetius*. The only difference between the structures of these two chemotherapy drugs is the side chain which terminates in primary alcohol for Dox, whereas it is a methyl group for daunorubicin (Minotti et al, 2004; Young et al, 1981). Despite this minor difference in the structure of these two anthracyclines, a major difference in their antitumor activity is evident (Minotti et al, 2004; Takemura and Fujiwara, 2007). While Daunorubicin is restricted for use in acute lymphoblastic or myeloblastic leukemias, Dox is a broad spectrum chemotherapy drug used for the treatment of a variety of cancers (Strauss, 1978; Takemura and Fujiwara, 2007).

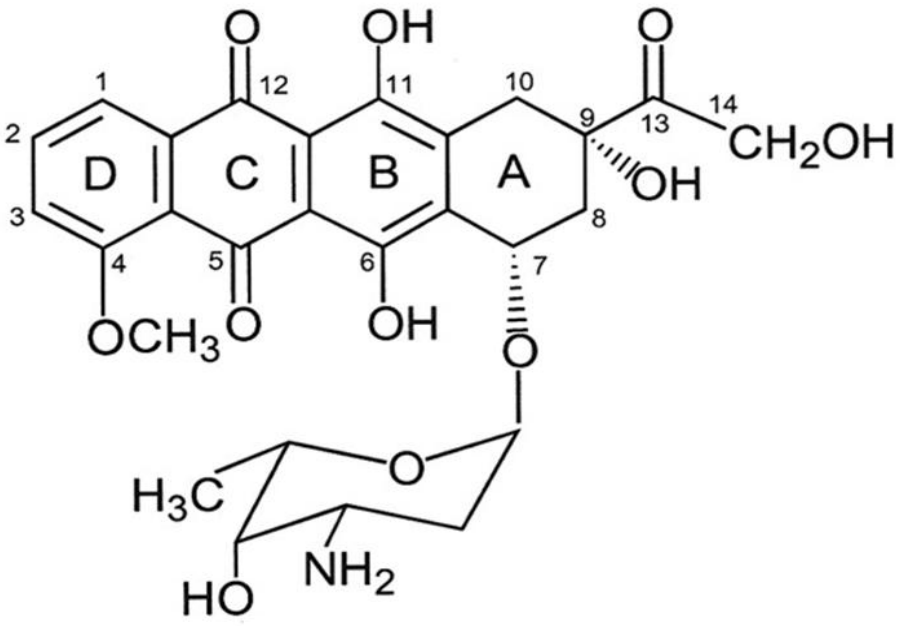

Figure 1: Chemical structure of Doxorubicin

(Minotti et al, 2004)

1.3 Pharmacokinetics

After the intravenous infusion of Dox, it has a multiphasic disposition as a result of rapid distribution phase and a slow clearance phase. It has a half-life of about 3-5 minutes in the plasma, indicating a rapid uptake of the drug in the cells and clearance from plasma. Dox, being lipophilic in nature, can enter cells via passive diffusion (Hilmer et al, 2004). The elimination of the drug from the tissues takes a far longer time as compared to its uptake (Minotti et al, 2004; Shi et al, 2011). It has a long half-life of about 24-36 hours in the body and about 48 hours in the heart. Intracellularly, the majority of the Dox is present in bound form to proteins and DNA thereby resulting in 10-500 fold increase in the intracellular concentration compared to the extracellular concentrations (Minotti et al, 2004). As a result of its intercalation in DNA, the concentration of nuclear Dox can reach to saturation level of 340 µM, about 50 fold higher than

the cytosolic concentration. Similarly, the concentration of Dox is 200-500 times higher in white blood cells and bone marrow as compared to the plasma (Strauss, 1978; Young et al, 1981).

Dox can accumulate in all the tissues except brain due to its inability to cross the blood-brain barrier. The highest accumulation of Dox is observed in liver most likely due to its role in metabolism and drug clearance (Kivisto et al, 1995). In liver, Dox undergoes biotransformation by chemical reduction of the ketone group at C-13 to hydroxyl group (Shi et al, 2011) as well as through a series of reactions such as glycosidic and reductive cleavage, O-sulfation, O-demethylation and O-glucuronidation (Tacar et al, 2013). These modifications are essential for the excretion of Dox, mediated through the hepatobiliary pathway (Licata et al, 2000). A small percentage, about 5-12%, of the drug is excreted in urine after its administration. The initial 10-20% of drug is excreted in faeces after 24 hours of infusion, about 50 % of the drug is excreted after about 150 hours; and the remainder of the drug is excreted over a period of about a week after the treatment (Maessen et al, 1988; Tacar et al, 2013). The alcohol metabolite of Dox, Doxorubicinol, is more toxic and results in irreversible toxicity (Licata et al, 2000; Minotti et al, 2000).

1.4 Anti-cancer mechanism

Dox-mediated cytotoxicity in tumor cells is multifactorial causing inhibition of DNA replication and RNA transcription, DNA cross linking, lipid peroxidation, free radical generation and inhibition of topoisomerase II (Top II) (Gewirtz, 1999; Minotti et al, 2004; Takemura and Fujiwara, 2007). However, inhibition of Top II is the basis of the anti-cancer action of Dox. Topoisomerases are evoultionary conserved enzymes that cause transient DNA breaks resulting in conformational change in DNA in all cell types (Minotti et al, 2004). Topoisomerase I (Top I) results in single strand breaks in DNA whereas, Top II results in double strand breaks in DNA.

This action of Top II causes uncoiling of super-coiled DNA which is crucial for DNA replication (Binaschi et al, 2001; Nitiss, 2009). Tumor cells being rapidly dividing cells have elevated expression of Top II (Vejpongsa and Yeh, 2014). Dox forms a ternary cleavage complex by binding both Top II and DNA (Tewey, 1984). The stabilization of the Dox-Top II- DNA complex interferes with DNA replication inducing DNA double strand breaks, the latter of which may trigger cell death in tumor cell (Tewey, 1984). Additionally, binding of Dox to DNA can also lead to cell death independent of inhibition of Top II (Swift et al, 2006). Furthermore tumor cells have higher accumulation of the drug compared to normal cells due to the higher rate of endocytosis, uptake as well as release of free Dox (Young et al, 1981).

1.5 Dox-induced cardiotoxicity

Although Dox generally targets rapidly dividing cells, being a lipophilic molecule it can easily penetrate, accumulate and exert its cytotoxic effects in all tissues except for the brain. (Tacar et al, 2013). However, the toxicity manifested in the heart is more pronounced and fatal compared to other tissues. The heart is exclusively dependent on the oxidative phosphorylation as its energy source has high generation of ROS. Therefore, the equilibrium between generation of ROS/RNS and their removal via antioxidant defense is tightly balanced in the heart (Ludke et al, 2009). This cardiac antioxidant balance is altered by Dox as a result of two synergistic events: i) enhanced generation of ROS as a result of cycling of Dox from its quinone to semi-quinone form and mitochondrial ROS production and ii) reduction in the antioxidant defense system of the heart (Jungsuwadee, 2016; Li et al, 2000). Dox decreases the protein levels and activities of various antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase responsible for scavenging free radicals (Li et al, 2000; Ludke et al, 2012[b]; Siveski-Iliskovic et al, 1994). This imbalance triggers activation of various cell death pathways. Thus

oxidative stress generated in the heart is about 10 times higher in comparision to other tissues such as liver, kidney and spleen (Davies and Doroshow, 1986).

Another important reason for the higher vulnerability of heart to develop Dox-mediated cardiotoxicity is due to drug accumulation in the mitochondria probably as a result of its ability to form complex with cardiolipin (Anderson and Arriaga, 2004; Parker et al, 2001). As cardiomyocytes have a high number of mitochondria comprising of about 40% of its total cellular volume, the concentration of Dox in mitochondria was observed to be hundred times higher than the plasma concentration (Sarvazyan, 1996). Hence mitochondria led increased generation of ROS via redox cycling of Dox (Jungsuwadee, 2016) and inhibition of electron transport chain (Rochette et al, 2015) becomes contributing factors to Dox-induced cardiotoxicity. Furthermore, cardiomyocytes are terminally differentiated cells with limited capacity to regenerate. Therefore, any loss of cardiomyocytes as a result of activation of various cell death pathways results in a permanent loss of cardiomyocytes. This deficit further contributes to the contractile dysfunction (Ludke et al, 2009).

1.6 Manifestation of cardiotoxicity

Cardiotoxicity is a continuous process initiated by cardiomyocyte injury leading to irreversible myocardial structural changes, and asymptomatic left ventricular dysfunction which progresses to overt heart failure (Cardinale and Cipolla, 2016). Alterations in cardiac functions as a result of Dox-induced cardiotoxicity affect morbidity in cancer survivors and can be a serious side effect associated with the use of Dox (Raj et al, 2014).

Electron microscopic examination reveals multiple levels of Dox-mediated injury affecting the structure of cardiomyocytes with a partial or total loss of myofibrils, distension of sarcoplasmic reticulum, vacuolization and chromatin disorganization (Billingham et al, 1978;

Bristow et al, 1981; Ferrans et al, 1997; Lefrak et al, 1973; Singal et al, 2000). These changes are progressive and lead to death of cardiomyocyte (Singal et al, 2000). Clinical presentation of cardiotoxicity can be categorized into acute and chronic cardiotoxicity.

1.6.1 Acute cardiotoxicity

Acute cardiotoxicity is transient and occurs during or immediately after the drug infusion. It is observed in about 11% of the patients (Lefrak et al, 1973) and is manifested as alterations in electrocardiogram such as changes in ST-T wave and QT prolongation. It can also cause hypotension as well as arrhythmia such as supraventricular tachycardia and ventricular premature beats. In rare conditions, it can cause pericarditis and myocarditis (Minotti et al, 2004; Schimmel et al, 2004). These cardiac alterations are either reversible or clinically manageable and usually resolve within days of cessation of Dox treatment (Lefrak et al, 1973; Wouters et al, 2005).

1.6.2 Chronic cardiotoxicity

The chronic effects of Dox are dose-dependent and irreversible with a poor prognosis (Lefrak et al, 1973; Von Hoff et al, 1979). The chronic effects can be classified as early or delayed onset cardiotoxicity. Early onset of cardiotoxicity is manifested within a year of cessation of Dox, whereas delayed onset cardiotoxicity can be manifested years after the administration of Dox (Takemura and Fujiwara, 2007). The effects are seen after discontinuation of the drug and are manifested as electrophysiological changes, decrease in left ventricular function and overt signs of CHF. The clinical symptoms of Dox-induced cardiomyopathy are similar to those observed for other types of CHF such as fatigue, sinus tachycardia, tachypnea, cardiac enlargement and pulmonary edema (Raj et al, 2014; Schimmel et al, 2004; Takemura and Fujiwara, 2007; Wouters et al, 2005). The incidence of Dox-induced cardiotoxicity could be as

high as 50% (Von Hoff et al, 1979). Late onset effects can occur up to even 20-30 years after a discontinuation of the drug (Goldberg et al, 2012; Steinherz et al, 1991). Thus long-term monitoring of the cardiac function is important for patients receiving chemotherapy with Dox as well as other anthracyclines (Singal and Iliskovic, 1998).

1.6.3 Risk factors

The development of Dox-induced cardiotoxicity is dependent on multiple factors such as the cumulative dose of the drug, age, prior irradiation, prevalence of other cardiovascular conditions and risk factors (Raj et al, 2014; Schimmel et al, 2004). Among these, cumulative dose is an important factor, showing a strong correlation with the incidence of CHF. Routinely Dox is administered intravenously at a dose of 60-75 mg/m^2 every three weeks (Von Hoff et al, 1979). The frequency of occurrence of CHF increases with the increased cummulative dose of Dox. The incidence is about 4% at a cummulative dose of 450-550 mg/m^2 of Dox whereas; increase in the dose to 600 mg/m^2 increased the incidence of HF to 36% (Lefrak et al, 1973; Singal and Iliskovic, 1998). Nevertheless, the signs of cardiotoxicity are also manifested at a cumulative dose of 300 mg/m^2 as a result of compounded effect of prevalence of other risk factors (Chung et al, 2013; Chung et al, 2016). Thus the minimum cumulative dose required to develop cardiotoxicity varies among different individuals depending on the presence of other risk factors (Ludke et al, 2009).

Radiation therapy used for the treatment of cancer also increases the risk of cardiotoxicity. Radiation may cause pericarditis as a result of enhanced fibrous thickening of the pericardium. It can also cause myocardial fibrosis resulting in diastolic dysfunction (Hong et al, 2010; Rochette et al, 2015). Simultaneous usage of radiation therapy and chemotherapy result in enhanced cardiotoxic effects (Rochette et al, 2015). The use of radiation therapy enhances the

probability of development of CHF in patients even at a low dose of 300 mg/m^2 of Dox (Schimmel et al, 2004).

Similarly age enhances the probability for incidence of CHF. The risk of developing cardiotoxicity was observed to be higher in older patient population as well as in patients younger than 18 years of age. Hence it is recommended not to exceed the maximum cumulative dose of 300 mg/m^2 Dox for patients younger than 18 years of age (Jungsuwadee, 2016; Lipshultz et al, 2014).

1.7 Monitoring and markers of cardiotoxicity

Clinical or sub-clinical myocardial dysfunction can be identified by routine use of cardiac imaging studies. Evaluation of cardiac function in patients receiving Dox needs to be frequently monitored during the treatment as well as long term followup after the cessation of treatment to minimize the development of cardiotoxicity (Takemura and Fujiwara, 2007). Endocardial biopsy determines anthracycline mediated cardiac damage via monitoring loss of myofibrils, distension of sarcoplasmic reticulum, vacuolization and fibrosis (Billingham et al, 1978; Bristow et al, 1981; Singal and Iliskovic, 1998). This technique provides an accurate guide for rational dose optimization and extent of myocardial damage (Bristow et al, 1981; Torti et al, 1983). Although endocardial biopsy has both high sensitivity and specificity; it is an invasive procedure. Additionally the expertise and cost required to perform this procedure limits its use for routine screening (Takemura and Fujiwara, 2007).

Angiocardiography with indium^{111}-labelled antimyosin monoclonal antibody is useful for detecting cell death. The uptake of antibody in Dox-induced cardiomyopathy is correlated to the total dose of Dox and the ejection fraction (EF). Hence this technique can be useful to detect EF as well as abnormalities associated with cardiac wall motion. Although the sensitivity of this test

is high, it is not very specific as it determines any type of cell death irrespective of its cause (Estorch et al, 1990; Singal and Iliskovic et al, 1998).

A more popular non-invasive method for detection of Dox-mediated cardiotoxicity is by evaluating systolic cardiac function via use of radionuclide angiography or echocardiography. Although the sensitivity of radionuclide angiography is higher than echocardiography, the latter does not involve ionizing radiation and is a more popular low cost method for the detection and consequent monitoring of Dox-induced cardiomyopathy (Bristow et al, 1981; Takemura and Fujiwara, 2007). However, the sensitivity of echocardiography for the assessment of cardiac function by monitoring LVEF is low for the detection of subclinical early cardiomyopathy in Dox-induced cardiotoxicity as extensive cardiomyocyte injury as well as cardiac damage has already occurred and apparent reduction in the ventricular function is manifested only after compensatory mechanisms are exhausted (Bristow et al, 1981; Cardinale and Cipolla, 2016; Takemura and Fujiwara, 2007). Nevertheless, echocardiography techniques such as tissue velocity imaging and strain imaging provide early detection of LV systolic dysfunction compared to conventional LVEF (Fallah-Rad et al, 2011; Jassal et al, 2009).

Estimation of cardiac damage markers in the plasma/serum is a feasible approach to monitor cardiac toxicity in patients at high risk. An increase in the levels of cardiac proteins such as Troponin T, atrial natriuretic peptide and brain natriuretic peptide is observed in conditions of cardiac damage such as myocardial infarction and Dox-mediated cardiotoxicity (Hayakawa et al, 2001; Morandi et al, 2001; Suzuki et al, 1998). Troponin is now considered as a gold standard biomarker for myocardial injury (Cardinale et al, 2000, 2004, 2006). Troponins have high cardiac specificity and sensitivity as well as it is minimally invasive and cheaper than other detection procedures (Cardinale and Cipolla, 2016). Elevated levels of Troponin have been

observed in patients administered high doses of anthracyclines and can be used as a marker for early detection of Dox-mediated cardiotoxicity (Cardinale et al, 2006; Ewer et al, 2011). Cardinale and Sandri (2010) proposed the use of Troponin as a marker for the assessment of cardiac risk of antineoplastic treatements.

2. Oxidative stress

Oxidative stress can be defined as a condition of imbalance between the generation of ROS and the organism's capacity to counteract their action by its' antioxidant protection systems. It has been implicated in the pathogenesis of several diseases such as atherosclerosis, diabetes, heart failure, myocardial infarction, neurodegenerative diseases, cancer and many more (Lu et al, 2010; Nimse and Pal, 2015; Valko et al, 2007). Free radicals are reactive chemical species with a lone electron in their outermost orbit. Molecules such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{-\bullet}$), singlet oxygen and hydroxyl radical ($HO^{\bullet}$) represent free and non-free radical ROS (Lu et al, 2010; Pisoschi and Pop, 2015). Low levels of ROS are generated in physiological metabolic process such as aerobic respiration and exposure to microbial infections act as an important signaling molecules required for various cellular processes such as regulation of cell proliferation, apoptosis and gene expression (Djordjevic et al, 2008; Valko et al, 2007). Physiologically redox homeostasis of the cell is maintained by its complex antioxidant defense system comprising of endogenous antioxidant enzymes such SOD, catalase and GPx as well as non-enzymatic compounds such as vitamin A, C and E, glutathione, co-enzyme Q and lipoic acid (Lu et al, 2010; Nimse and Pal, 2015; Pisoschi and Pop, 2015).

At higher concentrations, ROS targets all classes of biomolecules via the oxidation of lipids, oxidation of proteins resulting in protein carbonyl formation as well as DNA damage and strand break by alteration in the base structure and DNA- protein cross linking modulating the

cellular signaling. ROS mediated damage to mitochondria can in turn enhance generation of ROS and initiate apoptosis. ROS can also affect cardiac contractility by altering the function of ion channels (Droge, 2002; Pisoschi and Pop, 2015).

2.1 Effect of Dox on cardiac antioxidant defense

Low levels of SOD, catalase and GPx are present in cardiac tissue (Angsutararux et al, 2015). SOD catalyzes the conversion of $O_2^{-\bullet}$ to less reactive species H_2O_2 which is further reduced to $OH^{\bullet}$ radical by GPx and catalase and ultimately to H_2O (Angsutararux et al, 2015; Droge, 2002). In addition, the heart has other non-enzymatic antioxidants such as Vit C, Vit E, reduced glutathione (GSH), lipoic acid and carotene. However the levels of antioxidants in the heart are lower as compared to that of other tissues such as liver or brain. The heart has about 150 times less catalase and 4 times less SOD as compared to liver (Doroshow et al, 1980; Doroshow & Davies, 1986). The antioxidant defense of cardiac tissue might be just enough to counteract ROS generated due to high oxidative phosphorylation and cellular metabolism in the heart (Schimmel et al, 2004). Hence any additional generation of ROS or reduction in antioxidant defense causes deleterious effects on the heart.

Numerous studies have demonstrated decreased protein expression as well as activities of SOD, catalase and GPx in animals as well as in patients with the administration of Dox (Kasapovic et al, 2010; Li et al, 2000; Ludke et al, 2012b). Patients receiving Dox also showed a reduction in the total antioxidant capacity of plasma (TRAP), glutathione and Vit C (Fuchs-Tarlovsky et al, 2013; Papageorgiou, 2005; Wejil, 1998). In the presence of Dox, the amount of ROS generated was observed to exceed the capacity of cardiomyocytes to counteract via its antioxidant defense leading to condition of oxidative/nitrosative stress.

2.2 Dox-induced cardiac ROS generation

Production of ROS is initiated by electron reduction processes including one electron reduction of molecular oxygen (O_2) by oxidases. In this regard, NADPH oxidase, NADH oxidoreductase, xanthine oxidase and nitric oxide synthase (NOS) are the major sources of production of $O_2^{-\bullet}$ (Droge, 2002; Minotti et al, 2004; Octavia et al, 2012). Dox-induced cardiotoxicity involves increased generation of ROS in cardiomyocytes by multiple mechanisms. Mitochondrial ROS has been observed in failing hearts (Tsutsui et al, 2008) and is reported to be a major contributor to HF (Giordano, 2005). NAD(P)H oxidoreductases, in mitochondrial-complex I of electron transport chain, catalyze transfer of electron from NADH to O_2. In addition to enhanced $O_2^{-\bullet}$ production via mitochondrial electron transport chain, Dox forms an irreversible complex with cardiolipin in inner mitochondrial membrane. Physiologically, binding of cardiolipin to proteins of electron transport chain (ETC) is required for the function of ETC proteins, disruption of cardiolipin-ETC protein complex in the presence of Dox leads to increased production of $O_2^{-\bullet}$ (Octavia et al, 2012). Mitochondria are abundant in cardiomyocytes and through the electron leakage from the mitochondrial ETC leads to the generation of $O_2^{-\bullet}$. Addition of one more electron to superoxide results in the production of H_2O_2 and further one more electron leads to $HO^{\bullet}$ generation by Haber-Weiss reaction (Angsutararux et al, 2015).

Additionally, the expression and activity of transmembrane NADPH oxidase enzymes have been observed to be increased in various models of HF including Dox-induced cardiomyopathy (Qin et al, 2006; Zhao et al, 2010). The structure of Dox itself is a major source for the generation of $O_2^{-\bullet}$ due to the redox cycling between its quinone and semiquinone structure. The quinone group in ring C of Dox is reduced to semiquinone by transfer of electron from NADH by action of enzyme NADH oxidoreductase. This semiquinone recycles itself back

to quinone by donating an electron to O_2 and thus generating $O_2^{-\bullet}$. A large amount of ROS such as $O_2^{-\bullet}$ and H_2O_2 are generated in this futile redox cycling (Minotti et al, 2004; Tokarska-Schlattner et al, 2006; Young et al, 1981). NOS serves as another important source for Dox-induced generation of ROS. Binding of Dox to the reductase domain of NOS results in the generation of $O_2^{-\bullet}$ by one electron reduction of Dox to the semiquinone form (Vasquez-Vivar et al, 1997). Uncoupling of NOS switches it to $O_2^{-\bullet}$ producing enzyme and is associated with the progression of several cardiovascular diseases including Dox-induced cardiomyopathy (Neilan et al, 2007).

2.2.1 Role of iron

Transition metals, such as iron and copper, serve as catalysts and enhance the rate of $OH^{\bullet}$ production through kinetically slow Haber-Weiss reaction. Iron catalyzed Haber-Weiss reaction is also called as Fenton reaction (Angsutararux et al, 2015; Pisoschi and Pop, 2015). Under physiological conditions, cellular levels of free iron are almost negligible to catalyze free radical reaction (Cairo and Pietrangelo, 2002). The level of free iron within the cell is maintained by uptake of iron by internalizing iron laden transferrin by the action of transferrin receptor (TfR) and storage of excess iron bound to ferritin (Gamella et al, 2014; Minotti et al, 2004[b]). Both TfR and ferritin are regulated at the transcriptional and post-transcriptional levels by the interaction of iron regulatory protein (IRP) with iron responsive element (IRE) of TfR mRNA. When the cell needs iron, binding of IRP to IRE of TfR mRNA protects it from degradation whereas binding to ferritin mRNA inhibits its translation. When iron is depleted, the structure is disassembled and cytosolic aconitase switches to IRP (Gamella et al, 2014; Minotti et al, 1999, 2004; Xu et al, 2005).

Superoxide generated during redox cycling of Dox can penetrate through the transprotein channels of ferritin and reduce the iron core, enhancing the release of iron in its Fe (II) form (Minotti et al, 1999). Released Fe (III) forms complex with Dox. The conversion of Dox-Fe (III) to Dox-Fe (II), generation of $O_2^{-\bullet}$ and conversion of quinone to semiquinone are increased in the presence of reducing agents. The $O_2^{-\bullet}$ generated participates in Fenton reaction producing large amount of ROS (Minotti et al, 1999, 2004[b]). Another mechanism for iron mediated enhanced generation of ROS is by Doxorubicinol, a metabolite of Dox, which targets the [4Fe-4S] cluster, removes iron from it and forms complex with Fe-S group of cytoplasmic aconitase/IRP-1 preventing translation of iron sequestering proteins and subsequent increase in free iron (Cairo and Pietrangelo, 2000; Gamella et al, 2014; Minotti et al, 2004; Recalcati et al, 2010). Dox can also interact with iron response element (IRE) on mRNA hence altering IRP dependent regulation of many proteins involved in iron metabolism (Minotti, 2001; Xu et al, 2005). Unbound free iron participates in Fenton reaction generating significant amount of ROS (Gamella et al, 2014; Xu et al, 2005).

3. Nitrosative stress

In addition to oxidative stress, increasing evidence suggests involvement of nitrosative stress in the pathogenesis of Dox-induced cardiotoxicity. Nitrosative stress is a pathological condition arising as a result of increased generation of reactive nitrogen species (RNS) and is implicated in the progression of several pathological conditions such as diabetes, acute ischemia, sepsis, heart failure, myocardial infarction, cancer and other pathologies (Pacher et al, 2005[a, b]; Szabo, 2009). It is characterized by increase in RNS such as peroxynitrite, protein nitration and nitrosylation. The nitrosative stress is exacerbated by the oxidant environment in the cellular milieu. Cytokine mediated upregulation of inducible NOS (iNOS) expression promotes

formation of peroxynitrite and thereby results in NO/redox disequilibrium. In myocardium, nitrosative stress adversely affects cardiac performance by a disruption of NO mediated signaling of Ca^{2+} channels responsible for normal systolic and diastolic functions (Hare and Stammmer, 2005). It also enhances loss of cardiomyocytes via peroxynitrite mediated activation of apoptosis (Mukhopadhyay et al, 2009).

3.1 Peroxynitrite

Peroxynitrite is a strong biological oxidant and nitrating species formed by near diffusion limited reaction of NO with superoxide anion. Increased generation of peroxynitrite is a major mechanism associated with pathogenesis of a number of cardiovascular dysfunctions such as myocardial infarction, chronic HF, anthracycline induced cardiomyopathy and diabetes (Pacher et al, 2005a, 2006; Mukhopadhyay et al, 2009). Although peroxynitrite exerts its deleterious effects via targeting multiple signaling pathways in the cell, direct oxidation of cellular biomolecules such as lipid, protein and DNA are the basis for its cytotoxicity (Figure 2). Peroxynitrite mediated nitration of proteins at their tyrosine residues can result in the modification of proteins resulting in either inactivation or hyperactivation of their activity (Salvemini et al, 2006). Nitration can also prevent subsequent phosphorylation or alternatively enhance phosphorylation of proteins or alter degradation of proteins. Nitration is involved in the initiation and progression of a number of diseases (Turko and Murad, 2002).

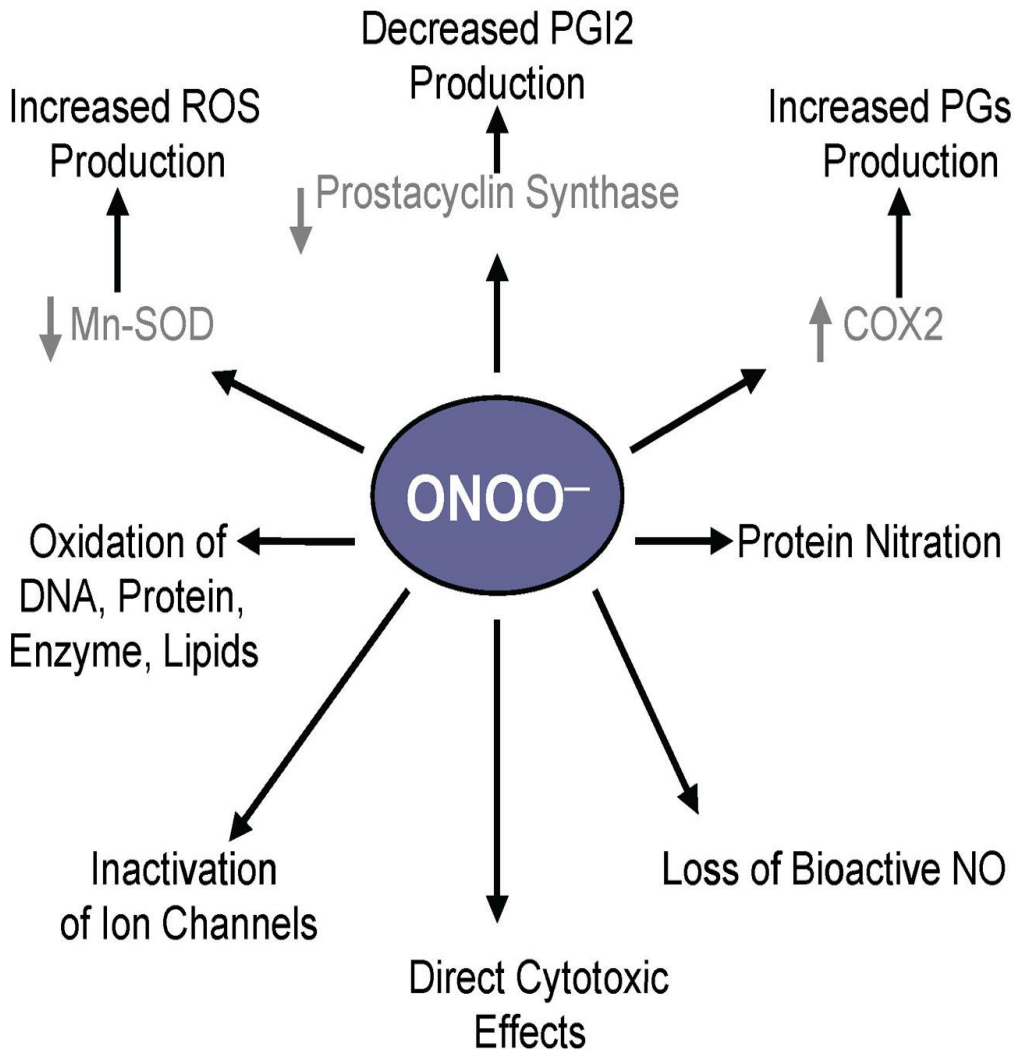

Figure 2: Peroxynitrite mediated cytotoxic effects.
Increased levels of peroxynitrite ($ONOO^-$) alter cellular function by targeting several biomolecules. Peroxynitrite can cause direct or indirect cytotoxic effects by protein nitration, loss of bioactive NO, oxidation of DNA, proteins and lipids, increased generation of ROS, reduction of antioxidant enzymes and inactivation of ion channels. (Modified from Salvemini et al, 2006)

Additionally, peroxynitrite acts as a major effector of apoptosis in cardiomyocytes via the activation of caspase-3 and PARP. Reduction in peroxynitrite suppressed protein nitration and apoptosis in H9C2 cells (Levrand et al, 2006). Peroxynitrite induced oxidation of sulfhydryl groups can inhibit important mitochondrial respiratory chain enzymes and irreversibly damage mitochondrial membrane resulting in excess generation of ROS (Brown, 2001; Pacher et al, 2005[a]; Salvemini et al, 2006). Furthermore, peroxynitrite impaired the antioxidant defense by inhibiting activities of SOD and GPx (MacMillan-Crow et al, 1996) as well as decrease the levels of other endogenous antioxidants such as Vit C and plasma thiols via enhanced nitration resulting in enhanced generation of ROS (Han et al, 2001)

Peroxynitrite impairs cardiac contractility in two ways: i) by enhanced nitration of various proteins such as myofibrillar creatine kinase (Mihm et al, 2002), Ca^{2+} handling proteins (Ryanodine receptor, Phospholamban) (Ungvari et al, 2005) and ii) by causing loss of bioactive NO via uncoupling of eNOS. Peroxynitrite can trigger eNOS uncoupling via nitration of eNOS and/or oxidation of its cofactor BH4 or alternatively via disrupting endothelial caveolae (Cassuto et al, 2014; Ungvari et al, 2005). Functional alteration of proteins through enhanced nitration has significant impact on pathogenesis of cardiovascular diseases.

4. Nitric oxide (NO)

NO or nitrogen monoxide is one of the oxides of nitrogen along with nitrous oxide (N_2O) and nitrogen dioxide (NO_2). N_2O, commonly known as laughing gas is popular for its medicinal use due to its anesthetic properties. In contrast, NO_2 is an air polluant also used as an oxidizer in rocket fuel as well as nitrating agent in chemical explosives. NO is a free radical and considered as an environmental pollutant at high concentrations, whereas at low concentration it is a crucial

signaling molecule with a key role in various physiological as well as pathological functions in mammalian system (Bian and Murad, 2014; Bruckdorfer, 2005).

Although initially recognized as endothelium derived relaxing factor involved in vascular functions, NO also plays an important role in cardiomyocyte contraction. It is produced in a variety of cell types such as endothelial cells, smooth muscle cell (SMC), cardiomyocytes, skeletal muscle, neuronal cells as well as inflammatory cells such as macrophage and monocyte (Bruckdorfer, 2005; Liu and Huang, 2008). Alterations in NO concentration are associated with progression of many conditions such as diabetes, cancer, atherosclerosis, hypertension, arthiritis and myocarditis (Liu and Huang, 2008).

Although the physiological importance of NO was recognized in 1998, medical use of nitrate containing compounds such as nitroglycerin has been practiced since 1895 for relief from angina pectoris (Bian et al, 2008). In 1977, Murad's laboratory demonstrated that NO mediated the activation of soluble Guanylate Cyclase (sGC) and the upregulation of cyclic guanosine monophosphate (cGMP) as being the mechanisms involved in the vasodilatory effect exerted by nitroglycerin and nitroprusside in vascular SMC relaxation (Katsuki et al, 1977). Furchgott and Zawakzki (1980) later identified NO as the endothelium derived relaxing factor. Three individuals, Robert Furchgott, Louis Ignarro and Ferid Murad were honored with Nobel Prize in Medicine or Physiology in 1998 for their contributions for the identification of crucial role of NO and NO was declared as "molecule of the year".

At physiological concentrations pico molar (pM) to low nano molar (nM), NO provides beneficial effects; whereas at higher μM concentration it can be toxic and proinflammatory resulting in deleterious effects (Aktan, 2004; Bruckdorfer, 2005; Colasanti and Suzuki, 2000;

Mocellin et al, 2007). NO is a transient free radical with half-life of about 5 seconds. This may be as a result of its high reactivity with proteins, lipids and DNA or reaction with ROS or interaction with sGC (Bruckdorfer, 2005). Given such a short half-life, spatially regulating NO production closer to its target molecule is important to facilitate its signal transduction and specific targeting as well as reduces its deleterious reactions (Villanueva and Giulivi, 2010).

Although the function of NO is less defined in the heart, it is a key regulator of excitation-contraction and hence myocardial contractility (Hare and Stamler, 2005; Massion et al, 2003; Ziolo et al, 2008). Ballingad et al (1993) first demonstrated the role of endogenous NO in mediating β adrenergic receptor (β-AR) mediated signaling. Low concentration of NO is produced in cardiac myocytes, in a pulsatile manner, in phase with the cardiac contraction cycle (Hare and Stamler, 2005; Massion et al, 2003; Ziolo et al, 2008).

4.1 Nitric Oxide Synthases (NOS)

Endogenously, NO is produced by the action of various NOS isoforms: neuronal NOS (nNOS) or NOS1, iNOS or NOS2 and endothelial NOS (eNOS) or NOS3 (Aktan 2004; Hare and Stamler, 2005; Liu and Huang, 2008). The names of the isoforms do not indicate exclusive localization of the isozyme in a particular cell type, but is indicative of the cell type where they were first discovered. NOS isoforms are encoded by different genes on separate chromosomes (Liu and Huang, 2008) and represent about 50-60% homology in their sequence with respect to cofactor binding regions (Singh and Gupta, 2011). Although all three isoforms have similar enzymatic reaction and cofactor requirement, each of the isoform has distinct expression pattern, regulation of their activity and subcellular localization thereby distinct catalytic activity.

All the isoforms of NOS require L-arginine as a substrate and molecular oxygen (O_2) as well as NADPH as co-substrate. The catalytic activity of the enzyme also requires binding of

other cofactors such as FAD, FMN, (6R) 5, 6, 7, 8-tetrahydro-L-biopterin (BH4) and adenosine to the enzyme. Catalytically active enzyme converts L-Arginine to L-citrulline and NO (Forstermann and Sessa, 2012). NOS enzymes are usually present as two monomeric proteins bound together by BH4 and heme (Alderton et al, 2001) (Figure 3). Oxidation of BH4 results in the dissociation of NOS dimers leading to uncoupling of NOS and the monomeric forms are unable to produce NO (Alp and Channon, 2004; Channon, 2004; Heller et al, 2001). BH4 thereby has a crucial role in maintaining functionally active form of NOS (Alp and Channon, 2004; Channon, 2004). All of the NOS isoforms have a reductase and an oxygenase domain. While the substrate L-arginine, O_2 and BH4 bind to the oxygenase domain. Transfer of electrons occurs from NADPH in the carboxy reductase domain to the heme in oxygenase domain of the enzyme (Alderton et al, 2001; Forstermann and Sessa, 2012). The flow of electron is facilitated by conformational changes in NOS as a result of binding of Ca^{2+} to calmodulin in the enzyme (Figure 3). However, the requirement for the concentration of Ca^{2+}, facilitating the binding of calmodulin and the concentration of NO produced by different NOS isoforms varies (Aktan, 2004; Forstermann and Sessa, 2012).

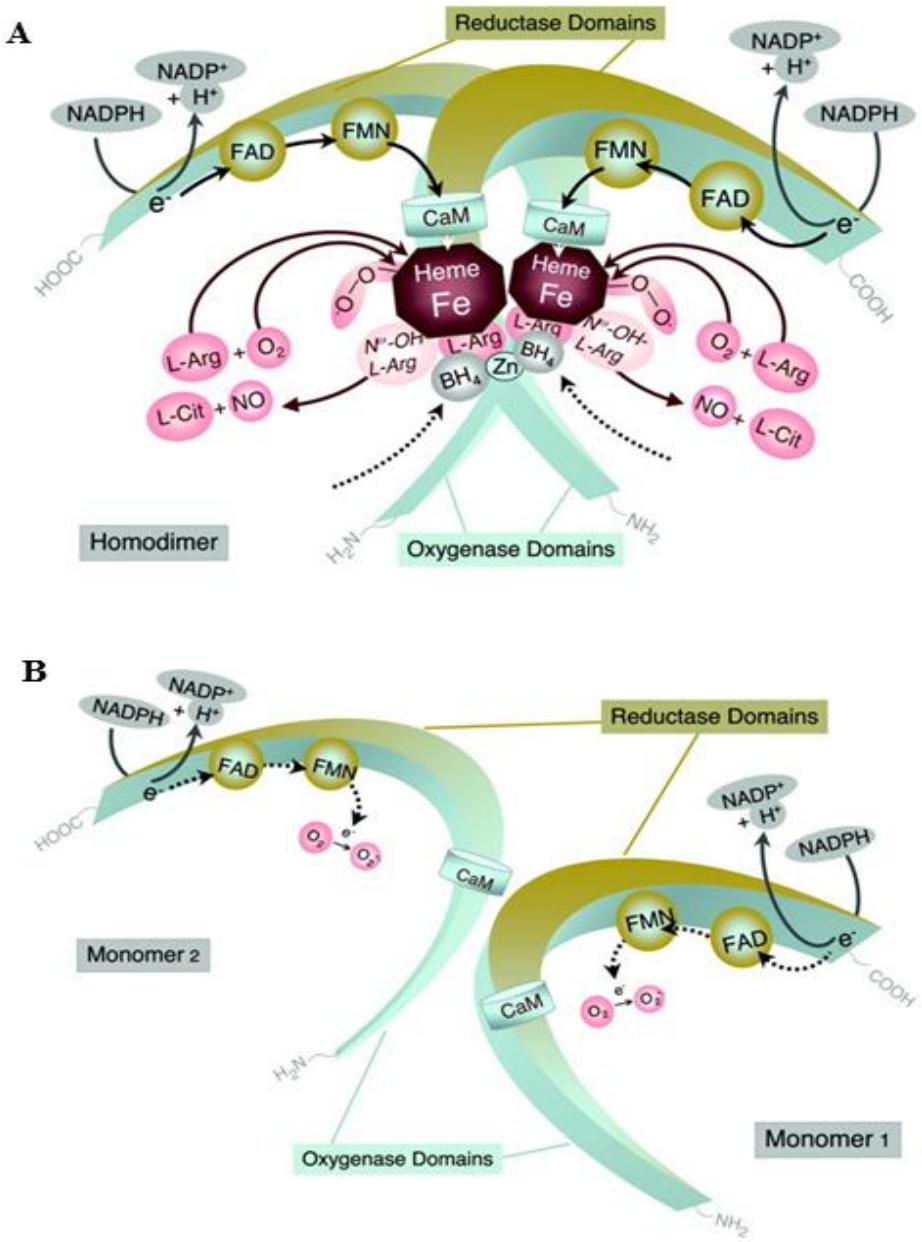

Figure 3: Nitric oxide synthase activity

A. Under physiological conditions, nitric oxide synthase (NOS) exists in dimeric (active) form, producing nitric oxide (NO) as a byproduct during the catalytic conversion of L-agrinine to L-citruline in the presence of cofactors such as NADPH, FAD, BH4, calmodulin and molecular oxygen. **B.** Pathological conditions such as oxidative stress leads to oxidation of cofactor BH4 resulting in the dissociation of dimeric form of NOS into monomeric (inactive) subunits. Monomerization of NOS leads to uncoupling of NOS thus generating superoxide instead of NO. (Forstermann and Munzel, 2006)

4.1.1 Endothelial Nitric oxide synthase (eNOS)

eNOS is a constitutively expressed, Ca^{2+}/Calmodulin dependent enzyme generating NO in pM-nM concentrations for a short period of time (Knowles and Moncada, 1994). Hence upon stimulation it is produced rapidly causing direct and short acting effects (Singh and Gupta, 2011). Even though initially thought to be exclusively present in endothelial cells, eNOS is found in a number of cell types such as cardiomyocytes, platelets, SMC and certain neuronal cells (Liu and Huang, 2008; Forstermann and Sessa, 2012). eNOS is involved in several cellular functions such as vasodilation, modulation of platelet aggregation, cardiomyocyte and SMC contraction, leukocyte-endothelial cell interaction as well as inhibition of SMC proliferation (Bian and Murad, 2007; Knowles and Moncada, 1994; Massion et al, 2003).

eNOS function is regulated via phosphorylation of the enzyme. Activation of the inactive eNOS dimer occurs via Ca^{2+} mediated protein modification through myristolyation, phosphorylation and palmitoylation resulting in a conformational change of the enzyme. Although phosphorylation of eNOS can occur at its multiple Serine (Ser) or Threonine (Thr) sites, phosphorylation at Ser1177 and Thr495 sites are more commonly studied and observed to be involved in its regulation (Fleming et al, 2003, 2010). While phosphorylation of Ser1177 is observed to activate enzyme activity, phosphorylation of Thr495 has inhibitory effect. Under non-stimulated condition Thr495 tends to be phosphorylated by Protein kinase C (PKC), which results in interference for binding of calmodulin to its binding site on the enzyme resulting in inactivation of eNOS (Fleming, 2010; Lin et al, 2003). In contrast, phosphorylation at Ser1177 stimulates the flux of electrons within the reductase domain thereby activating the enzyme (Fleming 2010; Forstermann and Sessa, 2012)). Although Ser/Thr kinase (Akt) and adenosine monophosphate activated protein kinase (AMPK) are involved in the phosphorylation of

Ser1177, Akt is the major regulator of phosphorylation in response to various triggers such as estrogen, vascular endothelial growth factor (VEGF) and insulin (Forstermann and Sessa, 2012; Ladurner et al, 2012).

eNOS derived NO exerts its biological effects by targeting various Ca^{2+} channels such as L-type calcium channels (LTCC), ryanodine receptor (RyR), sarcoplasmic Calcium ATPase (SERCA) via c-GMP dependent and independent signaling pathways (Massion et al, 2003; Umar and van der Laarse, 2010; Xu et al, 1998), which is discussed later in this section. Nonetheless, for effective and targeted signaling, eNOS is primarily localized in plasmalemma caveolae in the spatial vicinity of its target proteins (Drab et al, 2001; Gratton et al, 2000; Massion et al, 2003). Myristolyation and palmitoylation of eNOS on glycine and cysteine target eNOS to plasmalemma caveolae. Alternatively, it can translocate to other subcellular compartments including Golgi apparatus, cytosol and endothelial cell junctions (Treuer and Gonzalez, 2015). Dissociation of eNOS from caveolin by interaction of proteins such as heat shock protein 90 promotes phosphorylation of eNOS by recruitment of Akt (Feron, 2006; Gratton et al, 2000; Massion et al, 2003; Liu and Huang, 2008). Heat shock protein 90 also plays an important role in maintaining dimeric form of eNOS (Chen et al, 2014).

As low level of eNOS derived NO is involved in maintaining various cardiovascular functions, loss of bioactive NO as a result of eNOS uncoupling is implicated in the progression of many cardiovascular dysfunctions (Cai and Harrison, 2000; Channon et al, 2004). Loss of cofactor BH4 leads to uncoupling of NOS converting eNOS from NO producing enzyme to superoxide producing enzyme (d'Uscio et al, 2003; Satoh et al, 2005).

4.1.2 Neuronal Nitric oxide synthase (nNOS)

nNOS, a 161 kDa NOS isoform, although first characterized in neuronal cells, is also expressed in several other cell types such as cardiomyocytes, smooth muscle and skeletal muscle. Similar to eNOS, nNOS is constitutively expressed where enzyme activity is regulated by Ca^{2+}/calmodulin to produce low (pM-nM) amount of NO (Massion et al, 2003; Ziolo et al, 2008). In cardiomyocytes, nNOS is localized in the sarcoplasmic reticulum (SR) membrane and nNOS mediated NO is involved in the regulation of Ca^{2+} handling proteins such as SERCA, LTCC and phospholamban (Umar and van der Laarse, 2010; Ziolo and Bers, 2003; Ziolo et al, 2008). In cooperation with eNOS, nNOS mediated NO plays an important role in β-AR mediated excitation-contraction coupling in cardiomyocytes (Ashley et al, 2002; Ziolo and Bers, 2003). Although the exact mechanism for the cardioprotective role of nNOS is unclear, genetic manipulations resulting in loss of nNOS manifested in blunting of βAR response (Ashley et al, 2002; Sears et al, 2003). However, nNOS mediated NO plays a major role in the regulation of blood pressure in the central nervous system (Forstermann and Sessa, 2012).

4.1.3 Inducible Nitric oxide synthase (iNOS)

This NOS isoform is not constitutively expressed, rather its expression is induced particularly in the presence of pathological stimuli such as cytokines, bacterial LPS or stress (Liu and Huang, 2008; Mocellin et al, 2007). Though initially recognized to be restricted to inflammatory cells, recent evidence confirms that the expression of iNOS can be induced in several cell types including cardiomyocytes (Forstermann and Sessa, 2012; Massion et al, 2003; Ziolo and Bers, 2003). Unlike eNOS and nNOS, once expressed iNOS is constantly active and produces μM concentration of NO. iNOS is active even in the absence of changes in Ca^{2+} and does not depend on post translational modifications or Ca^{2+} for the regulation of its activity

(Aktan, 2004; Mocellin et al, 2007). Although NO produced by iNOS has a crucial role in defense against pathogens, parasites, tumor cells and microbes; it also exerts deleterious effects on neighbouring healthy cells (Aktan, 2004; Colasanti et al, 2000).

The production of NO by iNOS is controlled at the level of transcription via nuclear factor κB (NFκB). Activation of pro-inflammatory cytokines such as TNFα and IL-1 also facilitates activation of iNOS via translocation of NFκB from the cytosol to nucleus or upregulation of IFNγ mediated Jak-STAT signaling (Hibbs, 1991; Mocellin et al, 2007; Rao, 2000). The promoter region of iNOS gene has binding regions for several transcriptional factors like NFκB, AP1, Jun/Fos, CREB and STAT family of transcription factors. The binding of NFκB and AP1 transcription factors to the promoter region mediate expression of various inducible genes such as iNOS, COX-2, ICAM-1 and VCAM-1 (Aktan, 2004; Xia et al, 2001).

iNOS mediated NO exerts deleterious effects in healthy cells via multiple mechanisms. NO can bind to iron and inhibit the activity of key iron containing enzymes such as mitochondrial electron transport chain proteins, cis-aconitase, enzymes of complex I and II as well as ribonucleotide reductase (Brown, 2001; De Alba et al, 1999; Kronke, 1997). NO at high concentration can also directly interfere with DNA resulting in strand break and fragmentation (Fostermann and Sessa, 2012; Kronke, 1997). It can also form peroxynitrite resulting in apoptosis through the activation of various caspases and PARP (Pacher et al, 2005[a]). iNOS upregulation can exacerbate the pathophysiological conditions of myocardium and can modulate cardiac contractility by targeting multiple Ca^{2+} handling proteins involved in EC coupling (Liu and Huang, 2008; Ziolo et al, 2008). Increased myocardial iNOS can also initiate various cardiac remodeling events such as ventricular hypertrophy and dilatation (Colasanti et al, 2000; Umar, 2010).

4.2 NO signaling

NO mediated intracellular signaling is mainly via two distinct pathways: cGMP dependent and cGMP independent. The initial discoveries identified increased cGMP as a critical mediator to carry out biological action of NO. NO induced increase in soluble guanylate cyclase (sGC) was observed to be crucial to mediate vaso-relaxation in response to an increase in NO (Bian and Murad, 2007; Murad, 2011). Constitutively active NOS mediated generation of NO interacts with heme moiety of sGC. The latter further causes activation of sGC leading to conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP) (Bryan et al, 2009). cGMP activates cGMP dependent kinases, protein kinase G (PKG) resulting in phosphorylation of a number of proteins involved in Ca^{2+} regulation, reducing the levels of $[Ca^{2+}]_i$ (Hare and Stamler, 2005; Ziolo et al, 2008). This stimulates muscle relaxation, increase in vascular permeability, anti-platelet and anti-oxidant effects through targeting protein kinases and ion channels (Monica et al, 2016; Singh and Gupta, 2011). The stimulatory effect of NO is abrogated by enzyme phosphodiesterase-5 which converts cGMP to GMP (Monica et al, 2016; Murad, 2011).

Another alternative mechanism for NO signaling was discovered to be independent of an activation of sGC. NO was observed to directly alter proteins by the formation of NO-protein adducts resulting in more stable complex (Lima et al, 2010; Murad, 2011). NO triggers nitrosylation of proteins by targeting the sulfhydryl groups of the proteins resulting in reversible nitrosothiol compounds or nitration of proteins at tyrosine residue (Singh and Gupta, 2011). These reactions are highly specific and require low concentration of NO compared to cGMP mediated alterations (Treuer and Gonzalez, 2015). However the propensity of nitrosylation depends on many factors including the redox microenvironment (Singh and Gupta, 2011). Upon

nitrosylation, proteins can change their properties similar to the effect of protein phosphorylation. Low level of nitrosylation is critical for the activation of a number of proteins (Bryan et al, 2009; Lima et al, 2010; Liu and Huang, 2008). In fact, s-nitrosylation in coordination with phosphorylation regulates the function of many Ca^{2+} handling proteins such as LTCC, SERCA, RyR and phospholamban involved in β-AR mediated cardiac contractility (Irie, 2015). The formation of S-nitrosothiols is maintained and cleared by enzyme S-nitrosothione reductase degrading the former into glutathione disulphide (GSSG) and ammonia (NH_3) (Hare and Stamler, 2015; Irie et al, 2015). Hence, deficiency of s-nitrosothione reductase results in enhanced levels of s-nitrosothiols in tissue and contributes to pathological signaling (Umar and van der Laarse, 2010). To achieve specificity, NO is produced in close vicinity of the target molecule allowing direct interaction between NO and its target. In fact NOS is believed to be part of protein complex in which s-nitrosothiol signaling occurs (Lima et al, 2010; Treuer and Gonzalez, 2015). Extensive protein nitration is involved in disease initiation and progression as a result of gain or loss of function by stimulation or inhibition of protein phosphorylation respectively (MacMillan-Crow, 1996; Turko and Murad, 2002).

4.3 Dox-induced nitrosative stress

Various experimental models demonstrated upregulation of iNOS expression by Dox administration (Andreadou et al, 2007; Andreadou et al, 2014; Mukhopadhyay et al, 2009; Oktem et al, 2012; Pacher et al, 2003; Sayed-Ahmed et al, 2001). An imbalance in the expression of iNOS and eNOS is observed after administration of Dox (Andreadou et al, 2014). Peroxynitrite plays a critical role in exacerbating Dox mediated cardiotoxicity. Reduction in peroxynitrite using peroxynitrite scavenger abolished Dox-induced apoptosis and NT formation (Mukhopadhyay et al, 2009). Peroxynitrite leads to increased NT formation after administration

of Dox in isolated cardiomyocytes and cardiac tissue of Dox treated animals (Andreadou et al, 2007; Mihm et al, 2002). Dox causes extensive protein nitration and nitrosylation of cardiac myofibrils and contractile proteins and affects cardiac contractility resulting in LV dysfunction (Weinstein et al, 2000). Thus reduction of Dox induced nitrosative stress is critical for attenuation of its cardiotoxicity.

5. Dox mediated activation of cell death pathways

The balance between cell death and survival is maintained by programmed cell death. Apoptosis, necrosis and autophagy are three different forms of cell death (Ouyang et al, 2012). Dox mediated oxidative/nitrosative stress, mitochondrial damage, cell membrane injury and DNA damage can trigger various cell death pathways such as apoptosis, necrosis and autophagy (Carvalho et al, 2014; Minnotti et al, 2004; Octavia et al, 2012; Schimmel et al, 2004). At lower concentration of Dox apoptosis was observed to be predominant, whereas higher concentration (>10 μM) induced necrosis (Guchelaar et al, 1998). Cumulative loss of cardiomyocytes via an activation of either type of cell death pathway impairs cardiac contractility and conductance resulting in ventricular dysfunction (Jungsuwadee, 2016). Although apoptosis, necrosis and autophagy have distinct morphological and biochemical changes, there can be cross talk between the three forms of cell death.

5.1 Apoptosis

Apoptosis is an evolutionary preserved, well regulated, ATP driven processs (Biala and Kirshenbaum, 2014; Zhang et al, 2009). It is important for the normal development and maintaining cellular homeostasis (Zhang et al, 2009) and can be triggered by various stimuli such as deprivation of growth factor, radiation and chemotherapy drugs (Kim, 2005). Apoptosis is characterized by specific changes in the structure of the cell such as cell shrinkage, nuclear

condensation and nuclear fragmentation as well as biochemical changes. Until recently, apoptosis was not considered as a possible mechanism for cell death in Dox-induced cardiotoxicity. However, numerous studies have now indicated apoptosis as a key form of cell death by Dox-induced cardiotoxicity (Childs et al, 2002; Zhang et al, 2009). Apoptotic cell death is evident in both acute as well as chronic model of Dox mediated cardiotoxicity (Ludke et al, 2012a; Ueno et al, 2006; Wang et al, 2004). However, apoptosis triggered by Dox is via distinctly different pathways in normal and tumor cells (Wang et al, 2004).

Apoptosis can be triggered by two pathways: i) the extrinsic pathway involving death receptor; and ii) the intrinsic pathway involving mitochondria (Biala and Kirshenbaum, 2014; Zhang et al, 2009). The extrinsic pathway is triggered by binding of plasma membrane death receptor like Fas or other similar receptors such as tumor necrosis factor- α receptor (TNF-αR) with its extracellular ligand Fas-L. In presence of death stimuli, a death complex is formed by the combination of Fas with Fas-L (Fas/Fas-L) with Fas associated death domain (FADD) or by TNF- α/ TNF-αR with TNF-αR activator death domain (TRADD). This forms a death inducing signaling complex (DISC) crucial for autocatalytic activation of caspase-8 and further activation of downstream caspases such as caspase-3 (Angsutararux et al, 2015). The intrinsic pathway is mediated by members of Bcl-2 gene family; where Bax/Bak has an important role in the initiaition of apoptosis and release of mitochondrial cytochrome C into cytosol. Bax is a cytosolic protein, however in the presence of DNA damage or cellular insult by ROS, it forms a homodimer or a heterodimer with Bak and is recruited to the outer mitochondrial membrane (OMM) promoting its permeabilization. This results in loss of mitochondrial membrane permeability (MMP) via opening of mitochondrial permeability transition pore (MPTP) and release of apoptogenic mitochondrial proteins such as cytochrome C (Cyt C) and Endo-G in the

cytosol. Released mitochondrial proteins recruit other proteins and form the apoptosome complex. Various caspases are subsequently activated resulting in apoptosis (Angsutararux et al, 2015; Biala and Kirshenbaum, 2014; Zhang et al, 2004).

5.2 Necrosis

In contrast to apoptosis, necrosis occurs under condition of low cellular ATP (Zhang et al, 2009). For many years necrosis was considered as a random, accidental, uncontrolled cell death event. Recently with the discovery of key mediators such as receptor interacting protein (RIP) kinase and PARP, it is also included as a form of programmed cell death (Ouyang et al, 2012). Necrosis involves cell swelling, organelle dysfunction and lysis of cell membrane resulting in the release of intracellular macromolecules into the interstitial space provoking inflammation (Biala and Kirshenbaum, 2014). Changes in the subcellular structures of cardiomyocytes including loss of myofibrils, vacuolization of the sarcoplasmic reticulum and the swelling of the mitochondria can result in necrotic cell death (Chung and Youn, 2016).

Ligands such as TNF-α and Fas result in the recruitment and formation of signaling complex necrosomes by assembly of caspase-8, FADD/ TRADD and RIP1. Activation of caspase-8 mediates cleavage of RIP1, and inactivates it. In contrast, under the condition of caspase-8 inactivation, RIP1 is activated resulting in necrosis. RIP interacts with metabolic enzymes such as glycogen phosphorylase, glutamate ammonia ligase and glutamate dehydrogenase to enhance metabolism accompanied by ROS production. Excess ROS leads to mitochondrial membrane permeabilization and subsequent necrosis via defects in IMM (Biala and Kirshenbaum, 2014). Numerous studies show increased levels of inflammatory cytokines, inflammatory cell infiltration and necrosis in Dox treated hearts (Ikegami et al, 2007; Riad et al, 2009). In addition to activation via death receptors, cellular oxidative stress can also trigger

necrosis. ROS can lead to necrotic cell death via Ca^{2+} overload mediated opening of MPTP and mitochondrial swelling, as well as activation of RIP (Kim et al, 2007; Ouyang et al, 2012; Zhang et al, 2009). Various proteins such as Bnip-3 and PARP-1 are believed to play a major role in triggering necrosis in Dox-induced cardiotoxicity (Dhingra et al, 2014; Shin et al, 2015). Free radical scavengers protect the heart from Dox-induced necrosis (Ikegami et al, 2007; Ludke et al, 2012a).

5.3 Autophagy

Autophagy is a conserved catabolic process essential for maintaining the cellular environment by degradation of the unwanted macromolecules, misfolded proteins and damaged organelles. It plays an important role in cell survival by recycling cellular contents under conditions of cellular stress or nutrient deprivation (Zhang et al, 2009). Although autophagy has a vital role in cell survival and plays an important role in maintaining tissue homeostasis, excessive cell death by autophagy is detrimental. As cardiomyocytes are susceptible to enhanced production of ROS and oxidative stress mediated injury, autophagy has a crucial role in maintaining healthy cardiomyocytes by the elimination of misfolded proteins and damaged mitochondria (Bartlett et al, 2017; Li and Hill, 2014). Thus, too little or too much autophagy in the heart is associated with cardiac dysfunction and HF (Bartlett et al, 2017).

Autophagy is characterized by the formation of large autophagic vacuoles in the cytoplasm. Autophagy is comprised of 4 steps: initiation, engulfment of cargo by double membrane vacuole autophagosome, fusion of autophagosome with lysosome and degradation of autophagosome by lysosomal proteases. Although autophagy differs from apoptosis, several factors that regulate apoptosis such as Beclin-1 play an important role in the early steps of the initiation of autophagosome. Proteins such as light chain 3-I (LC3-I) and p62 are associated with

the membrane of autophagosomes (Bartlett et al, 2017; Li and Hill, 2014; Zhang et al, 2016). Proteolytic cleavage of LC3-I results in the formation of membrane associated LC3-II. Hence proteins such as LC3-II, p63 and beclin-1 can be used as markers of autophagy (Bartlett et al, 2017; Tacar and Dass, 2013).

Although the exact effect of Dox on autophagy is inconclusive, Dox induced cardiac injury is associated with dysregulation of autophagic function (Dirks-Naylor, 2013; Li and Hill, 2014). Dox treated isolated cardiomyocytes demonstrated predominance of apoptosis over autophagy (Tacar et al, 2015). Cardiomyocytes exposed to Dox have depleted ATP (Ludke et al, 2012[a]) and enhanced protein degradation (Ranek and Wang, 2009). While Dox treated cardiomyocytes have enhanced ROS and dysfunctional mitochondria, the ability to clear damaged mitochondria is also compromised (Li and Hill, 2014). Dox exposed cardiomyocytes have alteration in proteolytic process resulting in accumulation of non-degraded and dysfunctional autolysosomes. The ratio of LC3-II/I can be observed to increase as a result of excessive generation of autophagosomes as well as insufficient clearance of autophagosome. Thus Dox disrupts the autophagic flux as well (Bartlett et al, 2017). Inhibition of basal autophagy by Dox has deleterious effects due to an accumulation of degraded and damaged proteins (Pizzaro et al, 2016).

6. Management/ Prevention of Dox-induced cardiomyopathy

Patients with medical history of obesity, diabetes, hypertension, previous or concomitant cancer treatment, irradiation, family history of cardiomyopathy are at high risk for the development of chemotherapy-induced cardiotoxicity. These patients should be monitored more closely and alternative approaches should be considered to reduce chemotherapy-induced cardiotoxicity (Truong et al, 2014). Thus cancer patients not only need to be monitored by an

oncologist for treatment of cancer but also by a cardiologist to monitor the cardiotoxicity developed during chemotherapy. This led to the development of the interdisciplinary field of "cardio-oncology" to foster interaction between two disciplines for optimal care of cancer patients' and development of various strategies to treat cancer while reducing cardiotoxicity (Albini et al, 2010; Hong et al, 2010; Minotti et al, 2010; Vincent et al, 2013). Various strategies such as use of Dox analogues, alternative drug delivery method, reduction in drug dosage, early detection, administration in combination with cardioprotective drugs and antioxidants have been attempted to reduce/ prevent Dox-induced cardiomyopathy (Minotti et al, 2004; Octavia et al, 2012; Schimmel et al, 2004; Singal and Iliskovic, 1998; Zhang et al, 2016).

6.1 Anthracycline analogues

A large number of analogues of anthracyclines have been synthesized as alternatives to Dox. However, limited success has been achieved in generating analogue with reduced cardiotoxicity without compromising its anti-tumor efficacy. None of the analogues synthesized till date have stronger anti-tumor efficacy than Dox. Analogues such as idarubicin and epirubicin although approved for clinical use, still present the issue of cardiotoxicity. To limit the total dose of Dox administered, chemotherapy is sometimes given as a "cocktail" i.e. a combination of different anti-tumor drugs such as cisplatin, vincristine and cyclophosphamide, for different types of tumors (Chamber et al, 1996; Singal and Iliskovic, 1998).

6.2 Alternative drug delivery approach

Controlling the peak concentration of the drug is another approach to reduce cardiotoxicity. A rapid continuous intravenous infusion (within 15-20 minute period) at the recommended dose (60-75 mg/m^2) every three weeks is the standard approach to the administration of Dox (Singal and Iliskovic, 1998). However, it is believed that administration of

the drug at moderate concentration for longer term would be safer compared to administration of drug at high concentration over shorter period of time (Feng and Chien, 2003). Evaluation of various dosing schedules such as: 1 bolus dose every week; 3 divided doses every week; 3 divided doses given for three consecutive days every 3 weeks revealed the beneficial effect of divided dose over bolus dose in reducing cardiotoxicity without altering the tumor responses (Vejpongsa and Yeh, 2014). Dox is better tolerated in slow infusion compared to the standard method (Singal and Iliskovic, 1998). While the anthracycline concentration in tumor tissue showed no difference between bolus and a continuous delivery, anthracycline concentration in the heart was much higher with the bolus dose (Pacciarini et al, 1978). Administration of Dox as a slow continuous infusion over 6 hours provided beneficial effect in reducing the cardiotoxicity as indicated by a lesser decrease in ejection fraction and voltage in QRS complex. However, not all cancers respond in similar manner; continuous infusion of Dox for 48-72 hours was effective for treatment of sarcoma and lymphoma but not for pediatric patients (Chung and Youn, 2016).

Another approach to reduce cardiotoxicity of chemotherapy drugs is via targeted drug delivery. Development of drugs with specific carriers that preferentially distribute anthracycline to the tumor cell or specifically recognize the tumor cell would effectively minimize cardiotoxic side effects (Minotti et al, 2004). Use of liposomes or nanoparticles, present a promising approach to address this issue. Liposomal Dox formulation not only reduces Dox-mediated cardiotoxicity by limiting its accumulation in healthy tissue but also prevents the high levels of free Dox and its metabolites seen with Dox alone (Weiss and Manthei, 1983) without compromising its anti-tumor activity (Gabizon et al, 2016). It can also modify the bio-distribution and metabolism of Dox. Due to the small size of the formulation of 20 nm-10 µm, the drug is unable to enter the cardiac tissue. Doxil, Caelyx or Lipodox, are the first US Food and

Drug Administration approved pegylated (polyethylene glycol coated) liposomal Dox (Barenholz, 2012). Pegylation involves covalent attachment of surface bound methoxypolyethylene glycol to liposomal phospholipid layer which masks liposomes from mononuclear phagocytes lowering their antigenicity and increasing the half-life. Other non-pegylated liposomal formulation with anthracycline includes Daunoxome and Myocet. Although antitumor efficacy has been reported for pegylated liposomal Dox for a number of cancers, its clinical use has not become as widespread to replace the conventional therapy due to its associated cost (Gabizon et al, 2016). Also it is approved for treatment of limited types of cancer such as ovarian cancer and Kaposi sarcoma (Chung and Youn, 2016; U.S. Food and Drug administration). Immunoliposomes are conjugates of monoclonal antibody of FAB fragment of cancer overexpressing proteins in liposomal drug delivery. It can be beneficial in targeting cancers which overexpress proteins such as HER2 overexpressing breast cancer (Minotti et al, 2004). An alternative to liposomal Dox is synthesis of prodrugs which can be activated by tumor cell specific released peptidase. An example for this is prodrug L-377,202 obtained by covalently linking Dox with N-glutaryl-[4-hydroxyprolyl]-Ala-Ser-cyclohexaglycyl-Glu-Ser-Leu which in the presence of prostate specific antigen (PSA) is activated to an active drug which can enter PSA positive cancer cells (Denmeade et al, 1998).

Nanoparticles with size of 20-200 nm are developed using biodegradable polymers that can encapsulate hydrophilic or hydrophobic drugs. Delivery of drugs using nanoparticles prolongs the time of drug in circulation and enhances the delivery. Nanoparticles can be prepared from carbon nanotubes, gold or quantum dots, but clinical use is restricted due to concern of biodegradability (Ludke et al, 2009). Hence nanoparticles prepared from biodegradable material can provide drug delivery with high therapeutic efficacy and reduced adverse effects (Italia et al,

2007). Dox nanoparticles have provided improved bioavailability with reduced cardiotoxicity. Thus recent advances have shown alternatives for reducing cardiotoxicity via improving the delivery methods without affecting the antitumor property of the drug.

6.3 Early detection

An inverse relation between the initiation of therapy for left ventricular dysfunction and improvement in EF was observed in patients with Dox-induced cardiotoxicity (Cardinale et al, 2010). Hence early detection of cardiotoxicity in its preclinical stage before cardiac damage becomes irreversible and overt; resulting in drop in left ventricular ejection fraction (LVEF), can be beneficial in reducing the development of Dox-mediated cardiomyopathy (Truong et al, 2014). Early detection and therapy using heart failure medications within 2 months of the end of chemotherapy resulted in the recovery of LVEF in 64% of the patients suggesting the crucial importance of early disease diagnosis and cardiac surveillance to prevent the damage when it is reversible and not extensive (Cardinale et al, 2010; Cardinale and Cipolla et al, 2016). Subclinical cardiotoxicity can be detected by an assessment of the biochemical markers such as troponin and non-invasive strain imaging echocardiography (Cardinale and Cipolla, 2016; Fallah-Rad et al, 2011). Many studies have shown elevated levels of troponin prior to reduction in LVEF in patients treated with Dox (Colombo et al, 2014; Christenson et al, 2015). Use of non-invasive cardiac imaging techniques such as multigated acquisition scan (MUGA) and two dimensional transthoracic echocardiography can also be useful to address the cardiac safety profile. Instead of conventional LVEF, tissue Doppler imaging and strain rate are more accurate to detect early subclinical cardiotoxicity induced by Dox (Fallah-Rad et al, 2011; Jassal et al, 2009). These methods detected changes in cardiac function which preceed a decrease in LVEF (Thavendiranathan et al, 2014; Zeglinksi et al, 2011). However, dependence on acoustic window,

difficulty to compare due to variation of software packages yielding different strain results, time consumed and off-line data analysis are major limitations of this technique which precludes it to be included in routine assessment of cardiac function (Thavendiranathan et al, 2014).

6.4 Treatment with cardiovascular drugs

Currently no specific treatment is available for attenuation of Dox-induced cardiomyopathy. Conventional heart failure drugs such as β-blockers and renin angiotensin system (RAS) inhibitors are used to support reduced cardiac function developed as a result of Dox-induced cardiomyopathy. Although the treatment with heart failure medications is not initiated until the detection of left ventricular dysfunction, early start of treatment is associated with better therapeutic response (Curigliano et al, 2012). Numerous animal as well as clinical studies have investigated the beneficial effect of conventional heart failure medications on cardiac function in Dox-induced cardiomyopathy. With the increasing evidence of activation of RAS in Dox-mediated cardiomyopathy, inhibition of RAS at various levels using various renin inhibitors, angiotensin converting enzyme inhibitor and angiotensin receptor blocker have provided cardioprotection in an animal model of Dox-induced cardiotoxicity (Akolkar et al, 2015). Clinical trials using RAS inhibitor alone or in combination with β-blocker such as Carvedilol or Bisoprolol suggested cardioprotective effects on heart failure as there was a lower incidence of death, heart failure and LV systolic dysfunction (Bosch et al, 2013; Cardinale et al, 2006; Dessi et al, 2011). However studies with a longer follow-up are required to determine the usefulness of these drugs in primary prevention (Vejpongsa and Yeh, 2014).

6.5 Cardioprotective agents

Alternatively various antioxidants and polyphenolic compounds are being investigated for their potential to reduce the cardiotoxic effects of Dox. Dexrazoxane, a cyclic derivative of

ethylenediaminetetraacetic acid (EDTA), has the ability to chelate iron and hence reduce the formation of ROS (Ludke et al, 2009). It is the only cardioprotective drug licenced in USA, Canada (Zinecard) and Europe (Cardioxane) for reduction of Dox-induced cardiotoxicity (Doroshow, 2012; Hasinoff and Herman, 2007). Chelation of iron was considered to be the primary mechanism of protection against Dox-mediated cardiotoxicity, however similar cardioprotection is not observed with other iron chelators. Dexrazoxane changes the configuration of Top II preventing the binding of anthracycline to Top II (Hasinoff, 2008; Lyu et al, 2007). This questions the possibility of interference of dexrazoxane with anti-tumor property of Dox (Singal and Iliskovic, 1998; Swain et al, 1997). Numerous studies have shown that administration of dexrazoxane at recommended time and dose, before Dox, does not interfere with the antitumor mechanism of Dox (Links and Lewis, 1999). Meta-analysis from seven randomized clinical trials suggested beneficial role of dexrazoxane in reducing Dox-induced cardiotoxicity in breast cancer patients (Vincent et al, 2013). The use of dexrazoxane is recommended in adult patients with advanced or metastatic breast cancer who have already received a minimum cumulative dose of 300 mg/m^2 of Dox whereas its use is not recommended in children due to concerns of secondary malignant disease (Hasinoff, 2008; Kremer and van Dalen, 2015). Despite the cardiotoxic protective effect, the use of dexrazoxane is questioned as a result of myelo suppression when administered in excess doses (Hasinoff, 2008).

As increased generation of ROS is recognized as the underlying cause for Dox-induced cardiotoxicity, prevention of the cardiotoxicity using a variety of natural or synthetic compounds with anti-oxidant properties has been investigated. Probucol, a lipid lowering drug, prevented Dox-mediated cardiotoxicity and HF without compromising the anti-tumor property of the drug (Siveski-Iliskovic et al, 1994). Several other natural compounds such as vitamins, resveratrol,

oleuoprein, polyphenols, and flavonoids have been investigated as adjunct therapies to prevent Dox-induced cardiotoxicity. Although these compounds have shown beneficial effects in reducing Dox mediated cardiotoxicity in animal models (Andreadou et al, 2007, 2014; Gu et al, 2015; Li et al, 2000; Ludke et al, 2012[a, b]; Oktem et al, 2012; Siveski-Iliskovic et al, 1994), clinical studies highlighting their protective effects are either not been carried out or have provided inconclusive results. Most of these studies were underpowered and large multicenter trials need to be carried out to draw conclusive information. As Dox treated patients have shown reduction in plasma levels of Vit C, effect of Vit C in Dox induced cardiotoxicity is the focus of this thesis.

7. Vitamin C

7.1 Background

Vit C, also known as ascorbic acid (L-ascorbic acid or ascorbate), is ubiquitous water soluble vitamin. It was first identified as an important dietary component in preventing scurvy by surgeon James Lind (Carr and Vissers, 2013). The term Vit C was introduced by Casimir Funk around 1920 by indicating it as

an essential nutritional factor. Later on in 1932, the structure of Vit C was identified by Walter Norman Haworth while Szent-Giorgyi associated Vit C as anti-scorbutic factor. Haworth and Szent-Giorgyi coined the term ascorbic acid for Vit C and were awarded the Nobel Prize in 1937 for their discovery of Vit C (de Tullio, 2012).

7.2 Structure and biosynthesis

Vit C, a small carbohydrate molecule, exists in two enantiomers; L and D-ascorbic acid. L-Ascorbic acid is the bioactive form which has more affinity for Vit C transporter proteins and has anti-sorbutic acitivity (Carr and Vissers, 2013). At physiological pH, ascorbate exists as

radical ion with an unpaired electron at C (4) (Lohmann, 1983). Ascorbate undergoes one or two electron oxidations to produce the ascorbyl radical and dehydroascorbic acid (DHA) respectively (Carr and Frei, 1999; Carr and Visserss, 2013) (Figure 4).

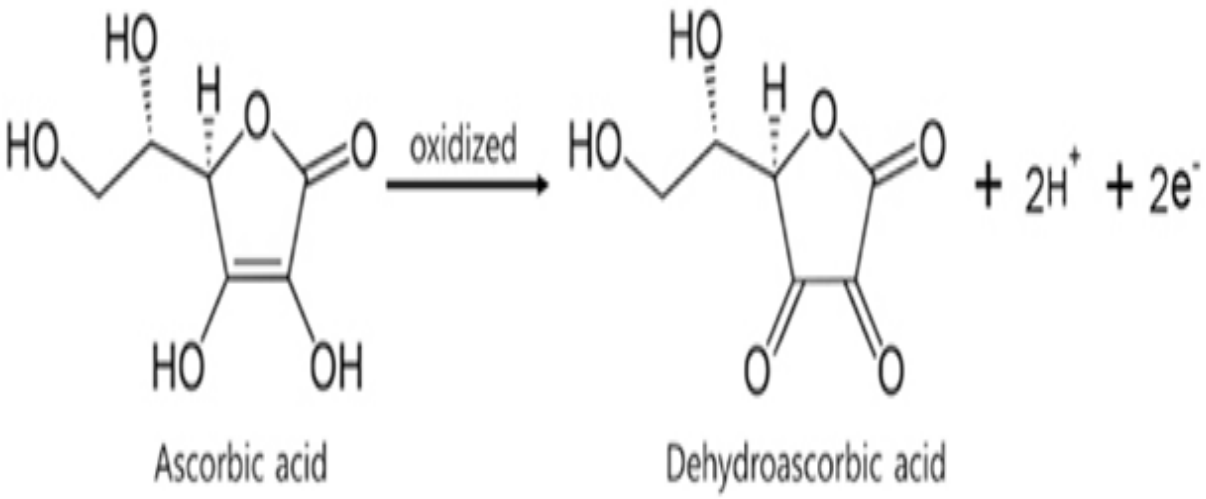

Figure 4: Chemical structure of ascorbic and dehydroascorbic acid
(Kim and Kim, 2016)

Vit C is synthesized from glucose in most plants and animals. Ascorbate synthesis occurs in kidney of fish, amphibians and reptiles, whereas it occurs in liver for mammals. However, humans along with other primates and some other mammalian species are not able to synthesize Vit C de-novo due to a mutation in the gene encoding L-gulonolactone oxidase, the final enzyme in ascorbate biosynthesis pathway. Hence dietary intake of ascorbate is vital for human health (Carr and Vissers, 2013; Corti et al, 2010; Du et al, 2012).

Humans obtain Vit C through consumption of fresh fruits and vegetables. However, the amounts of Vit C obtained are labile and depend on the storage and preparation of the food. The US Department of Agriculture, US National Cancer institute as well as Health Canada

recommend at least 5 servings of fruits and vegetables daily, estimating to 210 – 280 mg of Vit C. However, the amount of Vit C consumed by more than 25% of men and women is less than 2.5 servings daily (Levine et al, 1999).

7.3 Recommended Dietary Allowance and Plasma levels

The Recommended Dietary Allowance (RDA) for Vit C was set at 60 mg/d in 1989 (Carr and Frei, 1999; National Research Council, 1989). Plasma concentration of 24 μM is achieved by RDA of 60 mg/d. This is the concentration close to the Km for Vit C transport. Whereas, plasma concentrations of 66 μM, representing the Vmax for Vit C transport protein, is achieved at a dose of 200 mg/d and the Vit C transporter is saturated at this concentration. As Vit C does not exhibit any adverse effects at this dose, the ideal intake might be the amount that saturates the transporter (Levine et al, 1999). European nutritional societies are revising the RDA values for Vit C based on its metabolic losses, rate of absorption as well as urinary excretion. Based on these criteria, they have redefined the RDA to be 95 mg/d for women and 110 for adult men. The concentration of Vit C would be affected by smoking, pregnancy, infection, and age. Thus higher values are recommended for pregnant or lactating women and smokers (German Nutrition Society, 2015). Doses of Vit C up to 2g/day have not shown any deleterious effects are considered safe for consumption. However, studies indicate that single dose greater than 200 mg does not increase the relative bioavailability of Vit C, suggesting consumption of several smaller doses compared to single large dose. Slow release formulations or salts of Vit C may provide better bioavailability (Carr and Visser, 2013).

Vit C is present in the plasma mainly in its reduced form, ascorbic acid, at a concentration of 30-60 μM (Capellmann et al, 1994). Plasma ascorbate concentration of 20 – 50 μM indicates suboptimal status with increased risk of deficiency, whereas, concentration below

20 µM is associated with development of subclinical symptoms such as fatigue, muscle weakness, lethargy and anemia. Plasma ascorbate concentration increases in a sigmodial curve, with a steep rise up to 50 µM is achieved with an intake between 60-100 mg of Vit C. The curve plateaus at 70 -80 µM plasma concentration at a dose of approximately 250 mg of Vit C, (German Nutrition Society, 2015; Levine et al, 1999).

Vit C is accumulated by almost all human tissues. The levels of ascorbate is found to be in high mM range in metabolically active tissues as well as tissues exposed to or producing high concentration of ROS, such as erythrocytes, astrocytes, lymphocytes, neutrophils, adrenals, lungs, eye lenses and pituitary glands (Du et al, 2013; Horning, 1975). In fact the concentration accumulated in these tissues is higher than in the plasma (Horning, 1975).

7.4 Bioavailability and degradation/excretion

The bioavailability of ascorbate represents the portion of the dietary micronutrient absorbed by the intestine and is available for metabolic processes in the body (Carr and Vissers 2013). It is tightly regulated and determined by uptake through intestinal absorption, metabolism, renal reabsorption and excretion (Du et al, 2012; Carr and Vissers 2013). The bioavailability is reduced with increasing dose of Vit C, where 100 % bioavailability was observed at 200 mg dose. As the dose increased from 500 to 1250 mg, the bioavailability reduced from 73% to 49% respectively. Vit C undergoes glomerular filtration and concentration dependent renal reabsorption through Vit C transport protein. The kidneys do not have mechanism for reabsorption of DHA form of Vit C and hence it is excreted in urine. The reabsorption of Vit C occurs in its ascorbate isoform, which upon saturation of the transport protein, is excreted in urine (Graumlich et al, 1997). In contrast to bioavailability, urine excretion is directly proportional to the steady state dose. No urine excretion is observed for doses of less than 100

mg. However, as the dose increases from 100 to 250 mg, the renal excretion increases from 25% to 50%. Vit C doses of more than 500 mg have little effect on body stores as most of the vit C at those doses is excreted (German Nutrition Society, 2015; Graumlich et al, 1997; Levine et al, 2013; Rumsey and Levine, 1998). Vit C that is not absorbed by intestine is partially metabolized to organic acids and carbon dioxide and excreted via faeces. Ascorbic acid and its metabolites such as oxalic acid, L-threonic acid and L-xylose are excreted through urine (German Nutrition Society, 2015).

Vit C is well tolerated and no adverse effect have been reported up to a dose of 1 gm/d. There are a few adverse effects associated with the use of Vit C; however, they are dose dependent. Diarrhea or abdominal blotting can occur when several grams of Vit C are consumed at once, but consumption of such high doses of Vit C has not been reported (Levine et al, 1999). As Vit C enhances iron absorption, it is speculated that high consumption of Vit C can cause iron overload due to thalassemia and hemochromatosis. A dose of 2 gm of Vit C was unable to induce neither increased iron absorption nor increased iron stores in both healthy as well as iron replete subjects (Nienhuis, 1981). Hyperuricosuria was observed in some patients who were given a dose of 3 g/d as well as some patients treated intravenously with large dose of Vit C, while, dose of 1 gm/d was completely safe and no adverse incidences were reported at that dose (Herbert et al, 2006).

7.5 Transport

Ascorbic acid and DHA are absorbed from the lumen of the human intestine by enterocytes. Ascorbate is actively transported intracellularly by sodium dependent Vitamin C transporter (SVCT) protein. Two SVCT proteins, SVCT-1 and SVCT-2 are identified with a differential tissue distribution and uptake kinetics. SVCT-1 is expressed in epithelial lining and

plays a key role in determining the bioavailability by determining the intestinal uptake and renal reabsorption. Whereas, SVCT-2 is expressed in specialized and metabolically active tissues for delivery of Vit C for enzymatic reactions or protecting against oxidative stress (Corti et al, 2010; Rivas et al, 2008). SVCT has a high affinity for ascorbate (Km = 0.2 mM), while it takes up DHA with a low affinity (Km= 0.8 mM) (Savini et al, 2008; Wilson, 2002).

DHA, the oxidized form of ascorbate, competes with glucose for its uptake into the cells via glucose transporters (GLUTs). Variety of GLUT isoforms (GLUT 1-10) are involved in the transport of DHA in various tissues. GLUT 2 and 8 are present in small intestine and responsible for uptake of dietary DHA. GLUT 1 and 3 are present in a variety of cells for transport of DHA, whereas, cardiomyocytes express GLUT 4 (Rivas et al, 2008; Wilson, 2002). Following intracellular transport of DHA, it is rapidly reduced to ascorbate. Transport of DHA via GLUT occurs in competition with glucose, which occurs at relatively high concentration in the body. Therefore, despite the high concentration of DHA in dietary foods, transport is a critical limitation for its bioavailability (Harrison and May 2009). GLUT dependent uptake is influenced by various cellular factors such as oxidant levels, insulin and glucose levels, where uptake can become more intense in oxidative stress (Banhegyi et al, 2014) or impaired by excess glucose during uncontrolled diabetes (Wilson J, 2002). This would further impair regeneration of ascorbate and weaking of antioxidant defenses.

7.6 Functions

In addition to its function as an antioxidant and maintaining cellular redox homeostasis by scavenging various free radicals as well as ROS/RNS and recycling of other cellular antioxidants such as Vit E and GSH (Padayatty et al, 2003; Traber and Stevens, 2011), Vit C is a crucial cofactor for many copper and iron dependent enzymes. Ascorbate is crucial for

preserving iron in the Fe^{2+} state and hence retains the catalytic activity of members of Fe^{2+} dependent dioxygenases such as hydroxylases, histone demethylases and cytoplasmic prolyl hydroxylases. The latter has a key role in regulating hypoxia-induced transcription factor (HIF), which is involved in the activation of more than 800 genes involved in maintaining cellular functions such as angiogenesis, cell growth, cell survival, iron homeostasis, glucose uptake and glycolysis (De Tullio, 2012; Du et al, 2012). Low ascorbate content could activate molecular responses to hypoxia, while ascorbate supplementation decreased the HIF protein by enhanced degradation (Padayatty and Levine, 2000). It is also required for catalytic activity of hydroxylases involved in collagen synthesis metabolism (Geesin et al, 1988). Histone demethylases are involved in methylation of histones. Ascorbate supplementation has been shown to increase histone acetylation, thus epigenetic status of the tissue depends on ascorbate to keep Fe^{2+}- dioxygenases functional (Du et al, 2012). Vit C also has a key role in preserving the enzyme activity of NOS by preventing oxidation of its cofactor BH4 (Heller et al, 2001). Several clinical studies have demonstrated the ability of high dose intravenous Vit C to kill tumor cells and hence has been suggested as an adjuvant chemotherapy regime (Drisko et al, 2003; Ohno et al, 2009).

7.7 Antioxidant mechanism

Antioxidants are molecules that can accept or donate electron(s) to a free radical and thus eliminate the unpaired electron of latter and neutralizing it. While the antioxidant molecule may directly react with the free radical to eliminate unpaired electron, they themselves become free radicals in the process (Nimse and Pal, 2015). However the free radical generated from antioxidants is far more stable and less reactive than the free radical neutralized (Lu et al, 2010).

For example reaction of superoxide with Vit C results in the generation of less toxic species hydrogen peroxide or perhydroxyl radical (equation 1 and 2).

$O_2^{\bullet-} + AH_2 \rightarrow H_2O_2 + A^{\bullet-}$ (equation 1)

$O_2^{\bullet-} + AH_2 \rightarrow HO_2^{\bullet} + AH^-$ (equation 2)

Vit C is considered as an ideal antioxidant because of two major properties: i) it has low one electron reduction potential and ii) it has low reactivity and high stability even in its radical form. Vit C in its ascorbate and ascorbyl radical forms can react and reduce all physiologically relevant radicals and oxidants due to its low reduction potential. Ascorbate (AH^-) scavenges ROS or RNS and is in turn converted into ascorbate radical ($A^{\cdot-}$). The latter further readily dismutates to form ascorbate and DHA (A) (equation 3 and 4) or it is reduced back to AH^- by NADH dependent semidehydroascorbate reductase (Figure 5). DHA does not have any antioxidant capacity and hence can be reduced back to AH^- by glutathione dependent enzyme glutathione dehydroascorbate oxidoreductase or NADPH dependent thioredoxin reductase. Alternatively, DHA is rapidly and irreversibly hydrolyzed to 2, 3-diketogulonic acid (DKG) which can further dissociate to oxalate and threonate (equation 5) (Carr and Frei, 1999).

$AH^- \leftrightarrow A^{\bullet-} \leftrightarrow A$ (equation 3)

$A^{\bullet-} + A^{\bullet-} \rightarrow AH^- + A$ (equation 4)

$A \rightarrow DKG \rightarrow$ oxalate, threonate (equation 5)

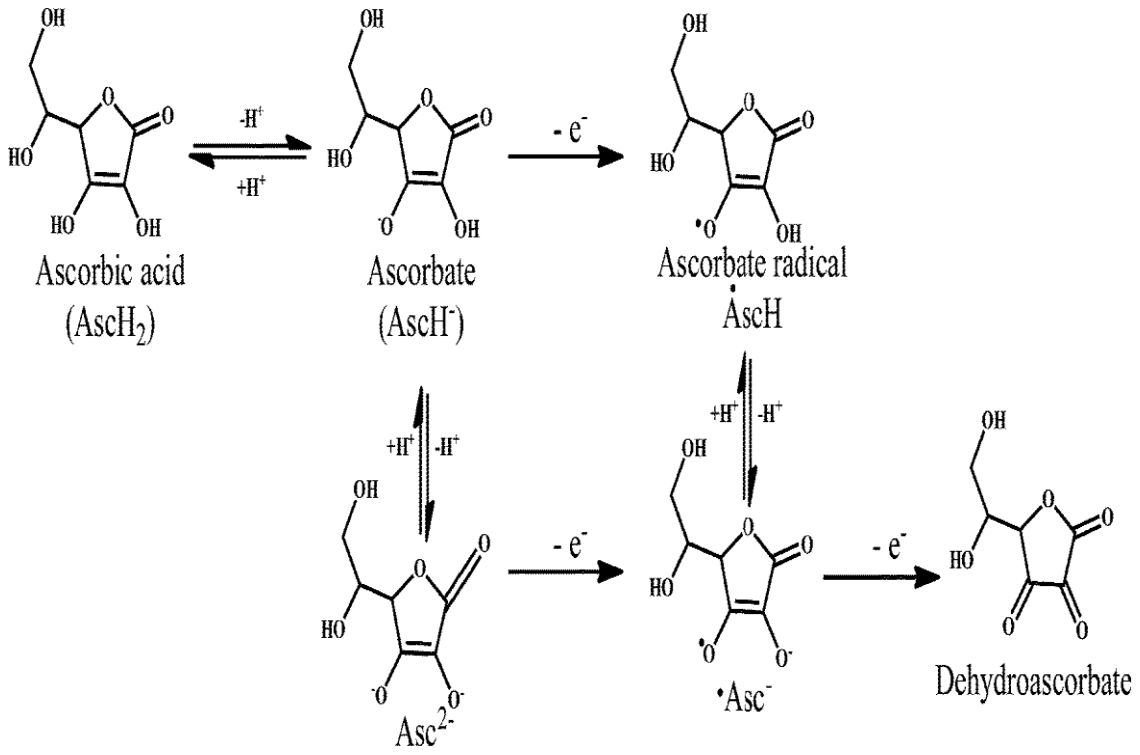

Figure 5: Free radical scavenging mechanism of Vit C

Ascorbic acid ($AscH_2$) reacts with free radicals and scavenges their unpaired electrons. Under physiological conditions, $AscH_2$ exists in the form of ascorbate ion ($AscH^-$). $AscH^-$ donates one or two electrons and is converted to ascorbate radical ($Asc^{-\bullet}$) or dehydroascorbate (DHA) respectively. DHA can be reduced back to ascorbate by glutathione dehydroascorbate oxidoreductase or NADPH dependent thioredoxin reductase. (Nimse and Pal, 2015)

Although not directly but in combination with tocopherol (TOH), ascorbic acid has a synergistic role in scavenging lipophilic peroxyl radicals (LOO) (equation 6). Ascorbic acid reacts with membrane bound oxidized tocopherol radical (TO) at lipid aqueous interphase and reduces it, to regenerate active TOH (Pisoschi and Pop, 2015) (equation 7).

$LOO\bullet + TOH \rightarrow LOOH + TO\bullet$ (equation 6)

$AH^- + TO \rightarrow A^{\bullet-} + TOH$ (equation 7)

7.8 Recycling of Ascorbate

Ascorbate radical and DHA are recycled by a variety of enzymatic and non-enzymatic pathways. Cellular ascorbate radical is reduced by NADH dependent cytochrome b_5 reductase. Ascorbate radical generated in the blood is reduced by plasma membrane ascorbate radical reductase, whereas thioredoxin reductase or glutathione dependent DHA reductase compliments as a cellular ascorbate recycling (May et al, 2000). DHA has a very short half-life of 5-15 minutes at physiological levels (Wilson J, 2002; Du et al, 2012). The concentration of DHA in plasma is less than 2 μM as it is either transported into cells by glucose transporters or reduced to ascorbate by cellular mechanisms. DHA reductase converts DHA to ascorbate in human enterocytes or it can be reduced to ascorbate non-enzymatically by GSH (Winkler et al, 1994). Liver protein disulfide isomerase is observed to react with GSH and DHA to catalyze reduction of DHA to ascorbate (Du et al, 2012). At physiological conditions, DHA which is not recycled back to ascorbate rapidly hydrolyzes to 2, 3-L-diketogluconate (2, 3-DKG). The latter being very unstable, rapidly decarboxylates into L-xylonate and L-lyxonate or degrades into L-erythrulose and oxalate (Du et al, 2012; Linster and Van Schaftingen, 2007). As DHA does not have antioxidant potential, efficient recycling of DHA to ascorbate ensures functioning of the latter as a reductant (Corti et al, 2010; Rumsey and Levine, 1998).

7.9 Pro-oxidant effects

In the presence of a catalytic metal ion, ascorbate exerts pro-oxidant effects. Ascorbate promotes recycling of Fe^{3+} to Fe^{2+}, which in turn catalyzes formation of highly reactive species. Ascorbate can reduce iron from its Fe^{3+} to Fe^{2+} state and is oxidized to ascorbate radical itself (equation 8). In the presence of O_2, Fe^{2+} can readily reduce it to superoxide radical ($O_2^{-\bullet}$)

(equation 9), which is further dismuted to H_2O_2 and O_2 (equation 10). Fe^{2+} can also react with H_2O_2 to generate Fe^{3+} hydroxyl radical ($HO^{\cdot}$) (equation 11).

$AscH^- + Fe^{3+} \rightarrow Asc^{\cdot -} + Fe^{2+}$ (equation 8)

$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\cdot -}$ (equation 9)

$O_2^{\cdot -} + O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$ (equation 10)

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\cdot}$ (equation 11)

The pro-oxidant effects of ascorbate principally depend on the availability of catalytic metal ions. In healthy individuals, iron is sequestered by iron binding protein transferrin and ferritin. This sequestered iron is redox inactive. Stored iron can be released from ferritin by reductants such as ascorbate, thiols and reduced flavins or by lysosomal degradation of ferritin (Minnoti et al, 1999). Excess free iron promotes the generation of ROS via the Fenton reaction. In pathological conditions, such as thalassaemia or hematochromatosis, non-transferrin bound iron is present, thus supplementation of ascorbate without the use of an iron chelator can lead to deleterious effects (Du et al, 2012).

8. Studies with Dox and Vit C

8.1 Animal studies

Administration of Vit C to Dox treated isolated primary cardiomyocytes or cardiomyocyte cell line such as H9c2 cells, demonstrated attenuation of oxidative stress via decrease in the generation of ROS whereas an increase in antioxidant enzyme activity was noted (Ludke et al, 2012[a, b]). Vit C pretreatment increased survival of isolated adult rat cardiomyocytes by reduction in an expression of the proteins associated with cell death such as apoptosis and autophagy (Ludke et al, 2012[a]). Vit C also increased the protein expression of Vit C transporter proteins SVCT-2 and GLUT-4, downregulated by Dox (Ludke et al, 2012[b]).

Supplementation of Vit C alone or in combination with other antioxidant improved the survival of Dox-treated rats (Fujita et al, 1982; Santos et al, 2007; Viswanatha Swamy et al, 2011; Wang et al, 2016). Vit C reduced the levels of Dox-mediated cardiac damage as evident by a decrease in enzyme biomarkers such as urea, creatine phosphokinase, lactate dehydrogenase, aspartate aminotransferase and alanine aminotransferase (Santos et al, 2007; Viswanatha Swamy et al, 2011). Dox mediated cardiac damage observed as loss of the myofibrils, vacuolization of cytoplasm, enlarged mitochondria and inflammatory cell infiltration was significantly attenuated by Vit C (Viswanatha Swamy et al, 2011). Electron microscopy revealed alterations in dilation of sarcoplasmic reticulum as well as transverse tubular system and appearance of large number of cytoplasmic fat droplets in cardiac tissue of Dox treated guinea pigs were significantly reduced by Vit C (Fujita et al, 1982).Vit C treated animals showed reduced ROS levels whereas an upregulation of antioxidant enzyme protein expression was noted (Viswanatha Swamy et al, 2011; Wang et al, 2016). Dox mediated alterations in

myocardial function were attenuated by Vit C by an increase in arterial pressure, heart rate and decrease in QRS, QT and ST intervals (Wang et al, 2016).

8.2 Clinical studies

Oxidative stress is linked to progession of a number of diseases including CVD. Plasma concentration of Vit C was inversely related to the mortality from CVD (Khaw et al, 2001; Ye and Song, 2008). Supplementation of ascorbic acid increased the plasma levels of ascorbic acid, and decrease in the levels of superoxide anion and lipid peroxidation as well as reduced risk of death from coronary heart disease (Enstrom et al, 1992; Weijl et al, 2004). A cardioprotective effect was observed by increasing the flow mediated dilation and endothelial derived NO action upon Vit C supplementation (Gokce et al, 1999).

Increase in oxidative/nitrosative stress markers such as increase in lipid peroxidation, nitrate/nitrite, $O_2^{-\bullet}$ and protein oxidation were observed in the plasma of patients with various cancers such as prostate, breast, ovarian and cervical cancers as well as during chemotherapy. Additionally, these patients also demonstrated a reduction in plasma antioxidant enzyme activities, total antioxidant potential, GSH and Vit C levels (Arsova-Sarafinovska et al, 2009, Fuchs-Tarlovsky et al, 2013; Kasapovic et al, 2010; Riordan et al, 2005; Weijl et at 1998; Yeh et al, 2005). The level of ascorbate was inversely dependent on the tumor burden and degree of inflammation in these patients (Mikirova et al, 2013). Hence increasing the levels of Vit C in cancer patients may improve immune system function and enhance patient's well-being (Mikirova et al, 2013). Studies involving supplementation of Vit C in cancer patients on chemotherapy with cisplatin observed increase in plasma antioxidant levels (Weijl et al, 2004). However, the protective effects exerted by Vit C raise concerns for antagonizing cytotoxic effects of antineoplastic drugs.

In vitro studies on leukemia and lymphoma cell lines indicated that Vit C supplementation affected the efficacy and therapeutic response of chemotherapy drugs by preserving MMP (Heaney, 2008; Subramani et al, 2014). In contrast, several *in vitro* and *in vivo* studies have dissociated anti-tumor mechanism of Dox from oxidative stress (Siveski-Iliskovic et al, 1995; Shimpo et al, 1991). Numerous clinical studies concluded that antioxidant supplementation does not alter the efficacy of chemotherapeutic regimens (Block et al, 2007; Drisko et al, 2003; Simone et al, 2007). Antioxidant supplementation increased survival times, tumor responses or both (Block et al, 2008; Simone et al, 2007). In fact antioxidant status can be a crucial factor in determining the responsiveness of chemotherapy for cancer patients. Increased lipid peroxidation and H_2O_2 is observed to interfere with the efficacy of chemotherapy drug by affecting cell death mechanism (Conklin, 2004; Conklin, 2009; Papageorgiou et al, 2005; Shacter et al, 2000) via inhibition of caspases and apoptosis by oxidative stress mediated aldehyde production (Conklin, 2004).

Although the exact mechanism is unclear, ascorbic acid supplementation showed improved antineoplastic activity of Dox, probably by reducing oxidative stress mediated aldehyde production in cancer cells (Kurbacher et al, 1996). Hence high dose of Vit C is suggested as an adjuvant therapy for cancer patients. High dose of Vit C is cytotoxic to cancer cells but not to normal tissue. A clinical trial is undertaken to address the safety and efficacy of antioxidants when added to chemotherapy (Drisko et al, 2003). Clinical trial in ovarian cancer patients demonstrated Vit C administration reduced toxicity associated with chemotherapy (Drisko et al, 2003; Ma et al, 2014). An adjuvant therapy of Vit C and E along with Dox in breast cancer patients increased the patient survival by preservation of overall antioxidant status and reduction of lipid peroxidation and DNA damage (Suhail et al, 2012).

Although studies highlight protective effects of Vit C, there is no evidence for the beneficial effect of Vit C on nitrosative stress and inflammation in Dox-induced cardiotoxicity. Furthermore beneficial effect of Vit C on cardiac function needs to be investigated in Dox-induced cardiotoxicity. This thesis focuses on the study of the elucidation of mechanisms involved in Dox-induced cardiotoxicity as well as investigating the beneficial role Vit C.

III. RATIONALE, HYPOTHESIS, OBJECTIVES AND APPROACH

Rationale: Numerous studies have highlighted increased oxidative stress as one of the major mechanism involved in Dox-induced cardiotoxicity. Recent evidence suggests an involvement of nitrosative stress in Dox-induced cardiotoxicity by increased formation of peroxynitrite and upregulation of iNOS. Previous studies carried out in our laboratory elucidated the beneficial effect of Vit C in reducing oxidative stress, nuclear fragmentation, apoptosis and cell death in isolated rat cardiomyocytes. However, the effects of Vit C on nitrosative stress in Dox-induced cardiotoxicity in cardiomyocytes and whole animals are not known. Therefore, we sought to understand the effects of Vit C on Dox-induced nitrosative stress in isolated adult rat cardiomyocytes. Furthermore, we also investigated the role of Vit C on cardiac structure, function, oxidative/nitrosative stress, apoptosis, inflammation and Vit C transporter proteins in Dox-treated rats.

Hypothesis: Vit C reduces Dox-induced cardiotoxicity by decreasing nitrosative/oxidative stress and inflammatory cytokines by the upregulation of eNOS and downregulation of iNOS. Together these changes reduce Dox-mediated cell death and improve cardiac structure and function.

Objectives: Following objectives have been addressed:

Objective 1: Characterize nitrosative stress in Dox-induced cardiotoxicity and determine any beneficial effects of Vit C on such a change in isolated cardiomyocytes. Following aims were targeted for this objective

a) Investigate the effects of Dox on cell viability and cardiomyocyte oxidative/nitrosative stress with or without Vit C.

b) Examine the effect of Dox on NO generation and protein expression of NOS isoforms (eNOS and iNOS) with or without Vit C.

c) Determine the effect of Dox on the levels of cytokines: TNF-α and IL-10 in the presence or absence of VitC.

Objective 2: Examine the effects of Vit C in an animal model of Dox-induced changes in cardiac structure and subcellular biochemical as well as contractile function under the following aims.

a) Analyze the effects of Dox on systolic and diastolic cardiac function in the presence or absence of Vit C

b) Determine the effects of Vit C on Dox-induced structural changes in the heart.

c) Examine the effects of Vit C on Dox-induced changes in antioxidant enzymes and oxidative stress.

d) Understand the role of Vit C and Dox on NO levels, NOS activities, and protein expression as well as protein nitrosylation.

e) Determine the effects of Vit C on the expression of key signaling proteins for apoptosis in the presence or absence of Dox.

f) Examine the effects of Dox and Vit C on Vit C transporter proteins SVCT-2 and GLUT-4.

g) Determine the effects of Vit C on the levels of proinflammatory cytokines TNF-α, IL-1β and IL-6 as well as anti-inflammatory cytokine IL-10 in the presence and absence of Dox.

Approach: Our approach was to first characterize the effects of Dox *in vitro*, in isolated adult cardiomyocytes, followed by *in vivo* studies of the subchronic effects of Dox and Vit C in rat hearts:

***In vitro* studies:** We used isolated cardiomyocytes from adult Sprague-Dawley rats. Cells were treated for 24 hours with Dox, Vit C and Vit C + Dox (time and dose selected were based on our previous studies by Ludke et al (2012[a]). Different end points were analyzed: cell viability by MTT assay; superoxide and peroxynitrite levels using fluorescence dye; released and cellular NO; total NOS activity; protein expression of NOS using western blot; protein nitration and nitrosylation; and levels of cytokines TNF-α and IL-10 were determined by ELISA.

***In vivo* studies:** Adult male Wistar rats (250±10 gm) were randomly divided into four groups: (i) Control (saline injected); (ii) Dox (six equal doses of 2.5 mg/kg BW for three weeks with a total cumulative dose of 15 mg/kg BW) given intraperitoneally; (iii) Vit C (50 mg/kg B.W) orally; and (iv) Dox + Vit C group. Vit C was started prophylactically one week prior to the treatment of Dox and was continued for 5 more weeks. Diastolic and systolic cardiac function were analyzed by echocardiography; cardiac structural analysis using hematoxylin-eosin staining; antioxidant enzyme (SOD, catalase and GPx) activities; antioxidant enzyme protein expression by western blot; cardiac NO levels by Greiss reagent; cardiac NOS activity; NOS protein expression by western blot; protein nitrosylation; protein expression of various apoptotic (Bax, Bcl-xl, Caspase-3 and PARP) and signaling pathway proteins (Akt, p53, NFκB) and Vit C transporters (SVCT-2 and GLUT 4) by western blot and cytokines (IL-10, IL-6, IL-1β and TNF-α,) using ELISA.

IV. MATERIALS AND METHODS

1. Materials

All the chemicals used were purchased from Sigma Aldrich (Oakville, ON, Canada) unless stated otherwise. Doxorubicin hydrochloride was obtained from Pfizer (New York city, NY, USA); Collagenase was obtained from Worthtington Biochem (Lakewood, NJ, USA); laminin was obtained from Corning life sciences (Corning, NY, USA) and Fetal Bovine Serum (FBS) was purchased from Life Technologies (Carlsbad, CA, USA). Hydroxyphenyl fluorescein (HPF) and diaminofluorescein-2diacetate (DAF-2DA) were purchased from Cell technology (Mounain view, CA, USA). Protease inhibitor cocktail and 2, 2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) were purchased from Roche diagnostics (Mississauga, ON, USA). Enhanced chemiluminescence (ECL) and phosphatase inhibitor were purchased from Pierce-Thermo Fisher Scientific (Waltham, MA, USA). NO detection kit, NOS activity and Nitrotyrosine antibody were purchased from Oxford biomedical research (Oxford, MI, USA). Protein S-nitrosylation kit was purchased from Cayman chemicals (Ann Arbor, MI, USA). ELISA kit for estimation of cytokines TNF-α, IL-1β, IL-6 and IL-10 was purchased from Peprotech (Rocky hill, NJ, USA). Antibodies phospho eNOS Ser1177, phospho eNOS Thr 495, total eNOS, iNOS, β-actin, Catalase, Akt, NKκB, p53, Bax, Bcl-xl, Caspase-3 and Bnip-3 were obtained from Cell Signaling Technology (Mississauga, ON, Canada), whereas antibodies for SVCT-2, GLUT-4, GPx and SOD were obtained from Santa Cruz biotechnology (Dallas, TX, USA). Goat anti-rabbit immunoglobulin G horseradish peroxidase was purchased from Bio-Rad (Hercules, CA, USA).

2. Methods

All the animal studies carried out for the *in vitro* experiments were approved by the University of Manitoba Animal Care Committee and conform to the guidelines of Canadian Council of Animal Care. All the *in vivo* experiments described in this study were approved by ethical committee for the use of animals at the Universidade Nove de Julho, Sao Paulo, SP, Brazil (Protocol no. 0032/2014) and were conducted in accordance with the National Institute of Health Guide for the care and use of laboratory animals.

2.1 Cardiomyocyte isolation

Cardiomyocytes were isolated as previously described (Ludke et al, 2012[b]) from adult male Sprague-Dawley rats (250 ± 10 gm). Briefly, animals were anesthetized using a ketamine (80 mg/kg) and xylazine (12 mg/kg) cocktail at a dose of 0.1 ml/100 gm body weight (BW) intraperitoneally after which they were heparinized with 200 units of heparin. Hearts were excised and mounted on a Langendorff perfusion apparatus and perfused with modified Krebs buffer containing: 110 mM NaCl, 2.6 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, and 11 mM Glucose (pH 7.4) for 5 mins to wash blood. The Ca^{2+} free perfusion was then switched to recirculating mode with the buffer containing 25 µM Ca^{2+}, 0.1% w/v collagenase, and 0.25% w/v Bovine Serum Albumin (BSA), for 30-40 mins. Ventricles from the heart were collagenase-digested, chopped into small pieces and gently passed through pipette tips of progressively smaller diameter. Cell suspension thus obtained was incubated in buffer with increasing $CaCl_2$ and BSA concentrations. The suspension was passed through a nylon mesh (200 µm) and allowed to settle for 10 min. The supernatant was discarded and the cell pellet was resuspended in a medium M199 with antibiotics (streptomycin/penicillin, 100 µg/ml) containing 10% Fetal Bovine Serum (FBS). Cardiomyocytes (10^6 per dish) were plated in

laminin - coated (20 µg/ml) 100 mm polystyrene tissue culture dishes. Cells were incubated at 37°C under a 5% CO_2 and 95% O_2 atmosphere. This culture medium does not support the growth and survival of fibroblasts. After 2 hours, the culture medium was changed to remove unattached dead cells, and the attached viable cardiomyocytes were incubated overnight in M199 with 0.5% FBS under same culture conditions.

2.2 Cardiomyocyte treatment

These viable cardiomyocytes (95%) were randomly divided into four groups and treated as follows: (i) Control; (ii) Vit C (25 µM sodium ascorbate); (iii) Dox (10 µM of Doxorubicin hydrochloride); and (iv) Vit C (25 µM sodium ascorbate) + Dox (10 µM Doxorubicin hydrochloride). For the combination group of Vit C + Dox, cells were pretreated with Vit C for 1 hour prior to the addition of Dox. The concentration and the time of treatment were based on our previous studies (Ludke et al, 2012[a]).

2.3 Cell Viability

Viability of the cultured cardiomyocytes was determined by MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay. Living cells reduce the yellow MTT to purple formazan in the mitochondria. Cardiomyocytes were seeded in 96-well microplates with the same starting cell number per well (10^3 cells/well). After 24 hours of treatment, 5 µg/ml MTT was added to the media in each well and further incubated at 37 °C for 2 hours. All the remaining supernatant was removed carefully and 150 µl of dimethyl sulfoxide (DMSO) was added to each well and mixed thoroughly to dissolve the formed crystal formazan. After 10 mins of incubation, absorbance was recorded at 570 nm using plate reader (Dynex Technologies, Chantilly, VA, USA). Cell viability in treated groups was expressed as percent (%) control.

2.4 Superoxide anion detection

Isolated cardiomyocytes (10^4 cells/ well) were seeded in 24 well cell culture plates. After 24 hours of treatment, cells were incubated with 5µM dihydroxyethidium (DHE) for 30 mins at 37 °C (Saiki et al, 1986). Cells without DHE served as negative control. Cells were visualized by fluorescence microscopy using Olympus microscope (1X81) (Olympus America Inc, Melville, NY, USA) at excitation and emission wavelength of 515/605 respectively. Fluorescence intensity was determined using Image J software (National institute of health, Bethesda, MD, USA; http://imagej.nih.gov/ij/) and expressed as relative fluorescence intensity per myocyte area. The results were also expressed as % superoxide positive cells.

2.5 Peroxynitrite levels

Peroxynitrite levels in cardiomyocytes were detected using peroxynitrite specific dye hydroxyphenyl fluorescein (HPF). HPF by itself does not exhibit any fluorescence. However, in the presence of peroxynitrite, it is converted to compound that exhibits strong dose dependent fluorescence (Setsukinai et al, 2003). Isolated cardiomyocytes (10^3 cells/ well) were seeded in black 96 well plates. After 24 hours of treatment, cells were washed with phosphate buffered saline (PBS) and loaded with 10 µM HPF and incubated at 37 ºC for 1 hour in dark. Cells without HPF dye were used as negative control. The fluorescence was measured using fluorescence plate reader (Glomax multidetection system, Promega, Madison, WI, USA) at excitation and emission wavelength of 488 nm and 515 nm respectively. Peroxynitrite levels were expressed as % control.

Additionally cellular peroxynitrite levels were also monitored using fluorescence microscope (Olympus 1X81). Images were acquired at 40 x magnification at excitation and emission of 488 and 515 nm respectively. Images were taken randomly from 10 different fields.

Fluorescence intensity was determined using Image J software (National institute of health, Bethesda, MD, USA; http://imagej.nih.gov/ij/) and expressed as relative fluorescence intensity per myocyte area. Data was also represented as % peroxynitrite positive cells in each treatment group from three independent experiments done in duplicate.

2.6 NO levels

Intracellular levels of NO were measured using fluorescent dye diaminofluorescein-2diacetate (DAF-2DA). DAF-2DA is a non-fluorescent cell permeable reagent which upon its hydrolysis by cytosolic esterases, is converted into DAF-2 which in the presence of NO is further converted into fluorescent triole derivative, DAF-2T (Kojima et al, 1998). After 24 hours of treatment, cardiomyocytes were washed with PBS and then incubated with DAF-2DA (5 µM) at 37 ºC for 1 hour in dark. Fluorescent images were obtained using microscope (Olympus 1X81) at excitation and emission wavelength of 488 and 515 nm respectively. Images were taken randomly from 10 different fields from three independent experiments done in duplicate for each treatment group. Fluorescence intensity was determined using Image J Software (National institute of health, Bethesda, MD, USA; http://imagej.nih.gov/ij/) and expressed as relative fluorescence intensity per myocyte area.

Additionally, spectrophotometric quantification of nitrite was done using Greiss reagent. In aqueous solution, NO rapidly degrades to nitrate and nitrite. Nitrate is further enzymatically converted to nitrite by nitrate reductase (Schmidt H, 1995). Levels of NO released into the media and cardiomyocytes were examined using a commercially available kit from Oxford biomedical research. Briefly, after 24 hours of treatment, media as well as cardiomyocytes from different treatment groups were collected. Cardiomyocytes were washed with PBS and cell lysate was prepared. Nitrate reductase and NADH were added to samples (cell lysate or media) or nitrite

standard and incubated for 20 minutes at RT. Later Greiss reagent was added and incubated for 5 mins at RT. Absorbance was recorded at 540 nm using plate reader (Dynex Technologies, Chantilly, VA, USA) and concentration of the samples was determined using a standard curve.

2.7 NOS activity

Estimation of NOS activity in cardiomyocytes was done using a commercially available kit. L-Arginine is enzymatically converted to citrulline and NO by enzyme NOS in the presence of NADPH and oxygen. NO rapidly degrades to nitrate and nitrite. NOS activity was assayed spectrophotometrically by measuring the accumulation of its stable degradation products, nitrate and nitrite. For accurate assessment of total NO generated, nitrate is enzymatically converted to nitrite by enzyme nitrate reductase followed by quantitation of nitrite using Greiss reagent (Ghigo et al, 2006). Briefly, cardiomyocytes lysate was suspended in PBS, protein concentration was determined using a Biorad protein detection and 40 µg of protein lysate was added to reaction buffer in a microcentrifuge tubes. NADPH was added and the mixture was incubated for 3 hours at 37 ºC. Nitrate reductase was added to each sample and the microcentrifuge tubes were vortexed and incubated for 20 minutes at RT. Samples were then centrifuged at 12,500 rpm for 5 minutes at 4 ºC. 100 µl of this mixture of sample or standard were added to microplate. 50 µl of color reagent was added and the plates were shaken for 5 minutes at RT. Absorbance was recorded at 540 nm using microplate reader (Dynex Technologies, Chantilly, VA, USA). Concentration of the samples was determined by extrapolation from the standard curve. Samples were expressed as µmoles of NO produced/µg protein/min.

2.8 S-nitrosylation of protein

S-nitrosylation of proteins was determined using a commercially available kit from Cayman's using biotin switch method. In this method, free SH groups are blocked, S-NO bonds

are cleaved and the free thiol groups generated are biotinylated. Detection of biotin coupled proteins can be done using avidin conjugated reagents (Ckless et al, 2004). Briefly, cardiomyocytes from different treatment groups were washed with cold PBS; the cells were scraped and transferred in microcentrifuge tubes. Cell pellet was generated by centrifugation at 500 g for 5 mins at RT. Free thiol groups were blocked using blocking solution containing dimethyl formamide (DMF). Samples were incubated for 30 minutes at 4 ºC and cell lysate was generated by centrifugation of samples for 10 minutes at 4 ºC. Four volumes of ice cold acetone was added to supernatant and incubated at -20 ºC for 1 hour. Compact protein pellet was produced by centrifugation at 3000 g for 10 mins. The pellet was resuspended in reducing and labelling reagent provided in kit which cleaves S-NO bonds and biotinylates free thiol groups. The samples were incubated for 1 hour at RT. Acetone was added and samples were incubated for 1 hour at -20 ºC. A compact protein pellet was again generated by centrifugation of samples at 3000 g for 10 mins at 4 ºC. Minimal amount of buffer was added to resuspend the protein pellet. A small volume was reserved for protein determination whereas rest of the sample was stored at -80 ºC to perform western blot analysis.

Samples were run on SDS-PAGE and then transferred onto PVDF membranes. The membranes were blocked using 2% BSA and then incubated in s-nitrosylation detection reagent HRP for 1 hour. The membranes were developed on X-ray films using ECL reagent. The bands were quantified by image analysis software (Quantity One- Bio-Rad, Hercules, CA, USA).

2.9 Cytokine Analysis

Levels of cytokines such as Tumor necrosis factor-α (TNF-α), Interleukin-6 (IL-6), Interleukin 1β (IL-1β) and Interleukine-10 (IL-10) released from the cardiomyocytes into the medium were detected using commercially available sandwich Enzyme linked immunosorbent

assay (ELISA) development kit. Briefly, 96 well plates were coated with capture antibody overnight and then blocked with BSA to prevent nonspecific binding. Plates were washed with PBS and incubated with samples (cell lysate or tissue homogenate) for 2 hours followed by detection antibody. Plates were washed and incubated with Avidin-HPR conjugate for 30 mins. Following washing ABTS substrate was added and color development was monitored using ELISA plate reader (Dynex technologies, USA) at 405nm. Concentration of cytokines in treated samples is extrapolated from color development of a known standard concentration.

2.10 *In vivo* Treatment

Male Wistar rats (250 ± 10 gm) were obtained from Universidade Nove de Julho and maintained on a standard chow and water ad libitum. All animals were housed in a temperature controlled room (22 – 24 °C) in 12 hour light – dark cycle. A total of 46 animals were randomly divided into four different groups: i) control (6); ii) Vit C (7); iii) Dox (17); and iv) Vit C + Dox (16). Control animals were injected with normal saline. Animals in Vit C group were given Vit C (50 mg/kg body weight) as oral gavage daily for the entire treatment period. Animals in Dox treated group were injected with doxorubicin hydrochloride intraperitoneally for a cumulative dose of 15 mg/kg body weight given as 6 equal injections of 2.5 mg/kg body weight over 3 weeks (treatment dose and protocol was adopted from Iliskovic et al, 1994) (Figure 6A). Animals in Vit C + Dox group were treated with both Vit C and Dox as mentioned above. The combination group animals were treated with Vit C one week prior to treatment with Dox. Vit C was continued for two weeks post Dox treatment (Figure 6B). At the end of treatment period, animals (Control: 6; Vit C: 7; Dox: 11; and Vit C + Dox: 15) were anesthetized using ketamine (80 mg/kg) and xylazine (12 mg/kg) intraperitoneally and cardiac function was assessed by echocardiography. Animals were sacrificed by decapitation; hearts were removed quickly and

rinsed with cold saline. Atria and other connective tissue were removed and hearts were weighed. Heart tissue was rapidly frozen in liquid nitrogen and stored at -80 °C for further biochemical analysis. In order to avoid repeated freeze and thaw of the samples, ventricular tissue was homogenized and aliquoted in smaller volumes.

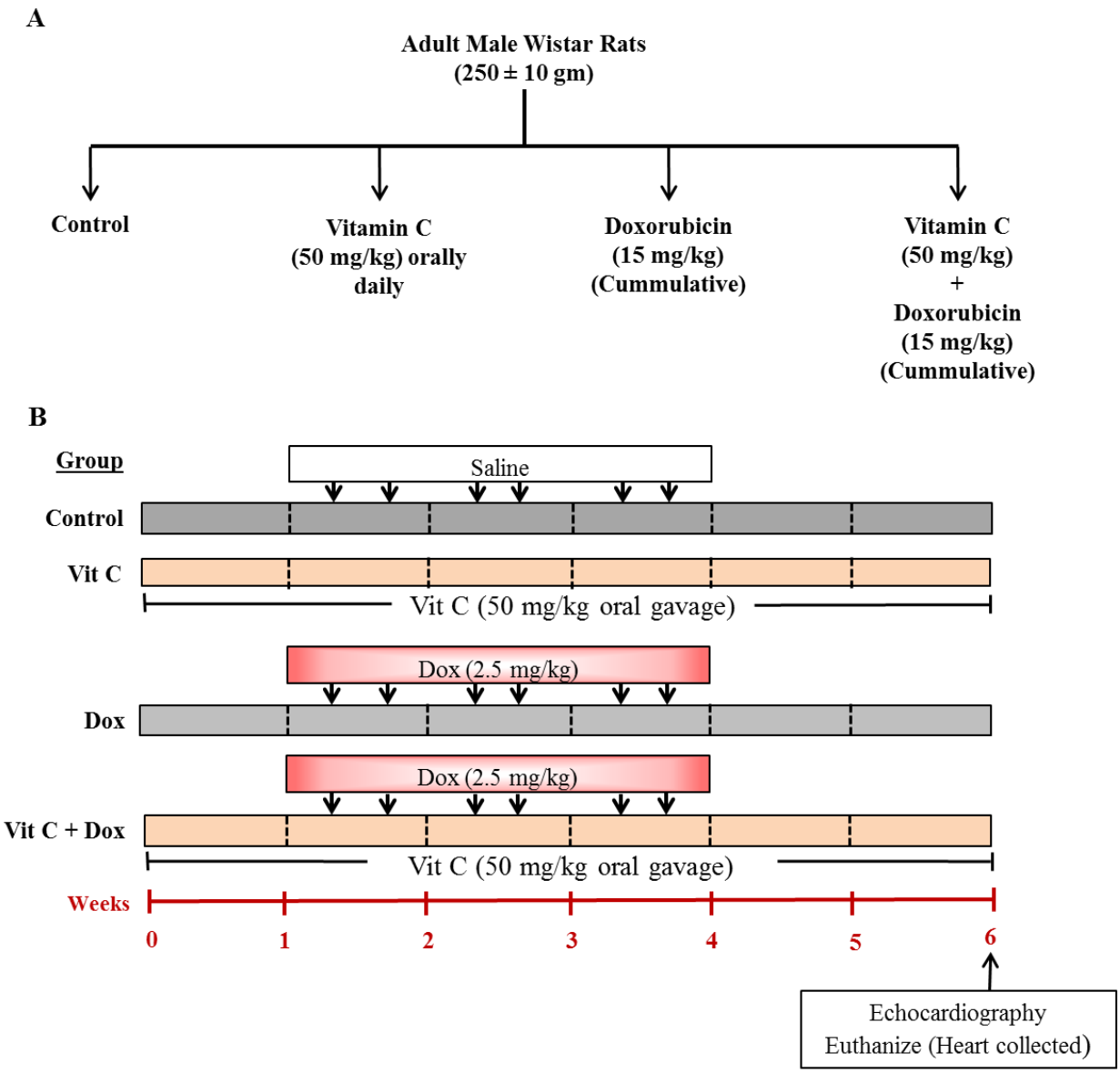

Figure 6: Treatment plan for the *in vivo* study

A) Adult male Wistar rats (250 ± 10 g) were randomly divided into four groups. Control animals were injected with saline. Dox and Vit C + Dox treated animals received intraperitoneal injection of 2.5 mg/kg BW Dox (six equal doses given for three weeks with a cumulative dose of 15 mg/kg BW). Vit C (50 mg/kg BW) was given by oral gavage daily to Vit C and Vit C + Dox group. **B)** Vit C treatment was started one week prior to Dox treatment and was continued for 5 more weeks. At the end of six weeks cardiac function was monitored in all the animals by echocardiography. Animals were euthanized and heart was harvested for histological and biochemical analysis.

2.11 Echocardiography

At the end of the treatment protocol, transthoracic echocardiography was performed at RT in all the groups. Rats were anesthetized (80 mg/kg ketamine and 12 mg/kg xylazine, ip), shaved and prewarmed echocardiography transmission gel was applied. Rats were covered with a surgical cloth and echocardiography analysis was done with a 10-14 MHz linear transducer in SEQUOIA 512 equipment (ACUSON, Mountain View, CA, USA). For the measurements of systolic function left ventricular (LV) fractional shortening (FS) and ejection fraction (EF) were evaluated. Diastolic function was assessed by evaluating LV isovolumic relaxation time (IVRT) and the ratio of early (E) to late (A) ventricular filling (Jorge et al, 2011). Each measurement was obtained with an average of three consecutive heart beats. The heart was first imaged in the parasternal long-axis followed by short axis two dimensionl views. Left ventricular dimension was measured at the level of the papillary muscles using 2D guided M-mode imaging. The diastolic function was evaluated at 4-chamber apical view. The echocardiographic observer was blinded to the various treatment groups.

2.12 Histological analysis

Following euthanasia, a part of ventricle was sliced and preserved in 10% formalin for structural analysis by histology. The formalin was removed by dehydrating through increasing concentrations of alcohol. The tissues are cleared of alcohol by passing through 100 % xylene and molded into paraffin blocks. Thin tissue sections (5 µm thick) were cut and mounted on microscope slides. Slides were deparaffinized and stained using hematoxylin and eosin. Tissue slides were observed at 63x magnification using Olympus DP70 (Olympus America Inc, Melville, NY, USA) microscope.

2.13 Oxidative stress profile

Frozen heart tissue was rinsed with saline and cut into smaller pieces and placed in ice cold phosphate buffer (pH 7.4) with 1mM phenylmethylsulfonyl fluoride (PMSF). Tissue was homogenized using ultra 80 Turrax blender (Ultrastirrer, Pilatusstrasse, Switzerland). The homogenate was centrifuged at 2500 g for 10 mins at 4°C. The supernatant was stored at -80 °C for analysis of oxidative stress parameters and antioxidant enzyme activities.

Protein carbonyl formation: Tissue samples were incubated with 2, 4-dinitrophenylhydrazine (DNPH) (10 mM) for 1 hour in dark vortexing every 15 mins. Trichloroacetic acid (TCA, 20%) was added to the samples and incubated for 10 mins on ice. Protein pellet was collected by centrifugation at 2000 rpm for 5 mins. The pellet was washed with TCA (10%) followed by 3 washes with ethanol/acetate (1:1) mixture. The final pellet was resuspended with Guanidine (6M) and incubated at 37 °C for 10 mins. The absorbance was measured at 360 nm. Protein carbonyl formation was expressed as nmol DNPH/mg protein (Reznick and Packer, 1984).

***Lipid peroxidation by thiobarbituric acid reactive substances (TBARS)*:** TCA (10% w/v) was added to tissue homogenate and incubated for 10 mins. The mixture was centrifuged to precipitate the proteins and thiobarbituric acid was added to protein free supernatant. The mixture was incubated in a water bath at 100 °C for 30 mins. Absorbance was measured at 535 nm. The amount of lipid peroxidation was expressed as µmol/mg protein (Buege and Aust, 1978).

***Superoxide anion*:** It was determined in ventricular tissue homogenates by calculating the rate of oxidation of adrenaline at 480 nm. Briefly tissue homogenate was added to glycine buffer followed by addition of catalase (494 µM). Absorbance was set to zero. Adrenaline (60

μM) was added and absorbance was recorded at 480 nm for 2 mins every 20 seconds (McCord and Fridovich, 1994). Levels of superoxide anion were expressed as $O_2^{-\cdot}$ mmoles /mg protein .

2.14 Antioxidant enzyme activities

Catalase activity was determined by reduction in the absorbance of H_2O_2. Tissue homogenate was added to phosphate buffer. H_2O_2 was added and reduction in the absorbance was recorded for 60 seconds at 240 nm wavelength (Aebi, 1984). Enzyme activity was expressed as nmol/mg protein.

Glutathione Peroxidase (GPx) activity is based on the conversion of NADPH to NADP. Briefly, tissue homogenate was added to phosphate buffer (pH 7.5) containing EDTA (1mM). To this mixture, glutathione reductase (10 U/ml), NADPH (10 mM) and reduced glutathione (10 mM) were added. The mixture was mixed and tert-butyl hydroperoxide (10 mM) was added. Absorbance was recorded at 340 nm wavelength for 60 seconds. GPx activity was expressed as nmol of NADH formed/minute/mg protein (Del Maestro R, 1985).

Superoxide dismutase (SOD) activity was determined by following the inhibition of pyrogallol auto-oxidation. Pyrogallol (24 mM) was prepared in 10 mM HCl. Tissue homogenate was added to Tris buffer with EDTA (1 mM) and Catalase (30 mM). Pyrogallol was added and the absorbance was recorded at 420 nm wavelength every 30 sec for 90 seconds. SOD activity was expressed as U/mg protein. One unit of SOD is the amount that shows 50% inhibition at pH 7.8 at RT (Mishra HP and Fridovich I, 1972).

2.15 Protein estimation

Cardiomyocytes from different treatment groups were washed with PBS and scraped. Cells were centrifuged at 2500 rpm at 4 ºC for 10 mins. The cell pellet was resuspended in ice

cold cell lysis buffer (1% TritonX-100, 1 mM EDTA, 1mM EGTA, 50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS and 0.2 mM Na_3VO_4) containing protease inhibitor cocktail, phosphatase inhibitor, 1 mM PMSF and 1mM Dithiothreitol (DTT). For *in vivo* studies, ventricular tissues from different treatment groups were washed with PBS and chopped into smaller pieces. Above mentioned lysis buffer was added to the chopped tissues and further homogenized using polytron homogenizer. The cell lysate/ tissue homogenate obtained was incubated on ice for 30 min and then centrifuged at 12000 rpm for 15 min at 4 ºC. The supernatant was collected and stored at -80 °C for further analysis.

The quantity of proteins in the samples was determined using dye binding assay (Bio rad). BSA was used as protein standard and the absorbance was determined in the range of 0- 1 mg/ml BSA. 5 µl of standard (BSA) and samples (cell lysate or tissue homogenate) were loaded in triplicate in 96 well plate followed by 245 µl of Bio rad dye (5x diluted) in each well. The plate was incubated for 10 minutes. Absorbance was recorded at 595 nm using microplate reader (Dynex Technologies, Chantilly, VA, USA). Concentration of samples was determined by extrapolation from the standard curve of BSA according to modified Bradford protein assay.

2.16 Western blot

Protein expression of NOS isoforms (phosphorylated (Ser 1177 and Thr 495) eNOS,total eNOS, and iNOS); nitrotyrosine (NT); antioxidant enzymes (SOD, GPx and catalase); Apoptotic proteins (Bax, Bcl-xl, Caspase-3, Bnip-3, and Bak); iNOS; Vit C transporter proteins (SVCT-2 and GLUT-4); signaling proteins (Akt, NKκB and p53) were determined using western blotting. The samples (cell lysate/ tissue homogenate) (30 µg protein) were subjected to electrophoresis and immunoblotting using specific antibodies for above mentioned proteins. Protein samples were subjected to one dimensional 8 to 15 % sodium dodecyl sulfate (SDS) polyacrylamide gel

electrophoresis (PAGE). Separated proteins were transferred to 0.45 µm polyvinylidene difluoride (PVDF) membranes for 90 mins at 300 mA. Non-specific sites on the membrane were blocked by incubating membranes with 10% skim milk, in Tris buffered saline with Tween-20 (TBS-T) containing 25 mM Tris, 150 mM NaCl and 2% Tween-20 (pH 7.4). Membranes were incubated overnight at 4 ºC with specified primary antibodies (1: 1000). Unbound primary antibody was washed with TBS-T. Membranes were placed in solution containing secondary antibody goat anti-rabbit immunoglobulin G horseradish peroxidase (1: 10,000) for 1 hour at RT. Unbound secondary antibody was washed using TBS-T. Detection of membrane-bound proteins was visualized by incubating the membranes with enhanced chemiluminescence (ECL) reagent, a substrate for horseradish peroxidise (HRP)-conjugated antibodies. The membranes were developed on X-ray film. The bands were quantified by image analysis software (Quantity One, Bio-Rad, Hercules, CA, USA). Equal protein loading for all the samples was confirmed using rabbit anti-GAPDH antibody (1:5,000) as a loading standard. Chemiluminiscence intensity of specific protein band in each lane was normalized to the intensity of GAPDH band in the respective lane.

2.17 eNOS monomer/dimer ratio

Low temperature (LT) electrophoresis was performed to determine the monomeric and dimeric forms of eNOS. Protein samples were incubated with laemmli buffer without β mercaptoethanol for 5 mins at 37 °C. Samples were loaded on 6% gel and SDS-PAGE at 4 °C. Transfer of proteins on PVDF membrane and probing of membrane with antibody was done as routine western blot analysis as described earlier. eNOS and GAPDH (Cell Signaling Technology, Mississauga, ON, Canada) were developed using ECL and the blots were analyzed

using Quantity One Software. Protein expression of eNOS was expressed as ratio of monomeric/dimeric form.

2.18 Statistical Analysis

All the *in vitro* experiments were performed in duplicate for each treatment group and repeated three times. For *in vivo* studies, data are expressed as Mean±SEM from 6-13 animals. Treatment groups were compared by One-way analysis of variance (ANOVA) and Tukey-Kramer's test was performed to identify differences between the groups. $P \leq 0.05$ was considered to be significant.

V. RESULTS

My approach was to first characterize Dox-mediated changes and their mitigation by Vit C in isolated cardiomyocytes, followed by *in vivo* studies in a whole animal model.

1. *In vitro* studies

Isolated cardiomyocytes were treated with a dose of 10 µM Dox and 25 µM Vit C for 24 hours to investigate the involvement of oxidative/nitrosative stress and inflammation in relation to cell viability and Dox-induced cardiotoxicity as well as its mitigation by Vit C. This dose as well as time for the treatment was based on the previous studies carried out in our laboratory (Ludke et al, 2012[a]).

1.1 Dox-induced reduction in cell viability and Vit C effect

Viability of isolated cardiomyocytes was determined in different groups using MTT assay. Conversion of the yellow colored MTT tetrazole dye to a purple formazan by cellular NADP(H) oxidoreductase indicates the metabolic activity of the cells and hence their viability. Viability of control cardiomyocytes was considered as being 100% and Vit C did not cause any significant difference in the viability (Figure 7). Dox treatment caused a significant reduction ($P<0.001$) in the viability of cardiomyocytes (63±3%) compared to Control and Vit C groups. Vit C treatment in Dox treated cardiomyocytes significantly ($P<0.001$) increased the viability to (87±2%) in Vit C + Dox group as compared to the Dox group (Figure 7).

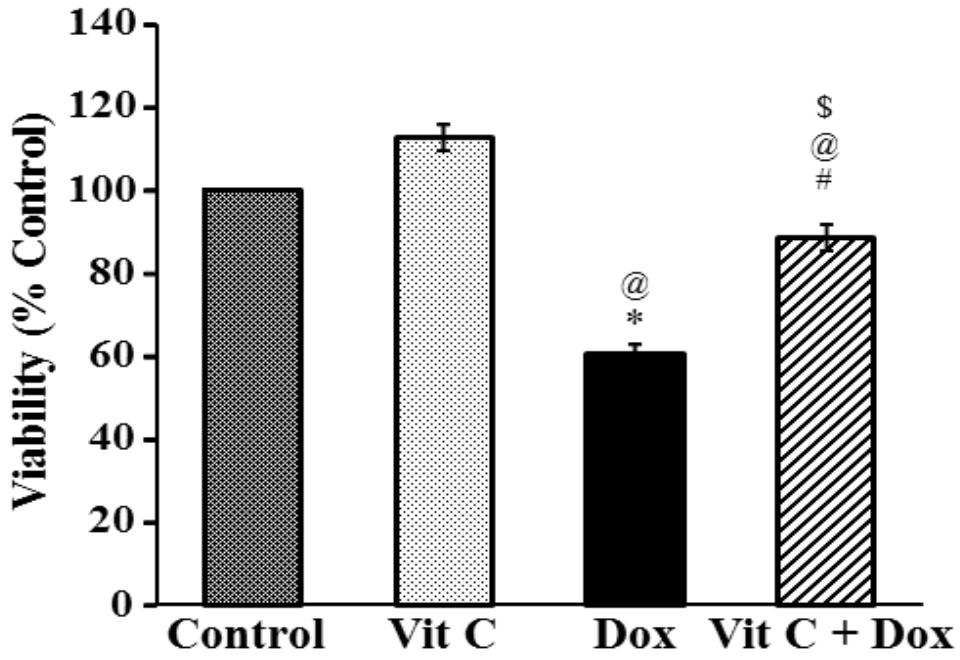

Figure 7: Viability of cardiomyocytes in different treatment groups

Viability of cardiomyocytes assessed by MTT assay after 24 hours of treatment with Dox and Vit C. Data are Mean ± SEM from three separate experiments done in duplicate. *P<0.001 compared to Control; $^{\$}$P<0.05 compared to Control; $^{@}$P<0.001 compared to Vit C and $^{\#}$P<0.001 compared to Dox.

1.2 Vitamin C reduces Dox- induced oxidative/nitrosative stress

Intracellular levels of superoxide anion were measured as an indicator of oxidative stress in cardiomyocytes from different treatment groups. Intracellular superoxide was measured by fluorescence microscopy using superoxide sensitive fluorescent dye, DHE. Intracellularly DHE reacts with superoxide anion resulting in the formation of red colored ethidium which intercalates into DNA. Increase in cellular superoxide anions is manifested as an increase in red fluorescence. Microscopy images showed an increase in superoxide anion as indicated by increase in red fluorescence in Dox treated cardiomyocytes compared to Control and Vit C treated cardiomyocytes (Figure 8A). Quantitative analysis of DHE fluorescence intensity per myocyte area demonstrated a significant reduction in the level of superoxide anion in Vit C + Dox group compared to Dox alone (Figure 8B). In addition to reducing the cellular levels of superoxide, Vit C + Dox treated cardiomyocytes also had significantly ($P<0.001$) lower number of total superoxide positive cells (58±3%) compared to Dox treated cardiomyocytes (78±3%) (Figure 8C).

As peroxynitrite is a potent reactive nitrogen species, intracellular levels of peroxynitrite were measured as a marker of nitrosative stress. Using peroxynitrite specific fluorescence dye HPF, intracellular levels of peroxynitrite were observed through fluorescence microscopy. Reaction of HPF, a non-fluorescent fluorescein derivative, with peroxynitrite exhibits green fluorescence. Control and Vit C treated cardiomyocytes had no detectable levels of peroxynitrite (Figure 9A). Compared to Control treated cardiomyocytes, an increase in the fluorescence intensity indicating increased levels of peroxynitrite per myocyte area was observed in Dox treated cardiomyocytes. Vit C + Dox treated cardiomyocytes showed significantly reduced levels of peroxynitrite per myocyte area compared to Dox alone (Figure 9A and B). Quantifying total

number of peroxynitrite positive cardiomyocytes indicated that Dox treatment caused a significant increase ($P<0.001$) in peroxynitrite positive cells (87±4%) compared to Control (Figure 9C). Vit C treatment to Dox treated cardiomyocytes significantly ($P<0.001$) reduced the number of peroxynitrite positive cells (62±3%) in Vit C + Dox group compared to Dox. Peroxynitrite levels were also quantified using a fluorescence plate reader. Quantitative analysis of fluorescence intensity confirmed that Vit C treatment significantly lowered the levels of cellular peroxynitrite compared to Dox alone (Figure 9D).

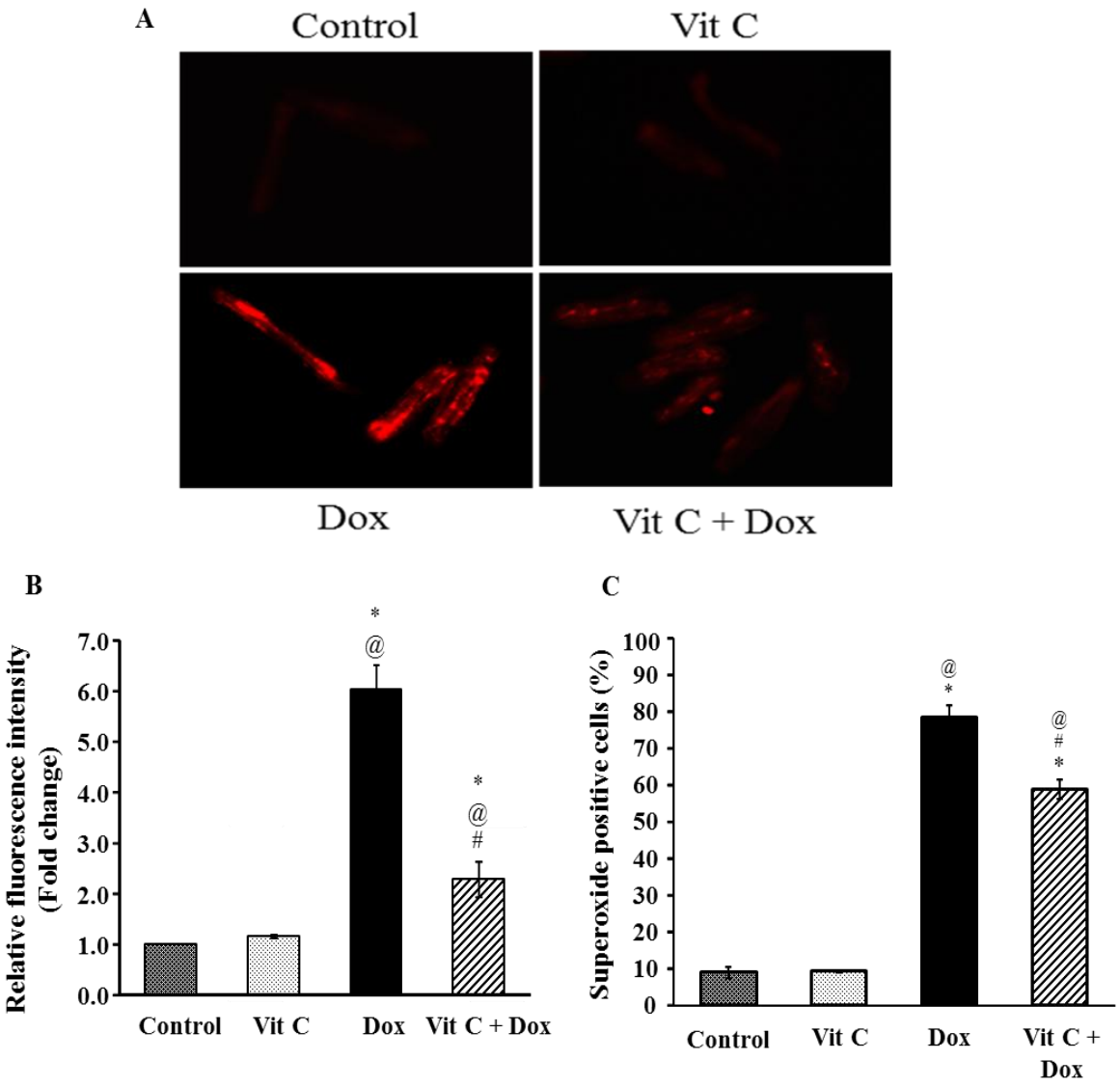

Figure 8: Superoxide anion levels in cardiomyocytes

A) Representative microscopy images of Dihydroxyethidium (DHE) fluorescence in cardiomyocytes; **B)** Quantitative image analysis of DHE fluorescence intensity/myocyte area; and **C)** Semiquantitative analysis of the number of superoxide positive cells (%). Data are Mean ± SEM from three separate experiments done in duplicate. *P<0.001 compared to Control; $^{@}$P<0.001 compared to Vit C and $^{\#}$P<0.001 compared to Dox.

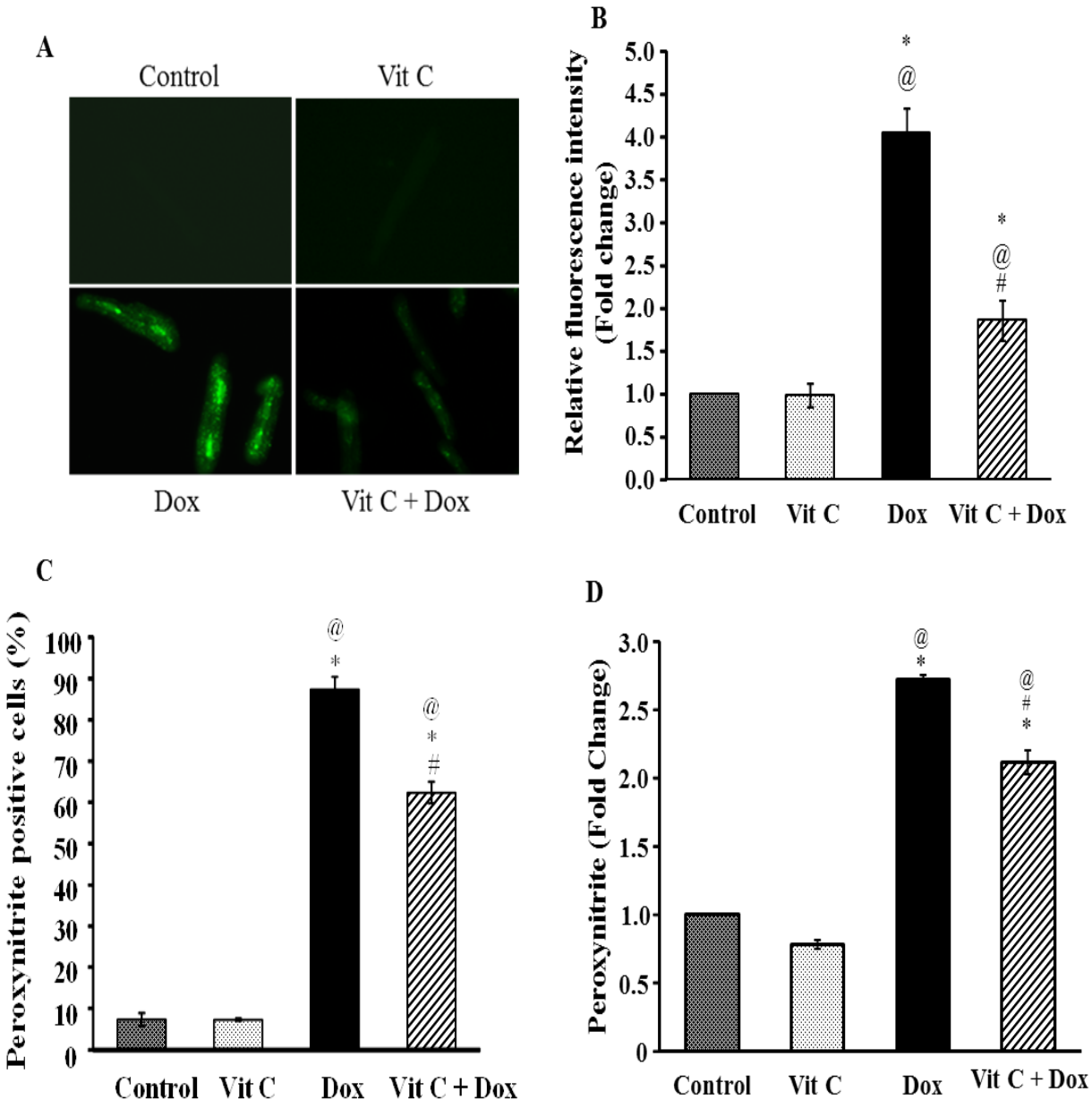

Figure 9: Peroxynitrite production in cardiomyocytes.
A) Representative fluorescence microscopy image of cardiomyocytes incubated with peroxynitrite specific dye hydroxyphenyl fluorescein (HPF); **B)** Quantitative analysis of HPF fluorescence intensity/myocyte area; **C)** Semiquantitative analysis of peroxynitrite positive cells (%); and **D)** Quantitative analysis of fluorescence intensity of peroxynitrite using fluorescence plate reader. Data are Mean ± SEM. from three separate experiments done in duplicate. $^{*}P<0.001$ compared to Control; $^{@}P<0.001$ compared to Vit C and $^{\#}P<0.001$ compared to Dox.

In addition to peroxynitrite levels, increased levels of protein nitrotyrosine and nitrosylation also serve as important markers for nitrosative stress. These protein modifications, caused by the formation of NO adduct to proteins on cysteine and tyrosine residues, are triggered by increased levels of reactive nitrogen species (RNS) such as peroxynitrite, inflammation or increased NO levels. Using western blot analysis, we determined protein nitrosylation and protein nitration in cardiomyocytes from different treatment groups. Western blot images indicated an extensive nitration and nitrosylation of a number of proteins by Dox as compared to Control and Vit C groups (Figure 10A and C). A significant reduction in these Dox mediated protein modifications was observed in Vit C + Dox treated cardiomyocytes (Figure 10A and C). Densitometric analysis indicated a significant reduction ($P<0.001$) in the levels of protein nitrosylation and nitrotyrosine by Vit C in Vit C + Dox treated cardiomyocytes compared to Dox. GAPDH was used as a loading control. Protein expression of nitrosylated and nitrated proteins was normalized to protein expression of GAPDH and represented as fold change (Figure 10B and D).

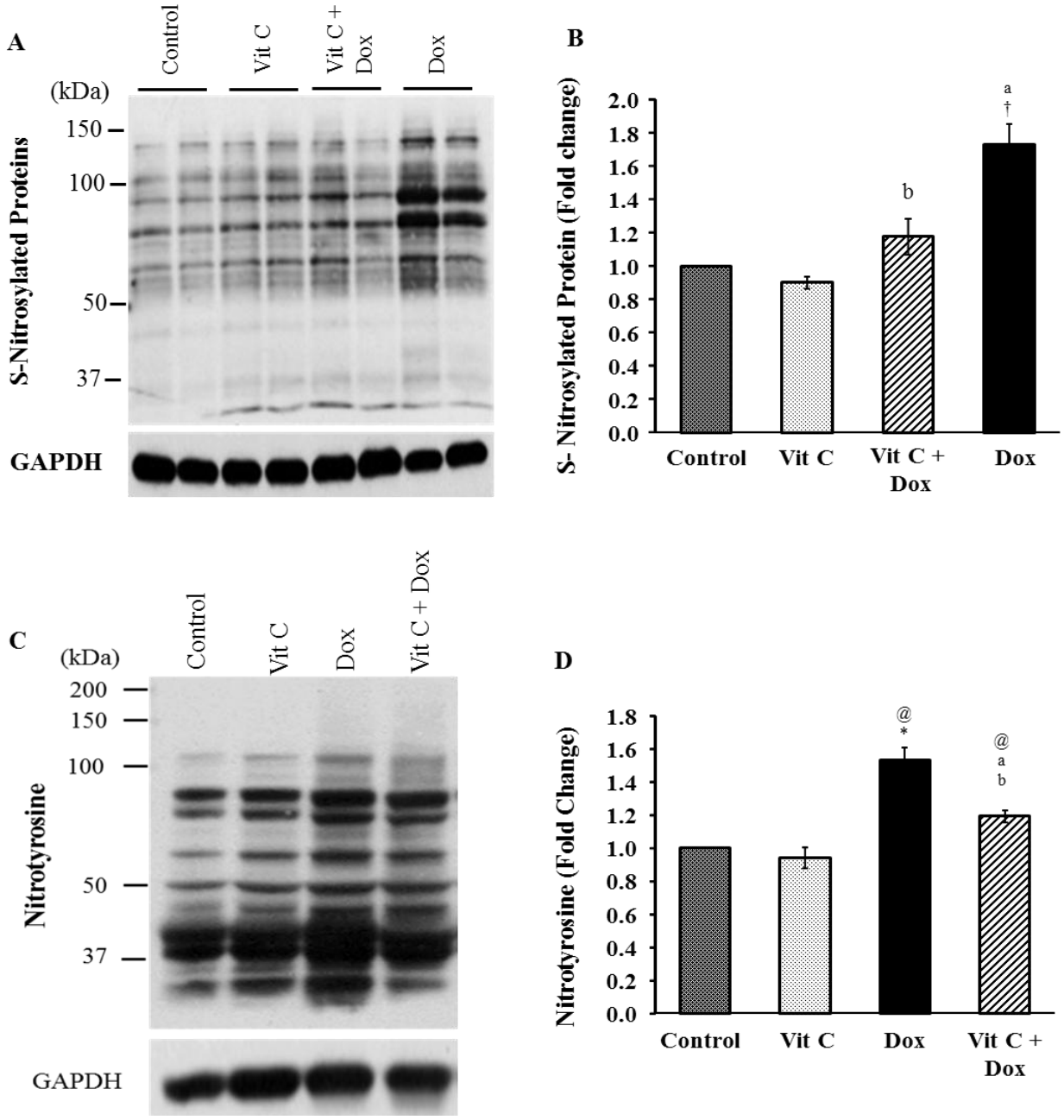

Figure 10: Protein modification in cardiomyocytes.

A) Representative western blot image of S-nitrosylated protein and GAPDH; **B)** Densitometric analysis of S-nitrosylated proteins represented as fold change; **C)** Representative western blot image of protein Nitrotyrosine and GAPDH; and **D)** Densitometric analysis of protein nitrotyrosine represented as fold change. Data are represented as Mean ± SEM from three separate experiments done in duplicate. $^{*}P<0.001$ compared to Control; $^{\dagger}P<0.01$ compared to control $^{@}P<0.001$ compared to Vit C; $^{a}P<0.05$ compared to Vit C and $^{b}P<0.01$ compared to Dox.

1.3 Vitamin C reduces Dox- induced changes in Nitric oxide (NO) production.

Since increased levels of peroxynitrite and protein nitration/nitrosylation are triggered through increased levels of NO, we wanted to investigate the effect of different treatments on the levels of NO. We measured the levels of cellular NO by fluorescence microscopy using a NO specific fluorescent dye DAF-2DA (Figure 11A). Fluorescence microscopy indicated increased green fluorescence in Dox treated cardiomyocytes representing increased cellular NO levels (Figure 11A). The fluorescence intensity of DAF-2DA was quantified per myocyte area. Vit C significantly decreased the levels of NO in Dox treated cardiomyocytes compared to Dox alone. There was no detectable level of NO in Control and Vit C treated groups (Figure 11A and B). The levels of NO were also quantified spectrophotometrically using the Greiss reagent. NO being an easily diffusible signaling molecule, the levels of both cellular and released NO was quantified in the form of more stable species of nitrate (Figure 11C and D). In comparison to Control and Vit C treated groups, Dox treatment caused a significant increase ($P<0.001$) in the levels of cellular as well as released NO of about 1.8 fold and 3 fold respectively. Vit C treatment of Dox treated cardiomyocytes caused a significant reduction ($P<0.001$) in the levels of cellular and released NO by compared to Dox treated cardiomyocytes (Figure 11C and D).

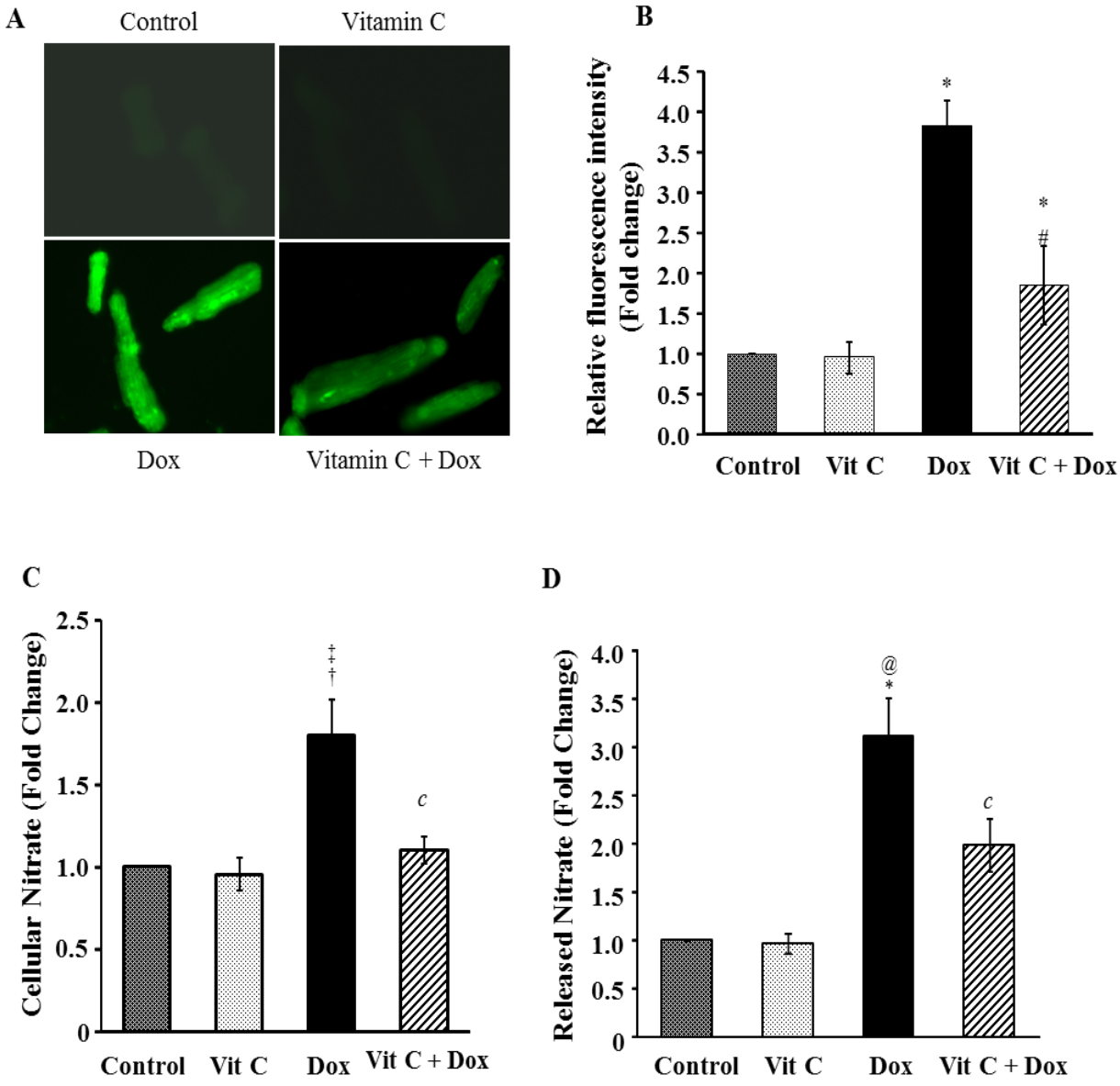

Figure 11: Nitric oxide (NO) production in cardiomyocytes.
A) Representative microscopy images of DAF-2DA fluorescence for Nitric oxide (NO) radical; **B)** Quantitative image analysis of NO fluorescence intensity/myocyte area. Levels of NO in **C)** cardiomyocytes; and **D)** released in the media. Data are expressed as Mean ± SEM from three independent experiments done in duplicate. *P<0.001 compared to control; †P<0.01 compared to control; $^{@}$P<0.001 compared to Vit C; ‡P<0.01 compared to Vit C and ^{c}P< 0.05 compared to Dox.

Endogenously, NO is produced by the action of the enzyme nitric oxide synthase (NOS). Therefore, we determined the effects of Vit C and Dox treatment on total NOS activity in isolated cardiomyocytes. Dox caused a significant increase ($P<0.001$) of about threefold increase in total NOS activity compared to Control and Vit C (Figure 12). Vit C prevented Dox mediated increase in NOS activity in Vit C + Dox treated cardiomyocytes. A significant reduction ($P<0.001$) in NOS activity of about 1.5 fold was observed in Vit C + Dox treated group compared to Dox (Figure 12). These alterations in NOS activity were reflective of the levels of NO as observed in Figure 11.

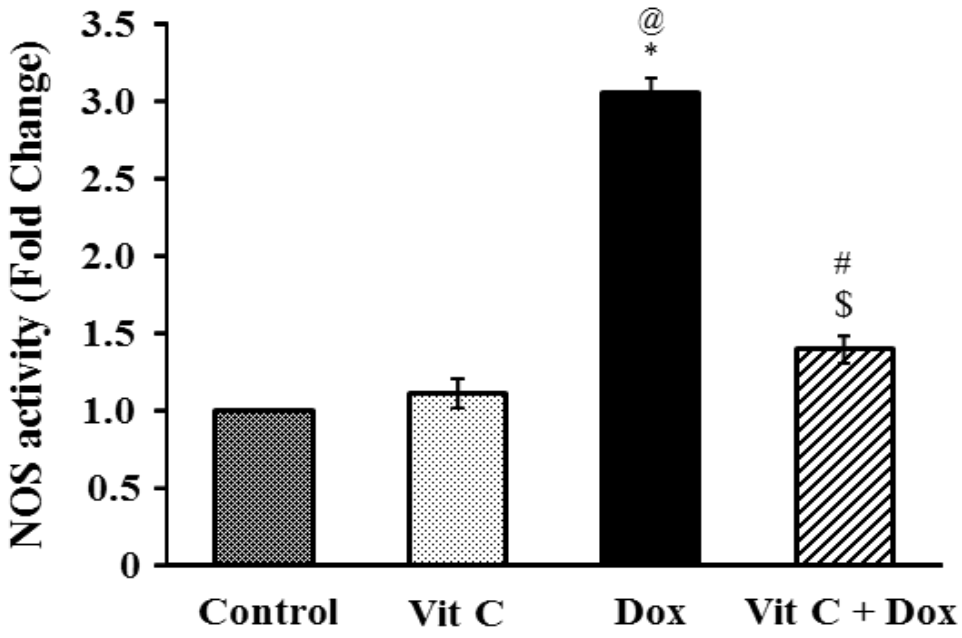

Figure 12: Nitric Oxide Synthase (NOS) activity in cardiomyocytes from different treatment groups.

NOS activity in cardiomyocytes treated with Vit C (25 µM), Dox (10 µM) or Vit C (25 µM) + Dox (10 µM) was determined at the end 24 hours of treatment. NOS activity was represented as fold change. Data are Mean ± SEM from three independent experiments done in duplicate. $^{*}P< 0.001$ compared to control; $^{\$}P<0.05$ compared to control; $^{@}P<0.001$ compared to Vit C; and $^{\#}P<0.001$ compared to Dox.

1.4 Dox- induced alterations in protein expression of NOS isoforms.

Different NOS isoforms: iNOS; eNOS; and nNOS have diverse physiological roles and are known to be activated by distinct stimuli. Since eNOS and iNOS have important functions in cardiomyocytes, using western blot analysis, we investigated the effects of Dox and Vit C on the protein expression of only iNOS and eNOS in isolated cardiomyocytes. Dox caused a significant upregulation ($P<0.01$) of iNOS protein expression as compared to Control and Vit C (Figure 13A). This Dox-mediated upregulation was prevented by treatment with Vit C in Vit C + Dox group ($P<0.001$). The protein expression of iNOS in Vit C + Dox group was not significantly different than that of Control and Vit C (Figure 13A).

eNOS is a constitutively expressed NOS isoform and its enzyme activity is regulated by differential activation or inhibition via phosphorylation at its activating site Ser1177 or inhibitory site Thr^{495} respectively. Dox caused an increased expression of phosphorylated form of eNOS at its inhibitory site Thr495. Vit C + Dox treated cardiomyocytes showed a reduced expression of phosphorylated eNOS at Thr495 as compared to Dox only (Figure 13B). In contrast, Dox treatment showed reduced expression of eNOS phosphorylated at its activating site Ser1177. Vit C significantly upregulated the phosphorylated form of eNOS at the activating site in Vit C + Dox group and Vit C only group. In fact, the protein expression of p-eNOS Ser1177 for both the Vit C treated groups was significantly higher compared to control (Figure 13C).

In addition to regulation of eNOS by phosphorylation, maintaining the dimeric form of the enzyme is essential for its functional activity. Dimeric form of eNOS is vulnerable to dissociation into monomers by oxidation of the protein and its cofactor BH4. Therefore, the effect of Dox and Vit C was further investigated on monomeric and dimeric forms of eNOS. Using low temperature (LT) electrophoresis, we determined the monomeric/dimeric and total

protein expression of eNOS. A significant increase in the monomeric form of eNOS was observed in Dox treated cardiomyocytes compared to Control and Vit C. As a result, a significant increase ($P<0.001$) in the ratio of monomeric/dimeric form of eNOS in Dox treated cardiomyocytes compared to Control and Vit C treatments (Figure 14A). Vit C protected against the Dox mediated dissociation of eNOS subunit as demonstrated by lower protein expression of monomeric form of eNOS in Vit C + Dox treated cardiomyocytes. Vit C + Dox treated cardiomyocytes showed a significant reduction in the ratio compared to Dox. There was no significant alteration in the protein expression of total eNOS between any treatment groups (Figure 14B).

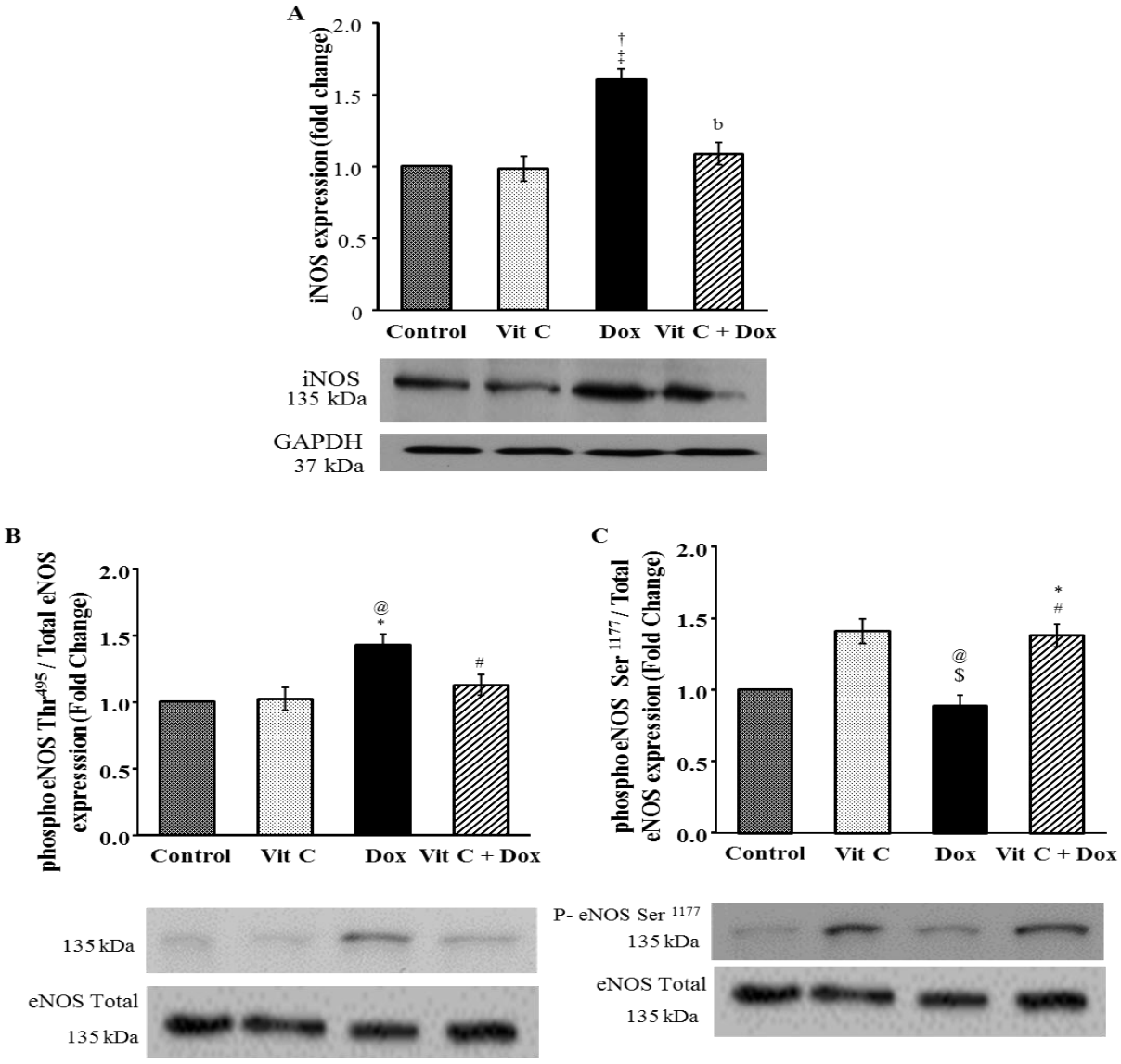

Figure 13: Protein expression of Nitric oxide synthase (NOS) isoforms in cardiomyocytes

Densitometric analysis of: **A)** inducible Nitric oxide Synthase (iNOS); **B)** phospho endothelial NOS (eNOS) Thr^{495} and; **C)** phospho eNOS Ser^{1177}. Data are Mean ± SEM from three separate experiments done in duplicate and represented as fold change. Lower panel in each figure is representative western blot images of specified protein. $^{*}P<0.001$ compared to control; $^{\dagger}P<0.01$ compared to control; $^{\$}P<0.05$ compared to control; $^{@}P<0.001$ compared to Vit C; $^{\ddagger}P<0.01$ compared to Vit C; $^{\#}P<0.001$ compared to Dox; and $^{b}P<0.01$ compared to Dox.

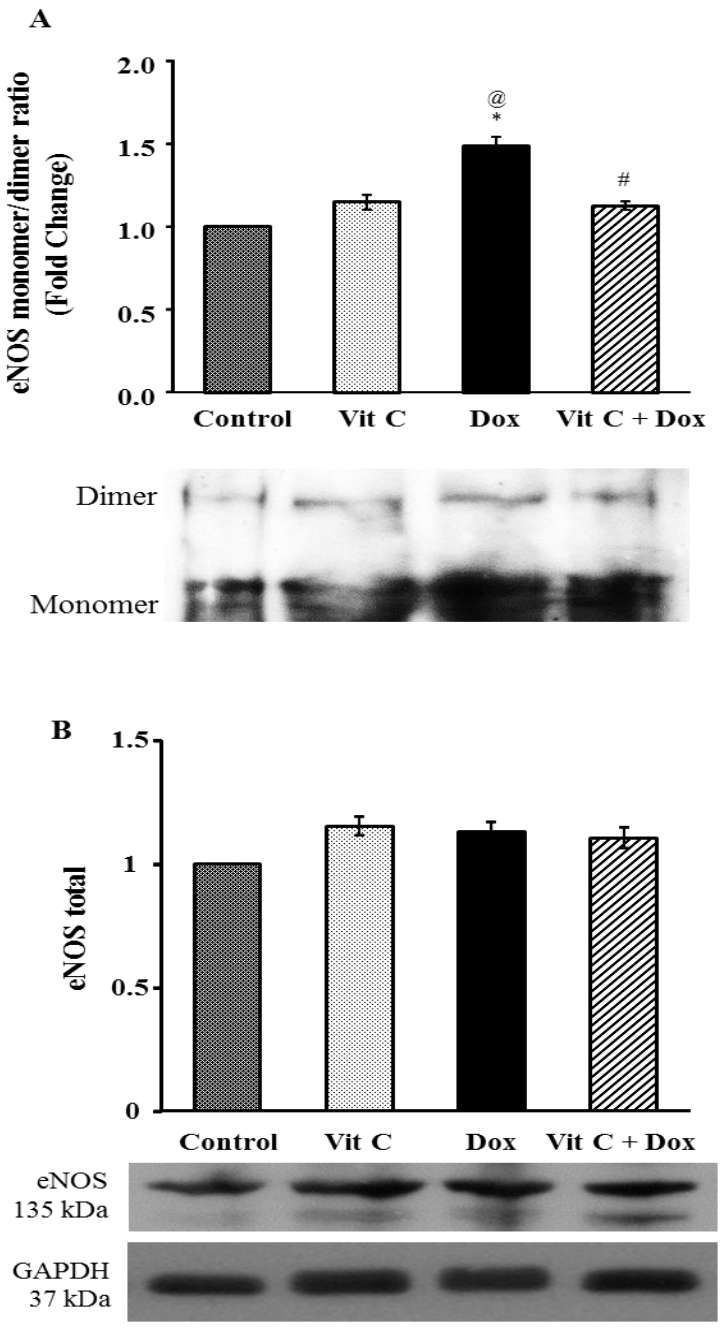

Figure 14: Endothelial Nitric Oxide Synthase (eNOS) monomer/dimer ratio. Protein expression of monomeric and dimeric forms of eNOS were determined using low temperature electrophoresis. Densitometric analysis of **A)** eNOS monomer/dimer ratio; **B)** total eNOS. Data are Mean ± SEM from three separate experiments done in duplicate and represented as fold change. Lower panel in each figure is representative blot image for specified protein. $^{*}P<0.001$ compared to control; $^{@}P<0.001$ compared to Vit C and $^{\#}P<0.001$ compared to Dox.

1.5 Vitamin C reduces Dox- induced alterations in levels of cytokines.

Increased levels of cytokines and inflammation are associated with the pathogenesis of various diseases. Cytokines are known to trigger the expression of iNOS. Therefore, we evaluated the levels of pro-inflammatory cytokine TNF-α and anti-inflammatory cytokine IL-10 in the media from different treatment groups. Compared to control, Dox significantly reduced the levels of IL-10, whereas an increase in the levels of TNF-α was observed (Figure 15A and B). Treatment with Vit C upregulated the levels of IL-10 in Vit C and Vit C + Dox group compared to Control. Vit C significantly ($P<0.001$) prevented Dox-mediated reduction in IL-10 in Vit C + Dox treated group compared to Dox alone (Figure 15A). There was no difference in the TNF-α levels between the Control and Vit C treatments. Vit C treatment of Dox treated cardiomyocytes significantly lowered the levels of TNF-α compared to Dox. In fact, the levels were even lower than Control and Vit C (Figure 15B). This can be due to synergistic action of Vit C in reducing pro-inflammatory cytokine as well as increasing the levels of anti-inflammatory cytokine as indicated by the ratio of TNF-α/IL-10. Vit C + Dox treated cardiomyocytes showed a significant reduction in the levels of TNF-α/IL-10 compared to Dox (Figure 15C).

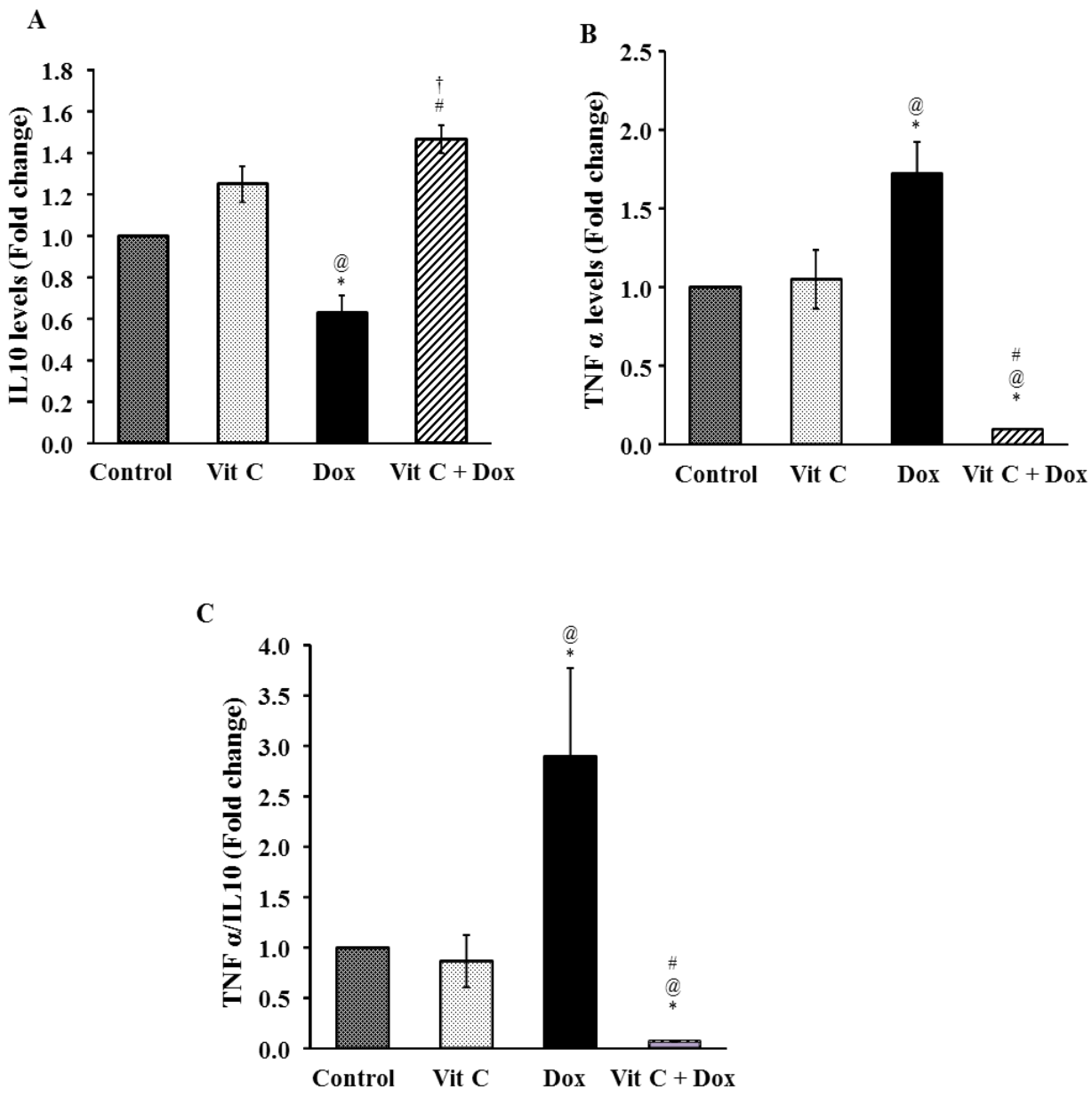

Figure 15: Levels of cytokines released from cardiomyocytes.
Changes in the levels of **A)** Interleukin-10 (IL-10); **B)** Tumor necrosis factor-α (TNF-α) released from cardiomyocytes were evaluated in the media; and **C)** Ratio of TNF-α/IL-10 released from cardiomyocytes. Data are expressed as Mean ± SEM and represented as fold change from three separate experiments done in duplicate. *P<0.001 compared to control; †P<0.01 compared to control; $^{@}$P<0.001 compared to Vit C and $^{\#}$P< 0.001 compared to Dox.

2. *In vivo* study

2.1 Survival and General Characteristics

In order to evaluate the translational value of the *in vitro* studies, the influence of Vit C in the modulation of Dox-induced cardiomyopathic changes was investigated in the animal studies. Male Wistar rats (250 ±10 g) were randomly divided into four different groups: Control, Vit C, Dox and Vit C + Dox. Dox was given intraperitoneally as six equal doses of 2.5 mg/kg body weight (BW) over three weeks with a total cumulative dose of 15 mg/kg BW. Vit C was given by oral gavage, daily for six weeks of treatment period. Dox treated animals showed reduced survival in a dose dependent manner (Figure 16A). At the end of six weeks of treatment, Dox treated animals had a 63% survival rate. Prophylactic treatment of Vit C in the combination group increased the survival rate of Dox treated animals to 93% (Figure 16B). Control as well as Vit C treated animals had 100% survival.

A gradual increase in BW was observed in Control and Vit C treated animals over the 6 weeks of treatment. Dox treated animals showed reduced BW in a time and dose dependent manner (Figure 17A). In addition, Dox treated animals also showed extensive loss of visceral fat and adipose tissue. No statistical difference was observed in the BW between Dox and Vit C + Dox treated animals (Figure 17A and B). This may be due to increased abdominal fluid accumulation in Dox treated animals. Dox treated animals also showed extensive fusion of the liver lobules. These Dox-mediated changes were reduced in the animals from Vit C + Dox group. Dox treated animals showed significant ($P<0.001$) reduction in the heart size and weight compared to Control (Figure 18A). In contrast, the lung weight was significantly ($P<0.001$) increased in Dox treated animals (Figure 18B). Vit C prevented these Dox mediated changes in heart and lung weight. The ratios of heart weight (HW) / tibia length as well as lung wet weight

(LWW)/ tibia length were calculated to determine the effect of Dox and Vit C on heart and lung weight respectively (Figure 18A and B).

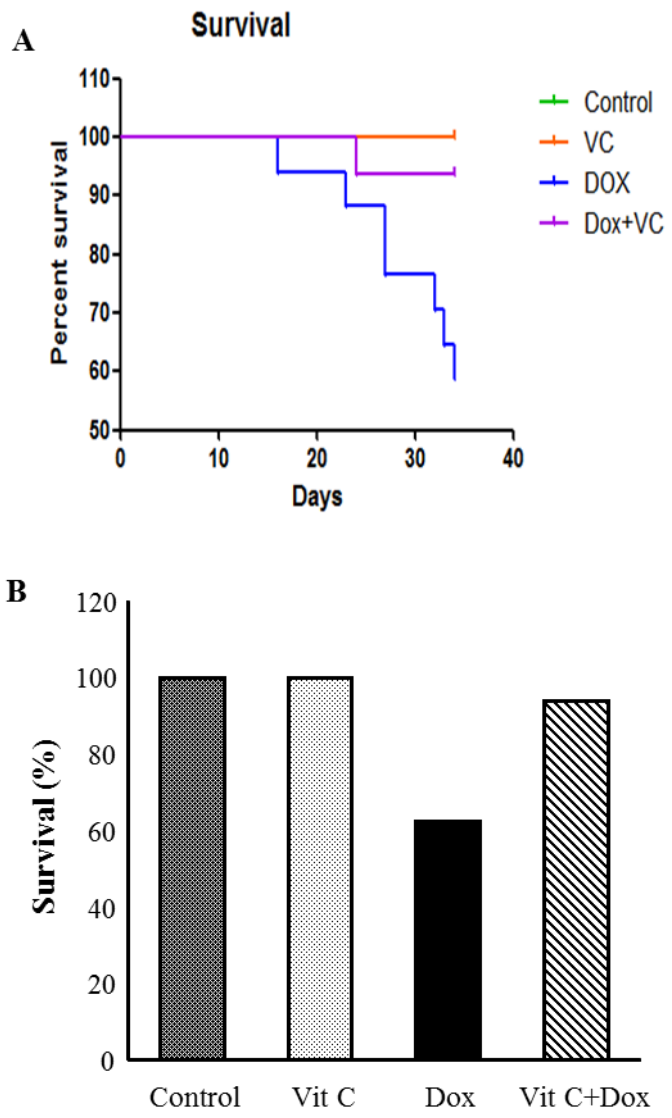

Figure 16: Percent survival

A) Percent survival of animals in different treatment groups: Control; Vit C (50 mg/kg bw); Dox (15 mg/kg); and Vit C (50 mg/kg bw) + Dox (15 mg/kg) during the entire course of treatment period. **B)** Percent survival of animals in different treatment groups at the end of 6 weeks of treatment. Number of animals survived in different treatment groups Control (6/6); Vit C (7/7); Dox (10/17); Vit C + Dox (15/16) at the end of treatment.

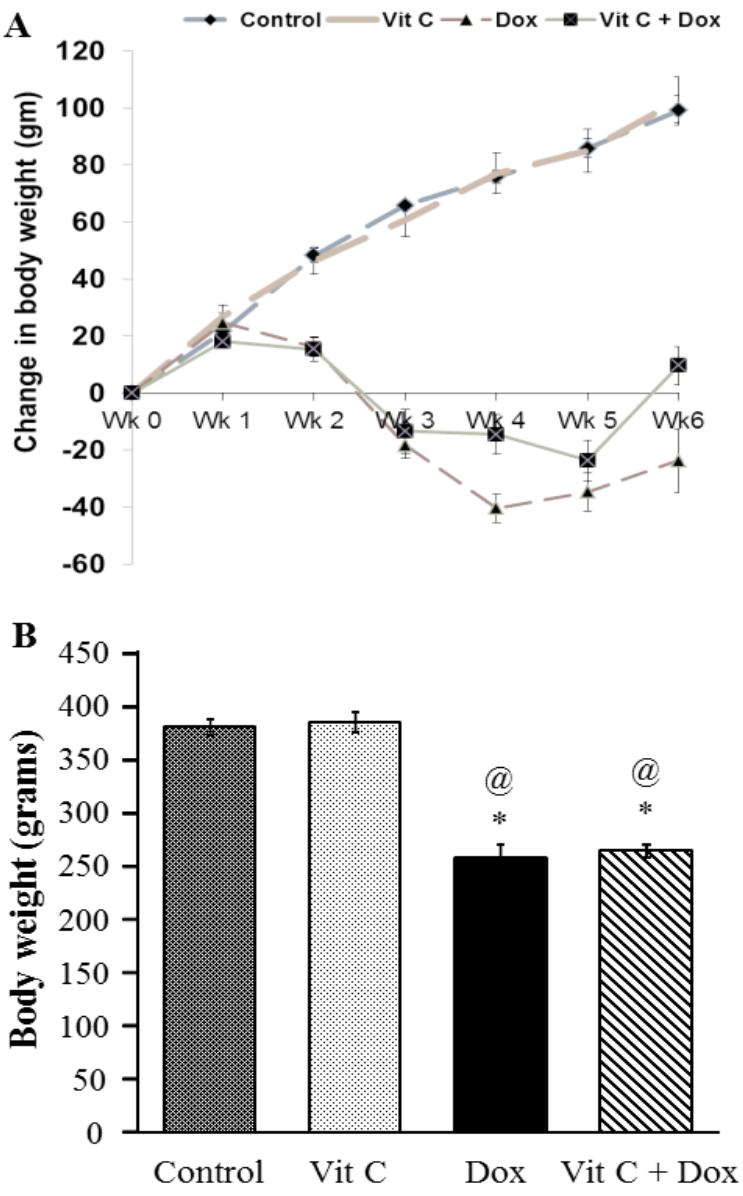

Figure 17: Body weight.
A) Time course for change in body weight over 6 weeks of treatment period. **B)** Body weight at the end of 6 weeks of treatment. Data are represented as Mean ± SEM. $^{*}P<0.001$ compared to control and $^{@}P<0.001$ compared to Vit C. Control (6); Vit C (7); Dox (9); Vit C + Dox (12)

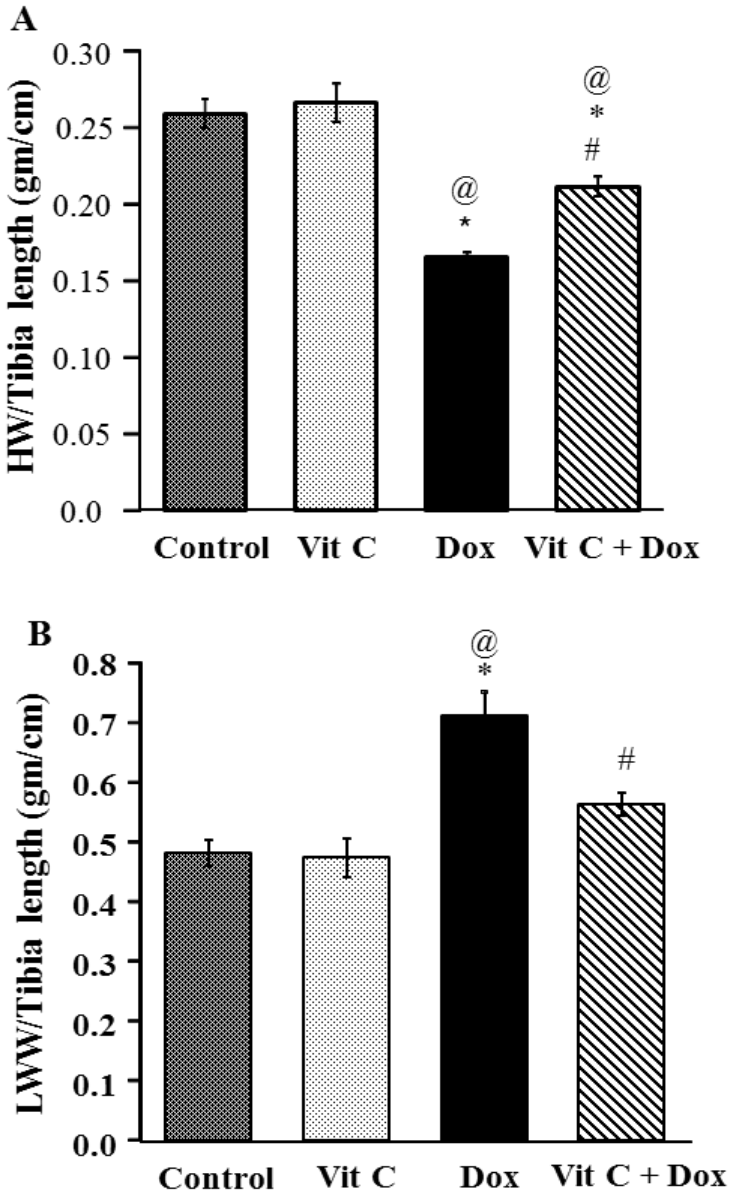

Figure 18: Changes in heart and lung weight

A) Heart weight (HW) / tibia length ratio; and **B)** Lung wet weight (LWW) / Tibia length ratio in animals from different treatment groups. Data are represented as Mean ± SEM. Number of animals: control (6); Vit C (7); Dox (9) and Vit C + Dox (13). $^{*}P< 0.001$ compared to control; $^{@}P<0.001$ compared to Vit C; $^{\#}P<0.001$ compared to Dox and $^{b}P<0.01$ compared to Dox.

2.2 Dox-induced cardiomyopathic changes and their mitigation by Vit C

For the characterization of cardiomyopathic changes, different parameters including heart rate (HR), cardiac function and cardiac histology were studied. Dox treated animals had increased HR (Figure 19). This increase was significantly higher by about 50 beats/mins as compared to control ($P< 0.001$) and Vit C ($P<0.01$) treated animals. This alteration observed in Dox treated group was prevented by Vit C treatment in Vit C + Dox treated animals ($P<0.01$). The HR observed in Vit C + Dox group was not significantly different than that of Control and Vit C treated animals (Figure 19).

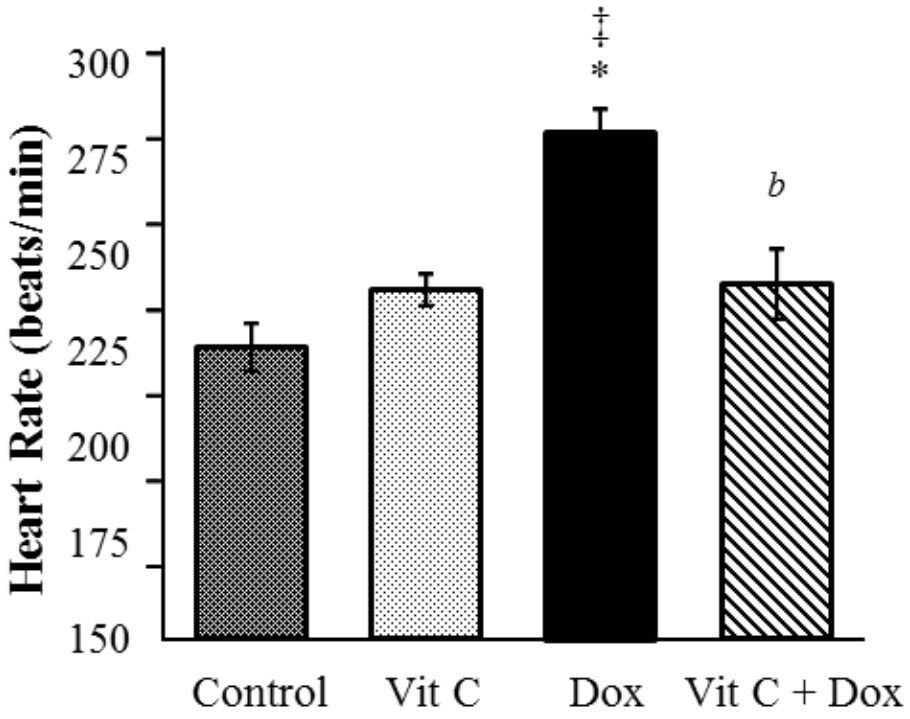

Figure 19: Heart rate
Data are expressed as Mean ± SEM from 6-11 animals. $^{*}P<0.001$ compared to control; $^{‡}P<0.01$ compared to Vit C and $^{b}P<0.01$ compared to Dox

Cardiac function was determined by echocardiographic analysis of left ventricular systolic as well as diastolic functions. Representative echocardiographic images from the Control, Dox and Vit C + Dox groups are shown in Figure 20A. Ventricular ejection fraction (EF) and fractional shortening (FS) were used as markers to determine systolic function whereas, diastolic function was determined by evaluating isovolumic relaxation time (IVRT) and the ratio of early/ active phase (E/A) of ventricular filling. Compared to Control animals, a significant ($P<0.001$) decline in EF and FS of approximately 20 % and 10% respectively was calculated in Dox treated animals. In fact, 7 of 9 Dox treated animals presented >15% decline of EF than control, but the function was still greater than 50%. In comparison to Dox alone, Vit C treatment in Vit C + Dox group significantly improved systolic function by increase in EF (%) ($P<0.001$) and FS (%) ($P<0.001$) (Figure 20B and C). All Vit C + Dox animals presented preserved EF (ie. <15% decline in EF compared to Control and the function remained >55%. On the other hand, Dox treated animals presented a diastolic dysfunction as indicated by an increase in the IVRT as well as E/A ratio in comparison of control animals. IVRT increased in Dox treated animals was significantly ($P<0.001$) reduced by Vit C compared to Dox alone (Figure 20D). Ventricular filling occurs in biphasic manner, where E indicates an early or passive filling whereas, A represents active filling phase of the ventricle. The normal range for E/A ratio is in between 1.5 - 2. Control and Vit C treated animals showed the E/A ratio to be in the normal range, whereas, Dox treated animals showed significant ($P<0.001$) increase in the ratio over 2.5 indicating restrictive filling of the ventricles. Vit C attenuated this Dox-mediated increase of E/A ($P<0.001$). This change was largely due to a bigger reduction in the A wave in Dox treated animals (Table 1). There was no significant difference between the E/A ratio from Vit C + Dox treated animals compared to Control and Vit C treated animals (Figure 20E amd Table 1).

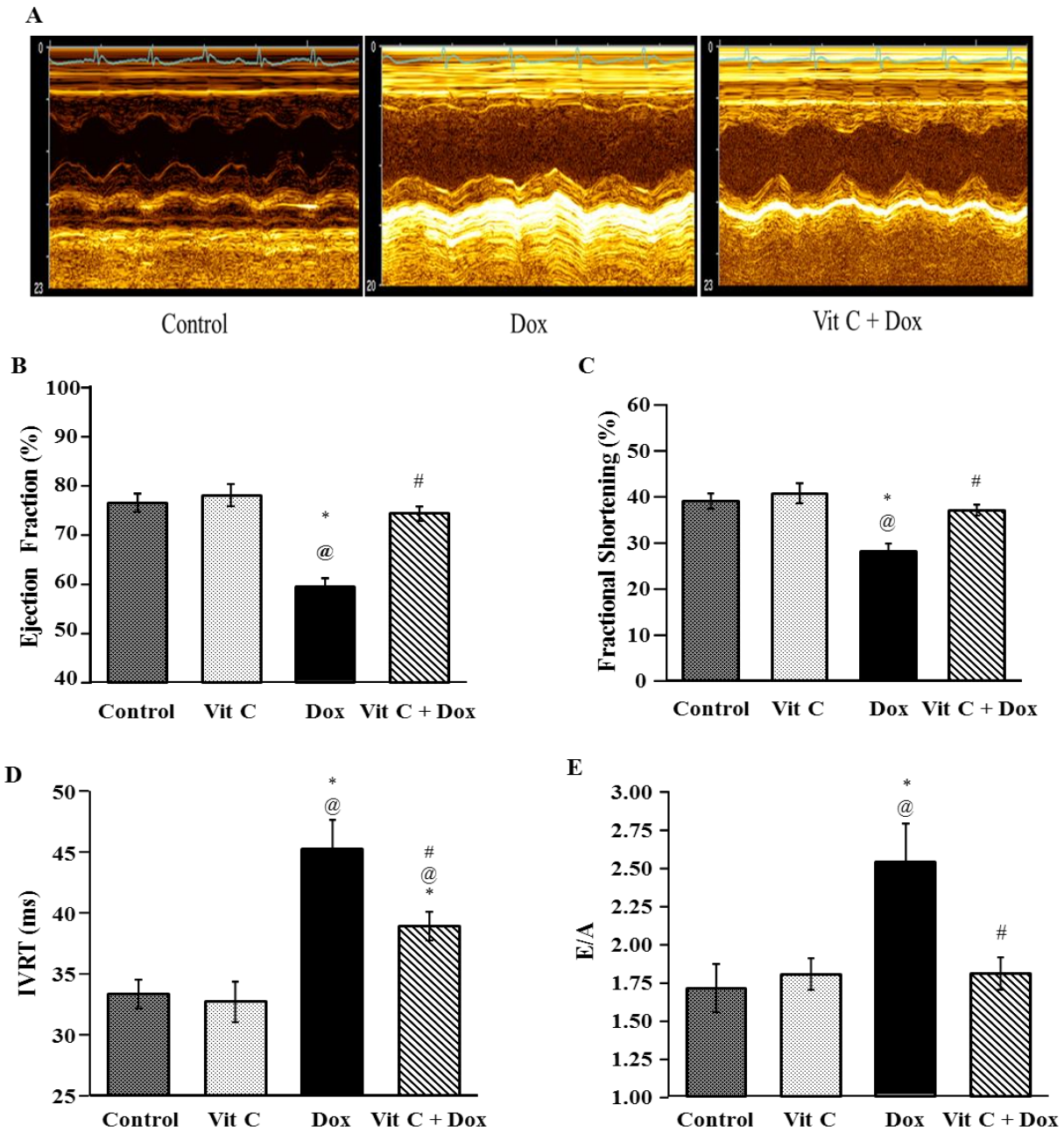

Figure 20: Cardiac function

A) Representative echocardiography images from the Control, Dox and Vit C + Dox groups. Systolic function was determined by evaluating **B)** Ejection fraction (EF); and **C)** Fractional shortening (FS). Diastolic cardiac function was determined by evaluating **D)** Isovolumic relaxation time (IVRT); and **E)** Ratio of ventricular filling in early and active phase (E/A). Data are expressed as Mean ± SEM from 6-11 animals. *P<0.001 compared to control; $^{@}$P<0.001 compared to Vit C and $^{\#}$P<0.001 compared to Dox.

Table 1: Effects of Vit C on Dox-induced changes in E and A waves as well as their ratio (E/A).

	E wave	A wave	E/A
Control (6)	0.56±0.036	0.345±0.043	1.70±0.156
Vit C (7)	0.602±0.027	0.34±0.017	1.78± 0.934
Dox (9)	0.402±0.016*	0.168±0.014*	2.53±0.249*
Vit C + Dox (13)	0.55±0.027#	0.312±0.019#	1.8±0.101#

Number of animals in brackets. *P<0.01 compared to control; # P<0.01 compared to Dox

Cardiomyocyte vacuolization is a salient feature of Dox-induced cardiotoxicity as is reported previously by us and others (Li et al, 2006; Singal et al, 2000; Wang et al, 2016). In this study, using hematoxylin-eosin staining, we confirmed the increased vacuole formation in the cardiomyocytes of Dox treated animals (Figure 21). In addition, disarray and loss of myofibrils were also observed in the histological profiles of hearts from Dox treated animals. Condensation and fragmentation of nuclei indicated apoptosis. In comparison with Dox treated animals, Vit C + Dox treated animals showed attenuation of vacuole formation and maintanence of myofibrils. Other cellular details in the cardiomyocytes were better preserved. Control and Vit C treated cardiac tissues showed linearly organized, binucleated and striated cardiomyocytes (Figure 21).

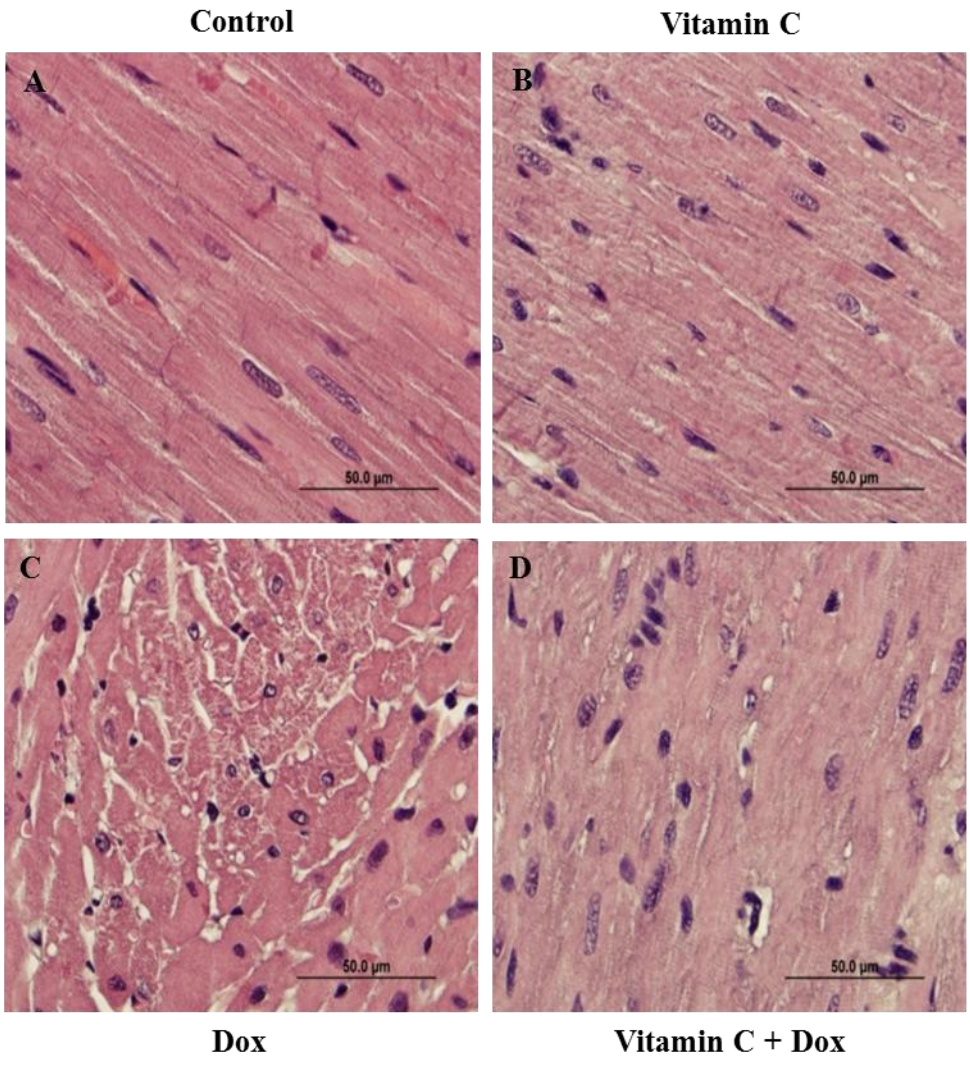

Figure 21: Histological analysis of cardiac structure
Representative histological image of cardiac structure using hematoxylin-eosin staining in animals from different treatment groups: Control; Vitamin C (Vit C); Doxorubicin (Dox) and Vitamin C + Dox (Vit C + Dox). Images were taken at X63 magnification.

2.3 Dox mediated cardiac oxidative stress and apoptosis is mitigated by Vitamin C

In vitro results indicated beneficial effect of Vit C in reducing Dox mediated oxidative stress. Increased oxidative stress is suggested to be one of the key mechanisms associated with Dox-induced cardiotoxicity. Oxidative stress is generated by an imbalance between the generation of ROS and cellular antioxidant defense mechanisms. Therefore, we evaluated the effect of Dox on the generation of ROS and on the cellular antioxidant defenses as well as their modification by Vit C treatment.

For the antioxidant defenses, endogenous antioxidant enzyme activity and protein expression of superoxide dismutase (SOD), Glutathione Peroxidase (GPx) and Catalase were determined in the cardiac tissue. Dox caused a significant ($P<0.001$) reduction in the SOD and GPx activities compared to Control and Vit C treated group, whereas these Dox mediated alterations were not observed for Vit C treated animals in Vit C + Dox group ($P<0.001$) (Figure 22A and B). There was no significant difference in the Catalase activity between any of the treatment groups (Figure 22C). The changes in enzyme activities were also reflected in protein expression of these antioxidant enzymes. Using western blot analysis, protein expression of SOD, GPx and Catalase was determined in cardiac tissue homogenates. In comparision to control and Vit C, a significant reduction in the protein expression of SOD ($P< 0.01$), GPx ($P<0.01$) and Catalase ($P<0.01$) was observed in Dox treated animals. Vit C treatment prevented Dox mediated decreases in the protein levels of SOD and GPx in Vit C + Dox treated group compared to Dox ($P<0.05$) whereas no significant effect was observed on protein expression of catalase (Figure 23A, B and C).

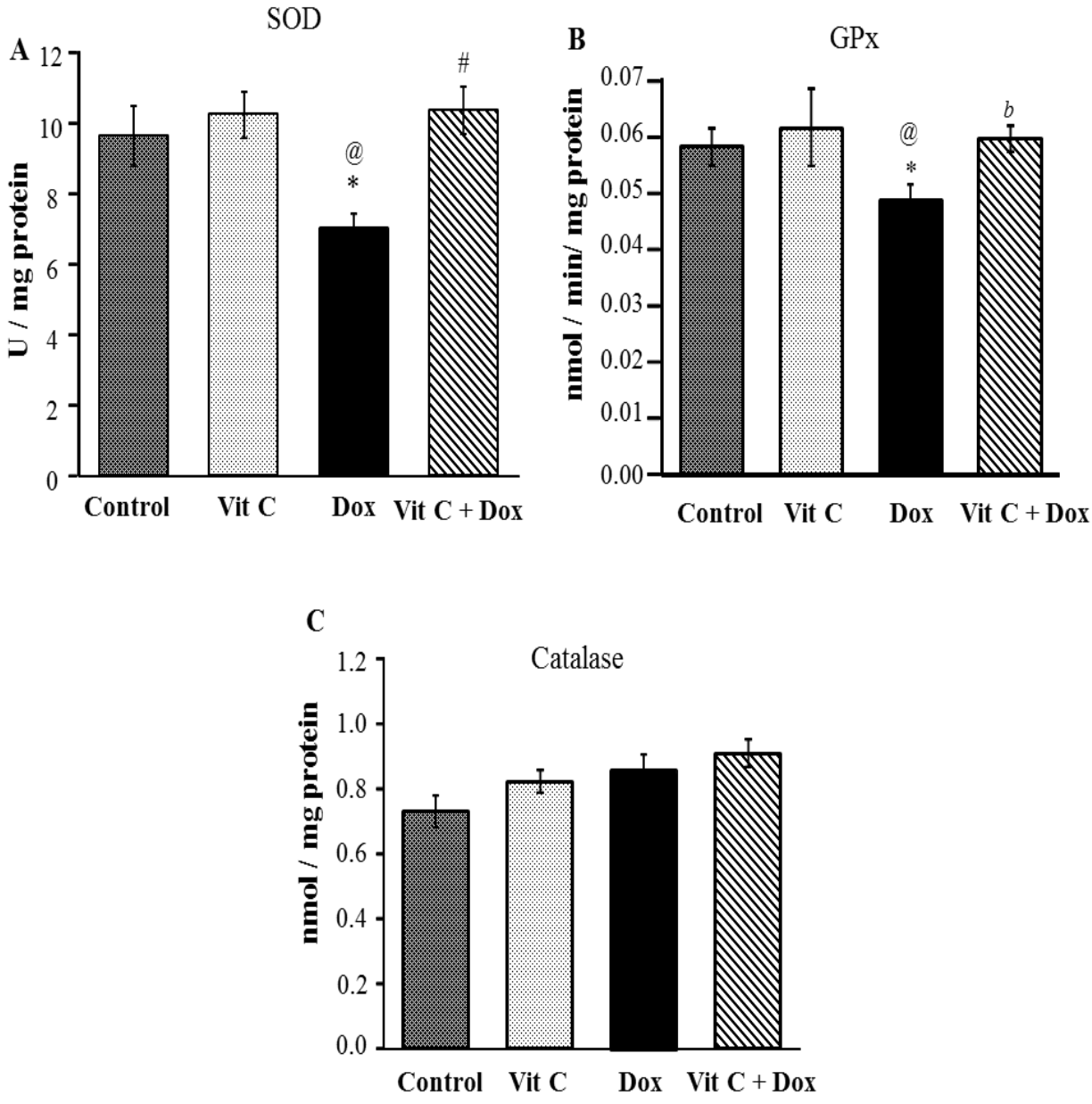

Figure 22: Cardiac antioxidant enzyme activities

Activities of cardiac **A)** Superoxide Dismutase (SOD); **B)** Glutathione Peroxidase (GPx); and **C)** Catalase. Data are represented as Mean ± SEM from 6-11 animals. $^{*}P<0.001$ compared to Control; $^{@}P<0.001$ compared to Vit C; $^{\#}P<0.001$ compared to Dox and $^{b}P<0.01$ compared to Dox.

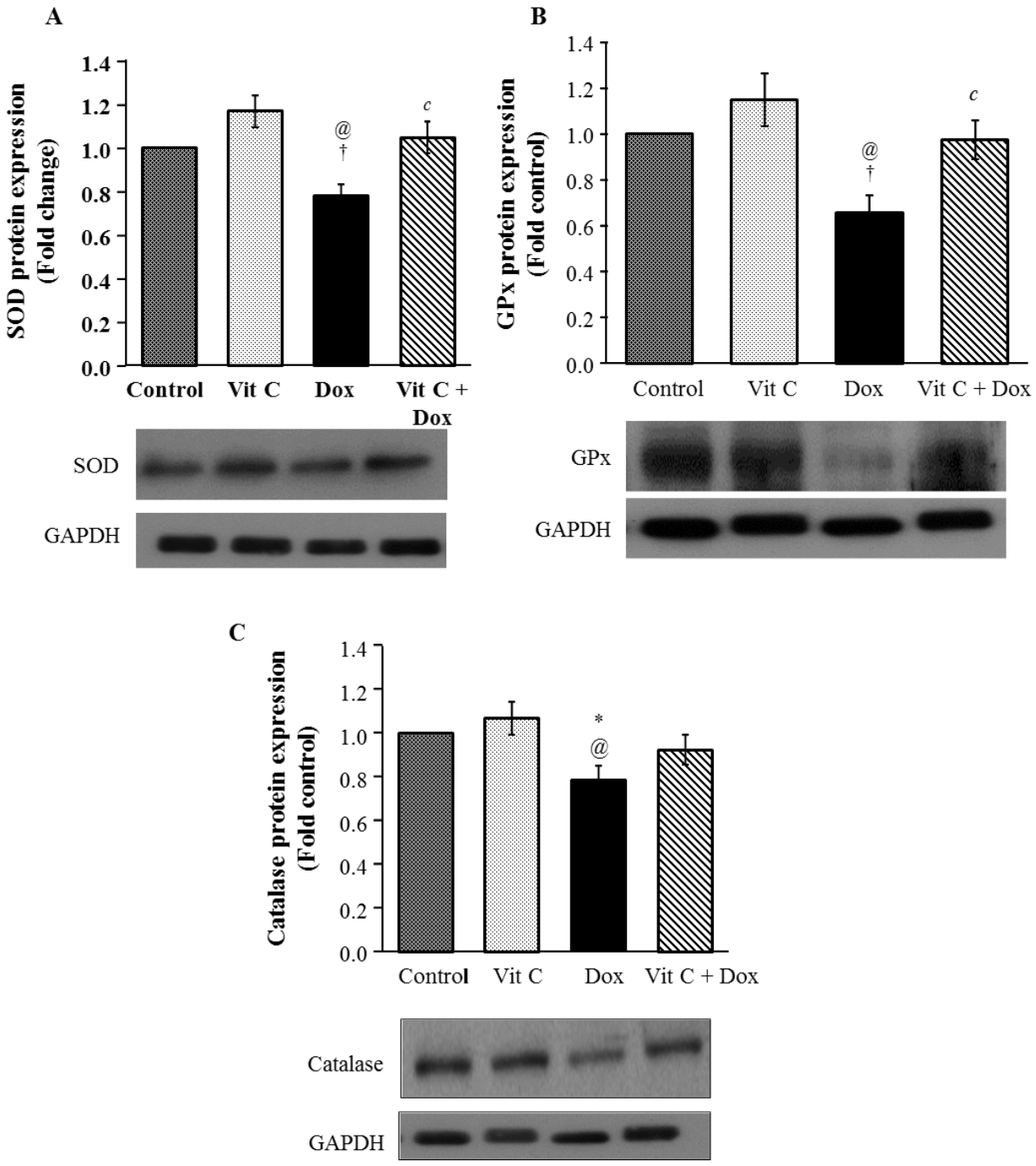

Figure 23: Cardiac antioxidant enzyme protein expression
Densitometric analysis of protein expression of cardiac antioxidant enzyme: **A)** Superoxide Dismutase (SOD); **B)** Glutathione Peroxidase (GPx); and **C)** Catalase. Lower panel in each graph shows representative blot for that specific protein. GAPDH is used as loading control. Data are represented as Mean ± SEM from 6 animals. $^{*}P<0.001$ compared to control; $^{\dagger}P<0.01$ compared to control; $^{@}P<0.001$ compared to Vit C; $^{c}P<0.05$ compared to Dox.

TBARS were analyzed for lipid peroxidation, protein carbonyl formation for protein oxidation and superoxide anion generation for ROS were evaulated as markers for oxidative stress. About a two fold increase in lipid peroxidation was observed in Dox treated animals compared to control ($P<0.001$) (Figure 24A). Vit C significantly lowered the levels of lipid peroxidation in Vit C + Dox treated animals compared to Dox alone ($P<0.001$). Dox resulted in an increase in cardiac protein carbonyls and superoxide anion by 1.5 fold compared to Control ($P<0.05$ and $P<0.001$ respectively). Vit C treatment in Vit C + Dox treated animals prevented this Dox-mediated increase in protein carbonyl and superoxide anion levels ($P<0.001$) (Figure 24B and C).

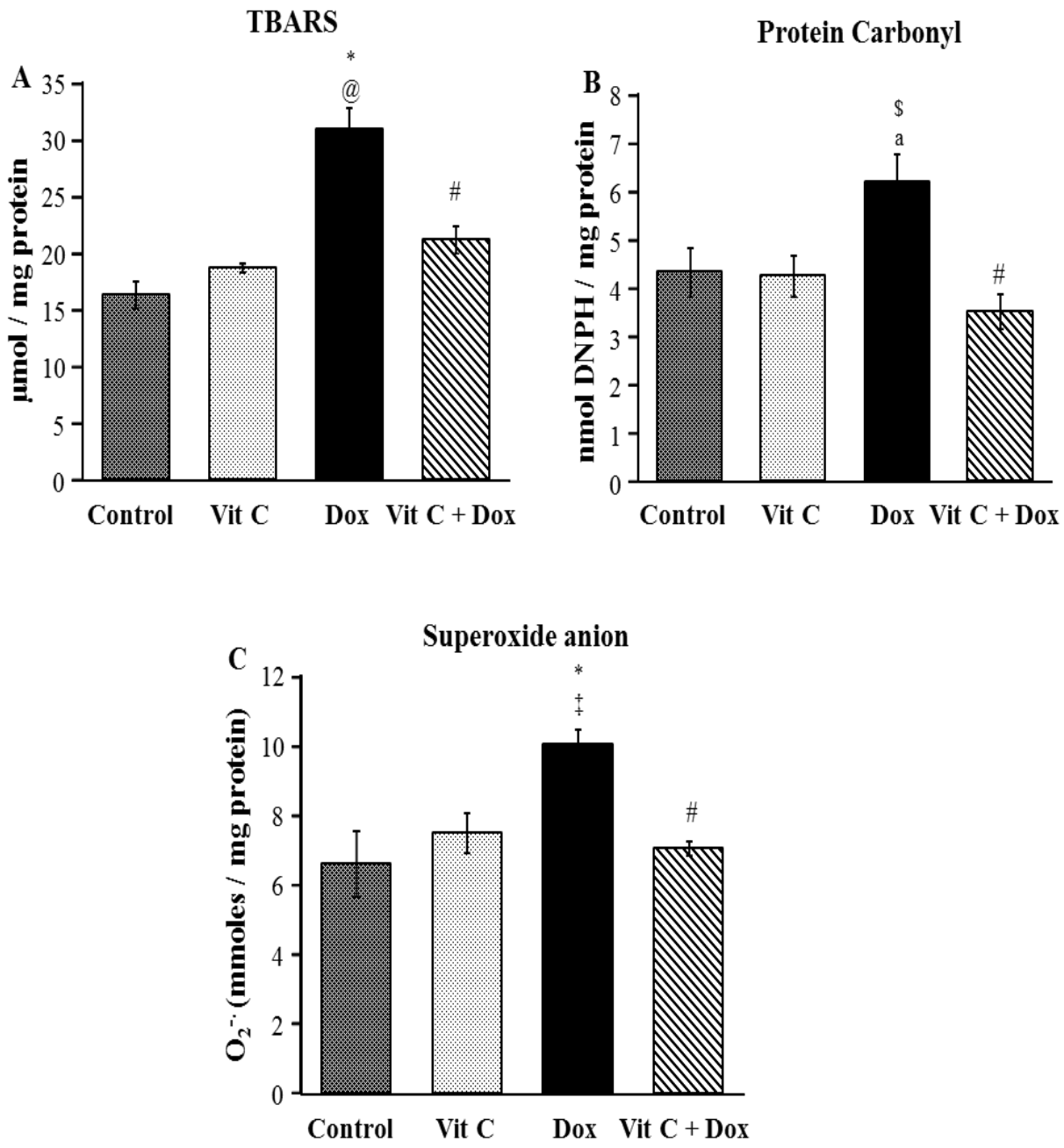

Figure 24: Cardiac oxidative stress markers

Cardiac oxidative stress was determined by evaluating: **A)** lipid peroxidation; **B)** protein carbonyl; and **C)** superoxide anion in cardiac tissue homogenate. Data are expressed as Mean ± SEM from 6-11 animals. $P<0.05$ is considered significantly different. $^{*}P<0.001$ compared to control; $^{@}P<0.001$ compared to Vit C; $^{‡}P<0.01$ compared to Vit C; $^{a}P<0.05$ compared to Vit C; and $^{\#}P<0.001$ compared to Dox.

It is well established that increased in oxidative stress is involved in activation of various cell death pathways. Previously our *in vitro* studies have determined the beneficial role of Vit C in reducing the expression of pro-apoptotic protein in Dox treated cardiomyocytes. Therefore we wanted to investigate the potential role of Vit C in preventing Dox-induced upregulation of apoptotic proteins in this *in vivo* study. Using western blot we determined protein expression of pro-apoptotic proteins Bnip-3 (Figure 25A), Bak (Figure 25B) and Bax (Figure 25C). A significant upregulation in the expression of Bnip-3, Bak and Bax proteins ($P<0.001$) was observed in Dox treated animals compared to control and Vit C. These alterations of protein expression were lowered by Vit C treatment in Dox treated animals in Vit C + Dox group compared to Dox alone ($P<0.001$) (Figure 25A and B). A ratio of pro-apoptotic protein Bax to anti-apoptotic protein Bcl-xl indicated beneficial effect of Vit C by significantly reducing the ratio in Vit C + Dox treated animals compared to Dox alone. Representative western blot image indicates the change in the ratio is mediated by reduction of pro-apoptotic protein Bax by Vit C (Figure 25C). We also quantified the protein expression of pro-apoptotic protein Caspase-3 in its total and cleaved form. Increase in ROS triggers upregulation and activation of Caspase-3. The activation causes fragmentation of total Caspase-3 into smaller fragments the latter of which is the active protein to further initiate apoptotic cascade. We observed activation of Caspase-3 resulting in fragmentation of 17 KDa protein from total Caspase – 3 (33 KDa) in Dox treated animals. This fragmented caspase was observed to be absent in Vit C + Dox treated animals (Figure 25D). Overall the protective effect offered by Vit C was two fold via reduction in generation of ROS as well as improving the antioxidant enzyme activity.

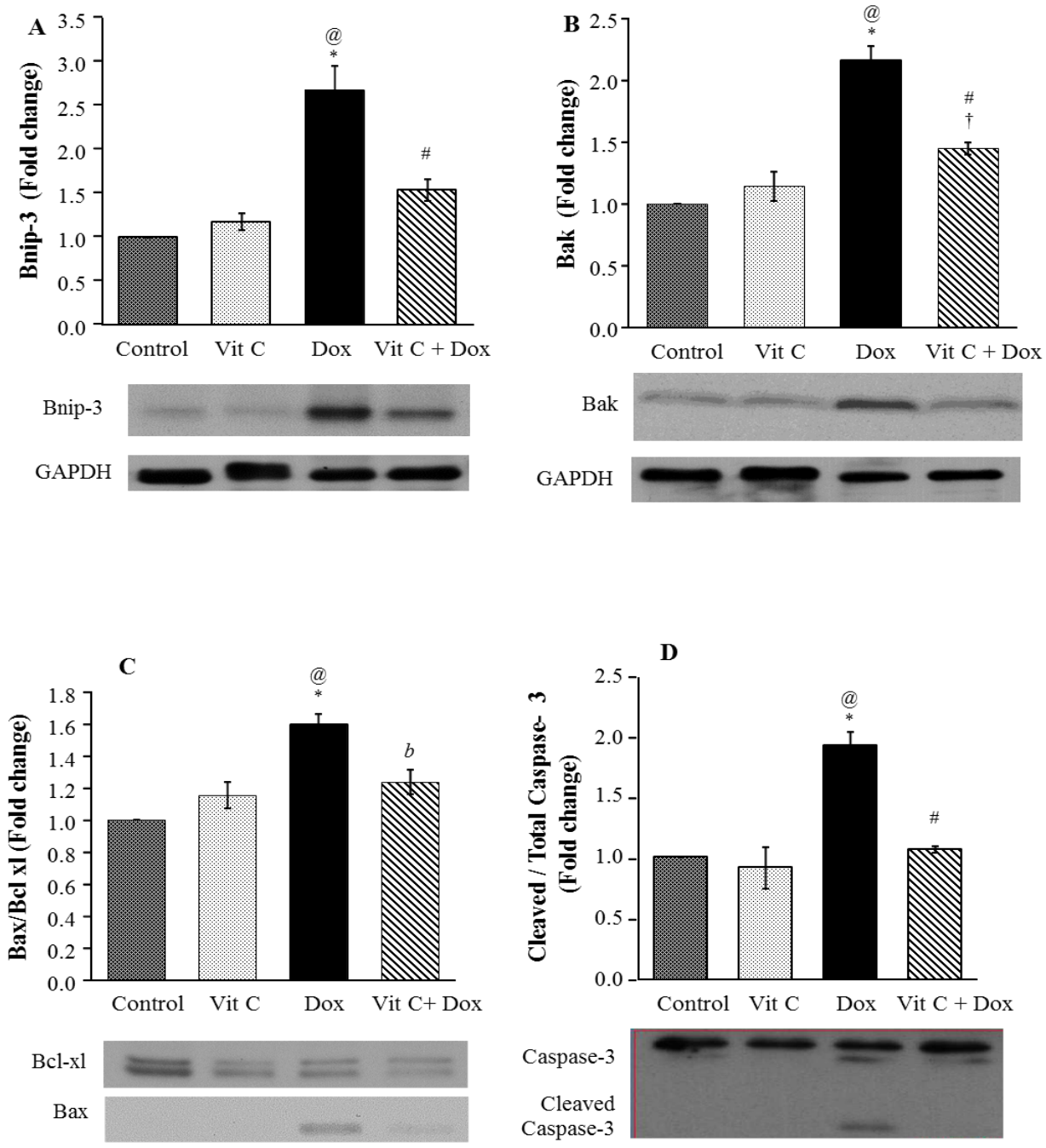

Figure 25: Expression of cardiac pro and anti-apoptotic proteins
Densitometric analysis of: **A)** Bnip-3; **B)** Bak; **C)** Ratio of pro-apoptotic protein Bax to anti-apoptotic protein Bcl-xl (Bax/Bcl-xl); and **D)** Ratio of Cleaved / Total Caspase-3. Lower panel in each figure shows representative blot of that protein. GAPDH is used as loading control. Data are represented as Mean ± SEM from 6 animals. $^{*}P<0.001$ compared to control; $^{\dagger}P<0.01$ compared to control; $^{@}P<0.001$ compared to Vit C; $^{\#}P<0.001$ compared to Dox; and $^{b}P<0.01$ compared to Dox.

2.4 Vit C reduces Dox-induced cardiac nitrosative stress

In addition to oxidative stress, Dox has also shown to be involved in nitrosative stress in many *in vitro* as well as animal studies. Our *in vitro* study also indicates an increased nitrosative stress in Dox-treated cardiomyocytes (Figure 10, Figure 11). Furthermore, we evaluated the role of Vit C in attenuating Dox-induced nitrosative stress in an animal model of Dox-induced cardiomyopathy. Cardiac NO levels and NOS activity were determined. Similar to our *in vitro* study, we observed an increase in the levels of NO in the cardiac homogenate from Dox treated animals. There was a significant increase ($P<0.001$) of about 2 fold in the levels of cardiac NO in Dox treated animals compared to Control. Vit C pre-treatment prevented against this Dox-mediated increase and significantly reduced the level of cardiac NO compared to Dox (Figure 26A). This reduction in NO levels can be attributed to reduction in NOS activity. Total NOS activity of cardiac homogenate showed a significant increase in Dox treated animals compared to Control ($P<0.001$). Vit C + Dox treated animals showed significantly ($P<0.01$) reduced NOS activity as compared to Dox (Figure 26B). Not only the enzyme activity was increased but, Dox caused a significant upregulation of iNOS protein expression also. Using western blot, iNOS protein expression was determined in cardiac tissue from all the treatment groups. Dox caused an activation and upregulation of protein expression of iNOS compared to control ($P<0.001$). Vit C + Dox treated animals demonstrated attenuation in iNOS protein expression compared to Dox (Figure 26C). Elevation in NO levels also resulted in increased formation of NO adducts to protein leading to formation of S-nitrosylated proteins in Dox treated animals (Figure 27). Densitometric analysis of western blot demonstrated about 3 fold increase in the nitrosylated protein in Dox treated animals which was significantly different ($P<0.001$) compared to Control and Vit C. A significant reduction in nitrosylated proteins was observed in Vit C + Dox treated animals compared to Dox alone (Figure 27A). Representative western blot images indicate

extensive S-nitrosylation of a number of proteins in Dox treated animals which is observed to be significantly reduced in Vit C + Dox treated animals (Figure 27B). Further analysis needs to be carried out to evaluate the effect of Dox on nitrosylation of specific proteins.

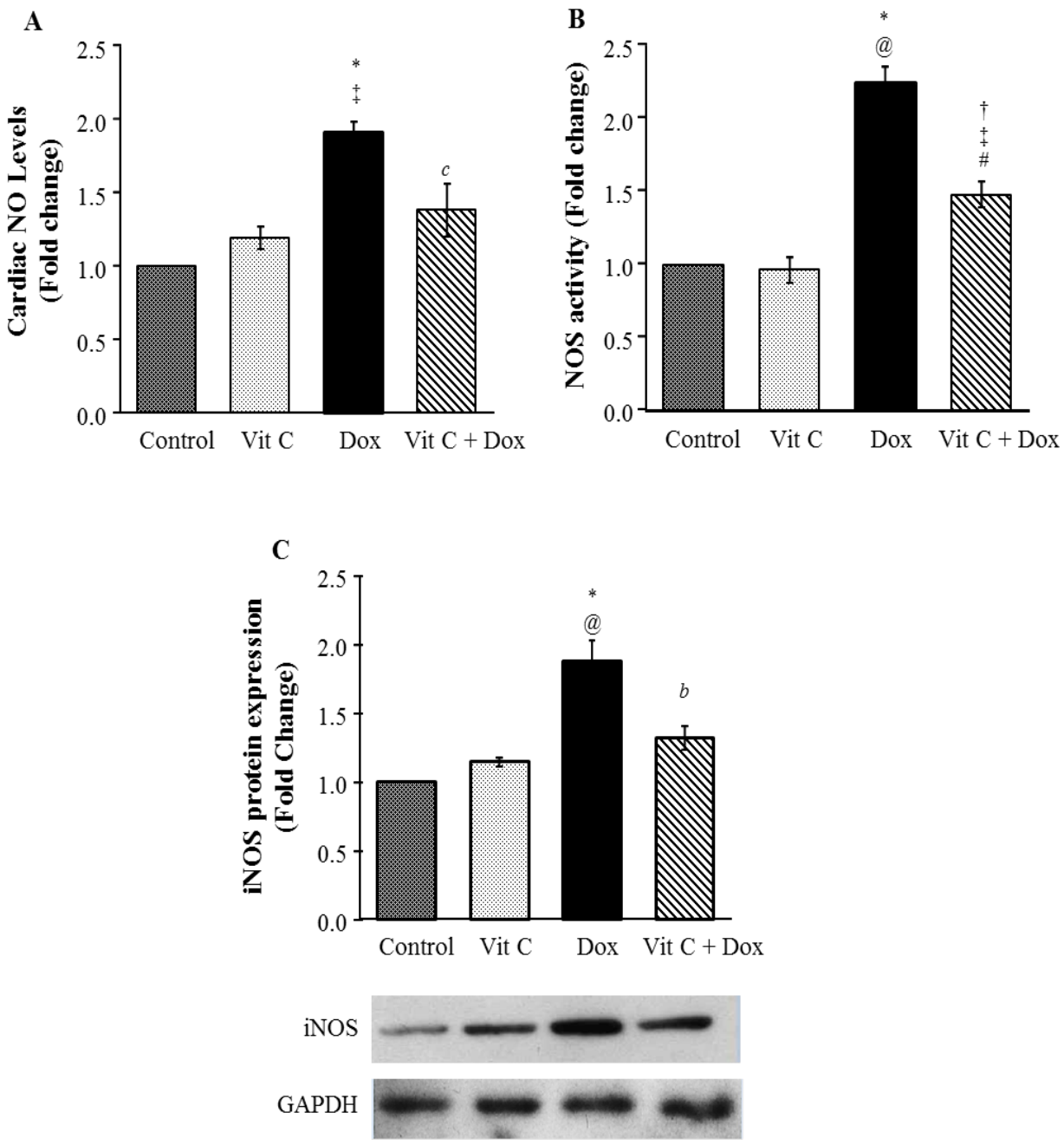

Figure 26: Cardiac Nitric oxide (NO)

Cardiac levels of **A)** Nitric Oxide (NO); **B)** Nitric Oxide Synthase Activity (NOS); **C)** Densitometric analysis of inducible Nitric oxide synthase (iNOS) protein expression. Lower panel indicates representative blot of iNOS. GAPDH was used as loading control. Data is represented as Mean ± SEM from 6 animals. $^{*}P<0.001$ compared to control; $^{\dagger}P<0.01$ compared to control; $^{@}P<0.001$ compared to Vit C; $^{\ddagger}P<0.01$ compared to Vit C; $^{\#}P<0.001$ compared to Dox; and $^{b}P<0.01$ compared to Dox.

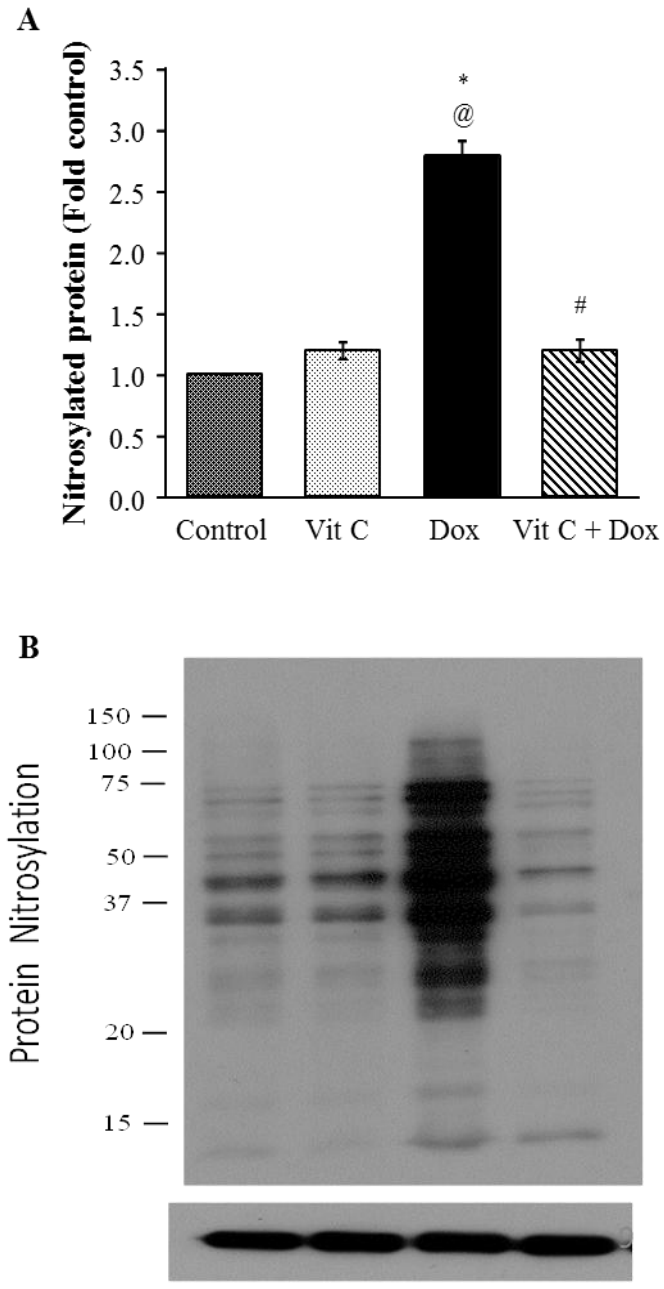

Figure 27: Cardiac protein nitrosylation

A) Densitometric analysis of S-nitrosylated proteins; and **B)** Representative western blot image of nitrosylated proteins. GAPDH is used as loading control. Data is represented as Mean ± SEM from 6 animals. $^{*}P<0.001$ compared to control; $^{@}P<0.001$ compared to Vit C and $^{\#}P<0.001$ compared to Dox.

2.5 Vitamin C reduces levels of pro-inflammatory cytokines

Elevation of inflammatory cytokines is implicated in the pathogenesis of a number of diseases. Our *in vitro* results demonstrate an increase in the levels of released TNF-α from Dox treated cardiomyocytes (Figure 15). Further, we wanted to investigate the effect of Vit C and Dox on the levels of cytokines in the cardiac tissue from an animal model of Dox-induced cardiomyopathy. Using ELISA, cardiac levels of IL-1β, IL-6 and TNF-α were evaluated as markers of pro-inflammatory cytokines, while IL-10 was determined as marker for anti-inflammatory cytokine. Compared to control, Dox significantly decreased the levels of IL-10 (Figure 28A). Vit C significantly increased the levels of IL-10 in Vit C + Dox treated animals compared to Dox group (Figure 28A). In contrast, a significant ($P<0.001$) increase in cardiac levels of IL-1β, IL-6 and TNF-α were observed in Dox treated animals. The levels of IL-6 as well TNF-α were increased by about 8 times and about 4 fold in IL-1β in Dox treated animal than that of control. Vit C showed beneficial effect in the reduction of these cytokines in Vit C + Dox treated animals (Figure 28B, C and D).

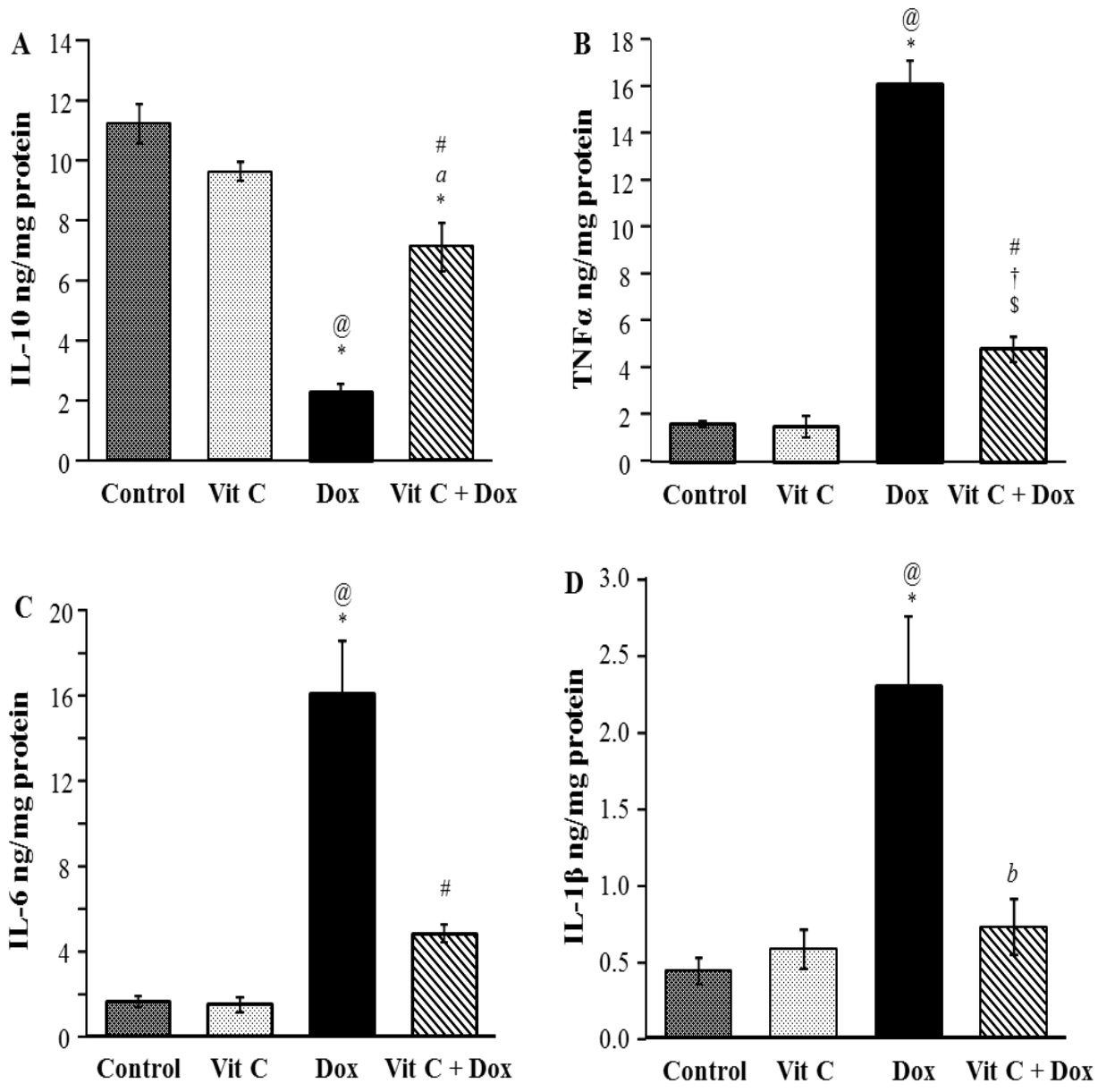

Figure 28: Levels of cardiac cytokines

Cardiac levels of: **A)** Interleukin 10 (IL-10); **B)** Tumor necrosis factor-α (TNF-α); **C)** Interleukin-6 (IL-6); and **D)** Interleukin-1β (IL-1β) were determined by ELISA. Data are represented as Mean ± SEM from 6 animals. *P<0.001 compared to control; †P<0.01 compared to control; $^{\$}$P<0.05 compared to control; $^{@}$P<0.001 compared to Vit C; ‡P<0.01 compared to Vit C; ^{a}P<0.05 compared to Vit C; $^{\#}$P<0.001 compared to Dox; and ^{b}P<0.01 compared to Dox.

2.6 Dox causes alteration in signaling proteins and Vitamin C transporters

Since cardiac tissue expresses GLUT-4 and SVCT-2 for the intracellular transport of Vit C, the effect of Dox and Vit C on the protein expression of these Vit C transporter proteins, was determined in cardiac homogenate using western blot. Our results indicate an upregulation of both the transporter proteins, SVCT-2 and GLUT-4, in Vit C and Vit C + Dox treated animals compared to control ($P<0.001$). In contrast, Dox led to down regulation of protein expression for GLUT-4 and SVCT-2 compared to Control ($P<0.05$). These Dox mediated alterations were blunted by Vit C in Vit C + Dox treated animals ($P<0.001$ for GLUT-4 and $P<0.01$ for SVCT-2) (Figure 29A and B).

Expressions of signaling proteins such as Akt, p53 and NFκB were determined in cardiac homogenate by western blotting. Akt, a serine/threonine kinase, plays a key role in cell survival and transcription. Activation of Akt was determined by a ratio of phospho/total Akt protein expression. Dox treated animals showed a downregulation in the protein expression of phosphorylated Akt. This Dox-mediated downregulation was improved by Vit C treatment in Vit C + Dox treated animals (Figure 30A). Vit C treatment resulted in a significant increase in the expression of phosphorylated Akt (Figure 30A).

Tumor suppressor protein p53 plays an important role in apoptosis and inhibition of angiogenesis. Dox treatment significantly ($P<0.001$) increased the protein expression of p53 compared to Control and Vit C (Figure 30B). Dox also increased the protein expression of NFκB, a protein complex involved in transcriptional control of cytokines and cell survival proteins (Figure 30C). Vit C treatment in combination with Dox reduced the protein expression of p53 and NFκB in Vit C + Dox group (Figure 30B and C).

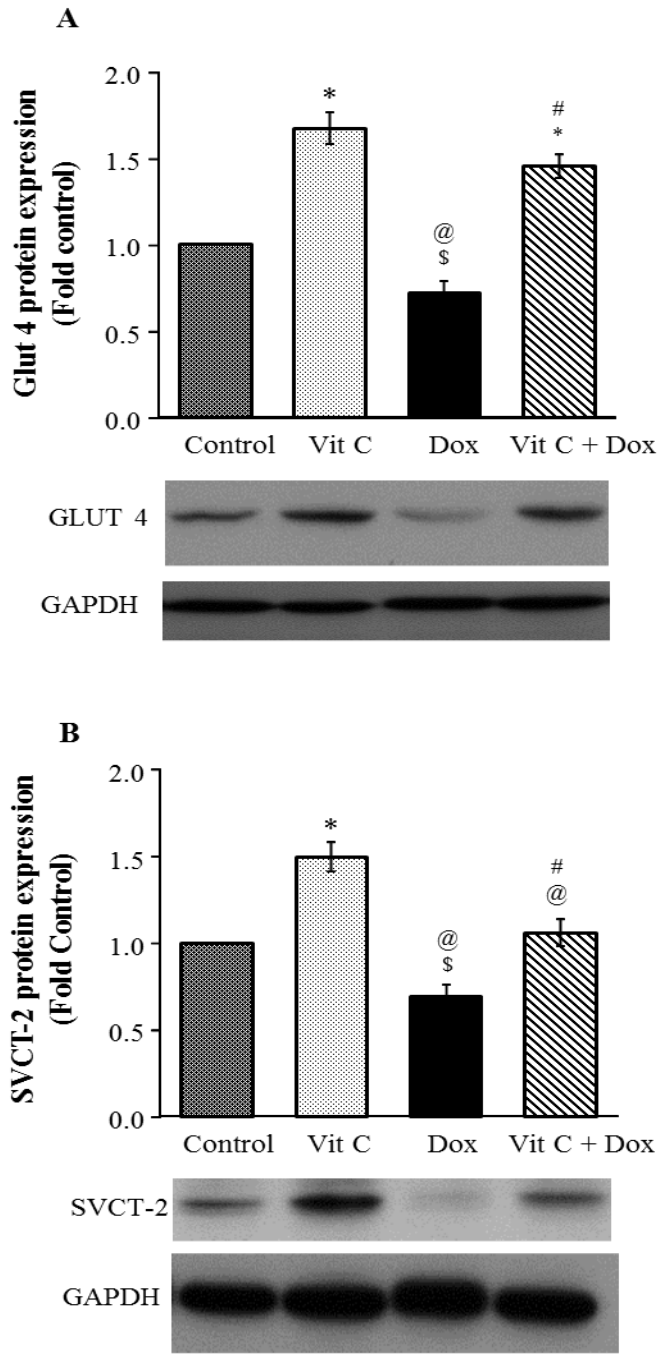

Figure 29: Expression of Vitamin C transporter proteins

Densitometric analysis of protein expression of: **A)** Glucose transporter (GLUT-4) and **B)** Sodium dependent Vitamin C transporter protein (SVCT-2) in animals treated with Vitamin C (Vit C) and Doxorubicin (Dox). Lower panel in each figure indicates representative blot of that protein. GAPDH was used as internal loading control. Data are represented as Mean ± SEM from 6 animals. $^{*}P<0.001$ compared to control; $^{\$}P<0.05$ compared to control; $^{@}P<0.001$ compared to Vit C; $^{\#}P<0.001$ compared to Dox; and $^{b}P<0.01$ compared to Dox.

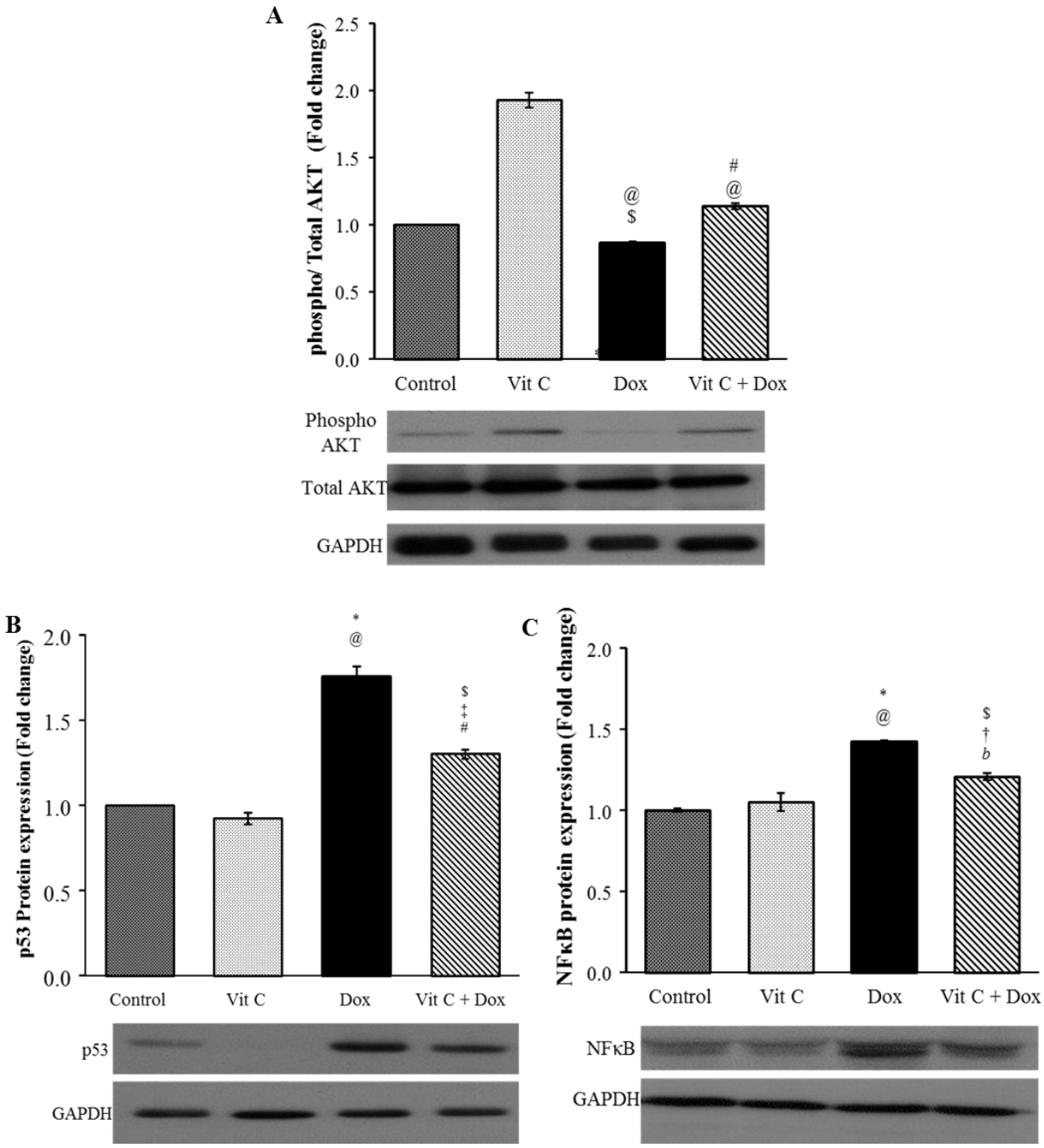

Figure 30: Expression of signaling proteins

Densitometric analysis of protein expression of **A)** AKT; **B)** p53; and **C)** NFκB. Lower panel in each figure indicates representative blot of that protein. GAPDH was used as internal loading control. Data are represented as Mean ± SEM from 6 animals. *P<0.001 compared to control; †P<0.01 compared to control; $^{\$}$P<0.05 compared to control; $^{@}$P<0.001 compared to Vit C; ‡P<0.01 compared to Vit C; $^{\#}$P<0.001 compared to Dox; and ^{b}P<0.01 compared to Dox.

VI. DISCUSSION

Despite the beneficial effects of Dox as a potent chemotherapy drug, the side effect of the development of cardiomyopathy leading to heart failure is a critical problem associated with its use in cancer patients. Thus understanding the molecular mechanism(s) involved in Dox-induced cardiotoxicity and identifying alternatives to reduce its cardiotoxicity is of clinical relevance. The present study investigated the molecular mechanisms of Dox-mediated cardiotoxicity by using adult rat cardiomyocytes followed by studies in an animal model. The data points to oxidative/nitrosative stress as the underlying cause in the progression of Dox-induced cardiomyopathy. This study reveals the details of biochemical and molecular changes leading to Dox-induced myocardial dysfunction. Increased generation of ROS is a widely accepted underlying cause for Dox-induced cardiotoxicity (Singal and Iliskovic, 1998). Although several agents are being investigated for their potential role in reducing Dox-induced cardiotoxicity, limited success has been achieved so far (Singal and Iliskovic, 1998; Quiles et al, 2002; Injac and Strukelj, 2008). Still we do not completely understand the biology of the pathogenesis of cardiac dysfunction caused by Dox. In this study, in addition to oxidative stress, we identify the occurence of Dox-induced nitrosative stress in isolated cardiomyocytes as well as in whole animals.

It is known that plasma levels of Vit C are decreased in cancer patients undergoing treatment with chemotherapy drugs (Mayland, 2005; Weijl et al, 1998). Reduced oxidative stress and improved antioxidant enzyme activities were observed in breast cancer patients under chemotherapy along with the use of Vit C (Mikirova et al, 2013; Suhail et al, 2012). A randomized trial was undertaken to examine the role of Vit C in the chemotherapy regime in newly diagnosed ovarian and breast cancer patients (Drisko et al, 2003; Suhail et al, 2012).

However, clinical trials using Vit C or other antioxidants in different types of cardiovascular diseases have provided inconclusive results. This inconsistency may be due to variations in the dose, duration of the treatment, end points evaluated, stage of the disease, and other comorbidities in the patient population involved. In this regard, the time of administration of Vit C has also been suggested to be critical (Farbstein et al, 2010). Moreover, most of the studies have investigated these effects in already sick or at risk populations, where extensive damage may have already occurred. Therefore, it is crucial to understand, if prophylactic treatment with Vit C can be cardioprotective in reducing Dox-mediated cardiac insults which was the objective of our study.

1. Dox mediated oxidative/nitrosative stress

Since cardiomyocytes heavily depend on oxidative phosphorylation for their energy supply to sustain their function; antioxidant reserve in the heart is tightly balanced with ROS production (Doroshow et al, 1980; Ludke et al, 2009). Therefore any additional insult leading to increased oxidative stress in the heart may lead to oxidative damage. Oxidative stress through increased generation of ROS and compromised antioxidant defense is well documented in both *in vitro* as well as *in vivo* models of Dox-induced cardiotoxicity (Li and Singal, 2000; Ludke et al, 2012a; Singal et al, 2000; Viswanatha-Swamy et al, 2011). This *in vitro* study, demonstrated Dox-mediated increased generation of superoxide anion in cardiomyocytes. Cardiac tissue of Dox treated animals in this *in vivo* studies also demonstrated oxidative stress as indicated by increased lipid peroxidation, protein oxidation and superoxide anion generation. Physiologically, the redox potential of the cell is maintained by antioxidant defense system via endogenous antioxidant enzymes such as SOD, catalase and GPx as well as non enzymatic antioxidants such as Vit C and E, carotenoids and phenolic compounds (Siti et al, 2015). Overexpression of SOD

attenuated oxidative stress mediated damage and apoptosis indicating a crucial role of this enzyme (Chen et al, 1998; Yen et al, 1999). Our *in vivo* study demonstrated a compromised antioxidant defense observed as a reduction in SOD and GPX enzyme activities in Dox treated animals. This was also associated with downregulation of protein expression of these antioxidant enzymes (SOD, GPx and Catalase). Downregulation of antioxidant enzymes has been observed to be initiated as early as 2 hours past Dox treatment in the animals (Li and Singal, 2000). Increased oxidative stress observed in Dox treated animals in our study indicates two prong damage: i) through increase in ROS and ii) via reduction in antioxidant enzymes.

In addition to oxidative stress, our *in vitro* studies demonstrated nitrosative stress by an elevation of cardiac NO levels and increased generation of reactive nitrogen species (RNS) such as peroxynitrite in Dox treated cardiomyocytes. Peroxynitrite is a strong biological oxidant and nitrating species are involved in the pathogenesis of a variety of cardiovascular pathologies such as myocardial infarction, chronic heart failure, diabetes, neurological diseases such as Alzheimer's and Parkinson's disease (ben Anes et al, 2014; Islam et al, 2015; Kouti et al, 2013; Pacher and Szabo, 2006). Increased plasma levels of NO and peroxynitrite in patients with cardiovascular pathologies including Dox-induced cardiotoxicity have been reported (Kouti et al, 2013; Sayed-Ahmed et al, 2001). These changes not only exacerbates the toxicity by direct oxidation of DNA, proteins and lipids (Levrand et al, 2006), but they also inhibit the activity of antioxidant enzymes (Han et al, 2001) and accelerates the production of ROS such as superoxide (Cassuto et al, 2014). As peroxynitrite is formed by near diffusion reaction of superoxide with NO (Islam et al, 2016; Levrand et al, 2006; Mukhopadhyay et al, 2009), it has been speculated that an increase in the generation of superoxide through the upregulation of NADPH oxidase, xanthine oxidoreductase would act as an initial trigger for the generation of peroxynitrite

(Ferdinandy et al, 2000). Further stimulation for the production of higher concentration of peroxynitrite is through increased generation of NO by upregulation of iNOS.

Additionally, peroxynitrite leads to nitration and nitrosylation of various proteins and thereby altering their function and downstream signal transduction. Although low level of nitrosylation has an important role in cardiovascular signaling and regulation of angiogenesis, cardiac contractility, vascular relaxation, apoptosis and inflammation (Beltran et al, 2000; Irie et al, 2015; Iwakiri et al, 2011; Lima et al, 2010), high levels of nitrosylation have inhibitory effects (Beltran et al, 2000). In this study, we observed extensive protein nitration and nitrosylation in both *in vitro* and *in vivo* models of Dox- induced cardiotoxicity. Although we have not identified individual proteins nitrated or nitrosylated, various studies have shown that increased peroxynitrite leads to nitration and nitrosylation of cardiac myofibrils, apoptosis, inflammation as well as calcium handling proteins resulting in ventricular dysfunction (Iwakiri et al, 2011; Mihm et al, 2002; Weinstein et al, 2000). Under the conditions of increased oxidative stress, high levels of NO had an inhibitory effect by increased nitrosylation of mitochondrial respiratory chain enzymes (Beltran et al, 2000). S-nitrosylation of proteins is dependent on the redox state of the cell and can be reversed in the presence of antioxidants such as glutathione, thioredoxin and ascorbic acid (Beltran et al, 2000; Ravi et al, 2004). In this study, we observed that the treatment with Vit C significantly reduced Dox mediated nitration and nitrosylation of proteins.

2. Dox- mediated modulation of NOS

Physiologically NO produced by different NOS isoforms has differential function as well as regulation. Since eNOS is compartmentalized in plasmalemma caveoli (Forstermann and Munzel, 2006; Massion et al, 2003) in proximity to the sarcoplasmic reticulum, eNOS derived NO in physiological levels (low concentration) plays an important role in cardiomyocyte

contraction (Fostermann and Sessa, 2012; Ziolo and Bers, 2003). As all forms of NOS are stable in their dimeric state, maintaining the dimeric form of eNOS is crucial for its catalytic activity (Andrew and Mayer, 1999; Forstermann and Munzel, 2006; Forstermann and Sessa, 2012; Zou et al, 2002). Nevertheless, dissociation of the dimeric form into monomeric form under the conditions of oxidative/nitrosative stress or cofactor oxidation can lead to uncoupling of enzyme resulting in the production of superoxide instead of NO (Chen et al, 2014; Forstermann and Munzel, 2006; Thum et al, 2007). Despite iNOS being a more potent source of generation of NO and superoxide than eNOS, uncoupling of eNOS is implicated in many pathological conditions. This is because of its inability to produce physiological levels of eNOS derived NO crucial for normal cardiovascular function (Forstermann and Munzel, 2006).

Uncoupling of eNOS is associated with the progression of many pathological conditions such as endothelial dysfunction, diabetes, obesity and aging (d'Uscio et al, 2003; Gamez-Mandez et al, 2015; Yang et al, 2009). Although the mechanism for uncoupling of eNOS is not clearly understood, it can be a consequence of monomerization of eNOS as a result of oxidation of the cofactor BH4, involved in binding of two monomers. However, conditions involving increased production of ROS can also trigger uncoupling of eNOS. In the present study, using LT non reducing western blot, we observed an enhanced disruption of dimeric form of eNOS into monomeric subunits in Dox treated cardiomyocytes (d'Uscio et al, 2003; Heller et al, 2001). Enhanced nitrosylation of eNOS also results in the disruption of dimer stability (Ravi et al, 2004). Monomerization and uncoupling of NOS results in synthesis of superoxide anion instead of NO (Heller et al, 2001). On the other hand, maintaining redox potential through an upregulation of thioredoxin reductase resulted in the protection of eNOS dimer and restoration of enzyme activity (Ravi et al, 2004). Furthermore, loss of eNOS in the eNOS knockout mice

provided cardioprotection against Dox-induced cardiotoxicity (Deng et al, 2009; Neilan et al, 2007).

eNOS being constitutively expressed, phosphorylation is the key mechanism for regulation of its activity. eNOS enzyme activity is regulated by differential phosphorylation of the enzyme at its activating site Ser1177 or inhibitory site Thr495. Phosphorylation at Ser 1177 enhances enzyme activity, whereas, phosphorylation at Thr495 reduces enzyme activity (Forstermann and Sessa, 2012; Ladurner et al, 2012). Phosphorylation of eNOS at its activating site leads to conformational change in eNOS resulting in its activation (Forstermann and Sessa, 2012). Under non-stimulated conditions, eNOS is phosphorylated at Thr495. Phosphorylation of eNOS is regulated by various factors such as estrogen, VGEF, bradykinin which activates various kinases such as Akt, AMP activated protein kinase (AMPK) and protein kinase C (PKC) as well as protein phosphatase 2A (PP2A) (Forstermann and Sessa, 2012; Ladurner et al, 2012). Akt dependent phosphorylation is required for the activation of NOS in endothelial cells (Dimmeler et al, 1999). In this study, we observed a reduction in the protein expression of phosphorylated eNOS at Ser1177, while an upregulation of phosphorylated eNOS at Thr495 in Dox treated cardiomyocytes. This may be possibly due to Dox mediated downregulation in Ser1177 activating kinases Akt and AMPK (Ludke et al, 2012[a]). In our animal model we found a downregulation of Akt by Dox which was prevented by Vit C.

In the present study we also investigated the effect of Dox on iNOS protein expression. An increased protein expression of iNOS in Dox-treated cardiomyocytes as well as in Dox treated animals was observed. Under physiological conditions, iNOS is usually not expressed in cells, but pathological stimuli such as bacterial lipopolysaccharide and stress can induce its expression (Treuer and Gonzalez, 2015). Upregulation of iNOS is implicated in various

pathological conditions including heart failure (Aldieri et al, 2002). Increased iNOS activity and expression were demonstrated in the myocardium and vasculature of both animals and patients with heart failure (Sayed-Ahmed et al, 2001; Ungvari et al, 2005). iNOS mediated enhanced NO levels in high μM range for prolonged period of time are responsible for enhanced peroxynitrite formation as well as protein modification by s-nitrosylation and nitration. Studies using genetic and pharmacological modulation of iNOS have highlighted the role of iNOS in the pathogenesis of heart failure (Liu and Huang, 2008). Disruption of iNOS has been shown to reduce protein nitrosylation (Ropelle et al, 2013) as well as improve cardiac function (Feng et al, 2001) via reduction in cardiac NO. Thus an increase in NO levels and total NOS activity by Dox treatment in isolated cardiomyocytes as well as in adult rat cardiac tissue in this study can be attributed to upregulation of iNOS protein expression and not via eNOS which is rendered inactive by the interplay of phosphorylation at the Thr495 and Ser1177 sites.

3. Dox-induced activation of cytokines

Inflammatory cytokines TNF-α, IL-1β and IL-6 are associated with the progression of a number of pathological conditions. Although these inflammatory cytokines are generally produced by inflammatory cells, increased levels are also observed in endothelial and myocardial cells under pathological conditions (Kaur et al, 2006). Involvement of inflammatory cytokines in Dox-induced cardiotoxicity is still not completely clear. Although Lou et al (2004), have reported no changes in the expression of TNF-α and IL-6 in the hearts of Dox treated rats, there is increasing evidence of involvement of cytokines such as TNF- α, IL-1 and IL-6 in the upregulation of iNOS in myocardial pathologies (Ferdiandy et al, 2000). Several studies have confirmed the involvement of these cytokines in Dox treated cardiomyocytes (Andreadou et al, 2014; Zhu et al, 2011). In addition to IL-1β, the expression of its receptor is also increased in

cardiac tissue from Dox treated animals (Zhu et al, 2011). High levels of NO can promote secretion of TNF-α, IL-1β and IL-6 in cardiomyocytes even in the absence of Dox (Maass et al, 2005). Nevertheless, under pathological conditions, it is crucial to maintain the balance between proinflammatory cytokine TNF-α and anti-inflammatory cytokine IL-10 (Khaper et al, 2010). In this study, we observed an imbalance between TNF-α and IL-10 in Dox treated cardiomyocytes in favour of TNF-α. Dox resulted in an increase in levels of TNF-α, IL-1β and IL-6, whereas there was a reduction in IL-10 levels. Elevated levels of TNF-α and IL-1β are associated with increased nuclear translocation of NFκB which further induces the production of NO via iNOS activation (Ing et al, 1999; Treuer and Gonzalez, 2014).

4. Dox-induced activation of apoptosis and signaling proteins

In our study, the MTT assay showed reduced cell viability in Dox treated cardiomyocytes. Histological analysis of cardiac tissue also revealed extensive loss of myofibrils and vacuole formation in Dox-treated cardiomyocytes. It is likely that oxidative/nitrosative stress seen in this study acts as a crucial trigger for the initiation of cell death pathways. It has been shown that increased cellular accumulation of various reactive oxygen and nitrogen species affect various organelles and initiate various cell death pathways such as apoptosis and necrosis (Mukhopadhyay et al, 2009). Apoptotic cell death is a complex interplay and requires a balance between pro-apoptotic and anti-apoptotic proteins (Reeve et al, 2007). In this study, we observed an increase in the expression of various pro-apoptotic proteins such as Bax, Bnip-3, Bak and Caspase-3 in Dox treated animals, whereas a downregulation of anti-apoptotic protein Bcl-xl was observed. Dox is known to activate all three forms of cell death pathways ie. apoptosis, necrosis and autophagy (Pizarro et al, 2016). Nevertheless, its role in the activation of autophagy is controversial. Some studies have actually found inhibitory effect of Dox on basal autophagy via

activation of Akt/mTOR signaling pathway and reduction in Beclin-1 in neonatal cardiomyocytes thereby preventing recycling of damaged cardiomyocytes (Pizarro et al, 2016).

Alteration of mitochondrial membrane potential by Dox and other insults results in the release of cytochrome C and activation of caspases. Caspase activation plays a crucial role in the initiation of apoptosis (Porter and Janicke, 1999). Inhibition of caspase prevents myocardial apoptosis as well as cardiac dysfunction in an animal model of sepsis (Neviere et al, 2000). In Dox treated animals, we observed an activation of caspase-3 further triggering apoptosis via mitochondria dependent pathway. Apoptosis can also be mediated through mitochondria independent pathway via the involvement of TNF-α. Additionally, nitration of various proteins involved in maintaining mitochondrial bioenergetics can lead to mitochondrial dysfunction which would further trigger apoptosis (Salvemini, 2006). Our Dox-induced cardiotoxicity model shows involvement of both mitochondria dependent and independent cell death pathways. Increased levels of peroxynitrite and superoxide act as a trigger for the initiation of apoptosis via changes in mitochondrial membrane permeability and opening of mitochondrial permeability transition pore (Mukhopadhyay et al, 2009) as well as activation of caspase-3 and PARP (Islam et al, 2015; Levrand et al, 2006). Peroxynitrite also triggers necrotic cell death (Islam et al, 2015) and peroxynitrite scavenger results in enhanced cardiomyocyte viability (Mukhopadhyay et al, 2009).

We observed a reduction in protein expression of Akt in cardiac tissue of Dox treated animals. Akt, a Ser/Thr kinase, also known as protein kinase B (PKB) is an important protein involved in downstream signaling of growth factor, cytokines and other cellular stimuli (Manning and Cantley, 2007). Akt is now considered as a major cell survival signaling pathway in the modulation of cardiomyocyte apoptosis (Kim et al, 2008; Matsui et al, 2005; Matsui and

Rosenzweig, 2005) as well as activation of key immune and inflammatory response (Ozes et al, 1999). Activation of Akt suppresses apoptosis by phosphorylating and inactivating apoptotic proteins such as BAD and caspase-3 (Brunet et al, 1999; Kim et al, 2001). Akt reduced Apoptotic signaling kinase 1 (ASK1) activity and reduced apoptosis (Kim et al, 2001). A reciprocal association is observed between the protein expressions as well as activation of Akt and ASK1 in Dox treated isolated cardiomyocytes. Dox was observed to reduce phosphorylation and activation of Akt whereas it upregulated the phosphorylation of ASK1 in Dox treated cardiomyocytes (Sharma et al, 2014). Therefore, manipulation of this signaling pathway can be beneficial in the attenuation of Dox induced apoptosis and improve cardiac function (Taniyama and Walsh, 2002). Additionally, Akt plays an important role in the phosphorylation and activation of eNOS (Dimmeler et al, 1999; Fulton et al, 1999). Peroxynitrite mediated inhibition of Akt phosphorylation and activity resulted in an inhibition of eNOS phosphorylation in bovine aortic endothelial cells (Zou, 2002). Henceforth, a reduction in the protein expression of phosphorylated eNOS at Ser1177 observed in Dox treated cardiomyocytes in our in vitro study may be a result of Dox-mediated downregulation of Akt expression (Ludke et al, 2012; Sharma et al, 2014).

The tumor suppressor protein, p53, plays a critical role in promoting cell death via stimulating the expression of Bax and suppressing the expression of bcl-xl (Miyashita et al, 1994) as well as via activation of Bnip-3 (Wang et al, 2013). We observed an increased expression of p53 in cardiac tissue of Dox treated animals. Wang et al. (2004) demonstrated differential mechanisms of Dox mediated apoptotic cell death between cardiomyocytes and tumor cells. Tumor cells showed early activation of p53, whereas in cardiomyocytes activation of caspase-3 preceded p53 stimulation. Activation of p53 is via oxidative damage to DNA and is

regulated by phosphorylation. Oxidative stress enhanced phosphorylation of p53 in neonatal cardiomyocytes (Long et al, 2007). Although Dox is shown to induce p53 activation (Spallarossa et al, 2009; Yoshida et al, 2009), mitochondrial/nuclear localization of p53 and its function in cardiomyocytes may be regulated through redox-dependent mechanisms (Nithipongvanitch et al, 2007).

We also observed increased protein expression of cardiac NFκB in Dox treated animals. NFκB is a dimeric transcription factor that regulates genes associated with stress responses including inflammation, oxidative stress and apoptosis (Bowie and O'Neill, 2000; Wang et al, 2002). Dox is implicated in the activation of NFκB in various cell types (Lin et al, 2008; Riganti et al, 2008; Yu et al, 2008). Inhibition of NFκB resulted in a decrease in the levels of IL-1β, IL-6, TNF-α and Cox-2 in H9C2 cardiomyocytes (Guo et al, 2013). Conversely, NFκB is activated by various agents such as IL-1β, IL-6, TNF-α and low levels of H_2O_2 whereas antioxidants suppress activation of NFκB (Schreck et al, 1992).

5. Vit C mediated protection against Dox-induced alterations

A reduction in the plasma levels of Vit C is observed in various pathological conditions such as myocardial infarction, gastritis, diabetes, stroke and critically ill patients including cancer patients (Kim, 2013; Langlois et al, 2001; Mayland, 2005; Schorah et al, 1996). This reduction can be attributed to excessive metabolic need to neutralize free radicals generated in these pathological conditions (Kim, 2013) or reduction in Vit C transporter proteins. Vit C being a water soluble compound is transported intracellularly via specific proteins such as SVCT-2 and GLUT-4 in its oxidized or reduced form respectively (Du et al, 2012; Rivas et al, 2008). Lack of SVCT-2 results in fetal death as a result of low Vit C levels and increased oxidative stress (Harrison et al, 2010[a]). Vit C being vital antioxidant in brain, SVCT-2 has crucial role in

transport of Vit C in neuronal and retinal cells (Harrison et al, 2010[b]). In isolated adult rat cardiomyocytes, Dox caused a downregulation of the protein expression of SVCT-2 and GLUT-4 (Ludke et al, 2012b). In our study, a similar effect was observed in the cardiac tissue of Dox treated animals. Furthermore, supplementation of Vit C along with Dox prevented this effect and upregulated the protein expression of SVCT-2. Thus it can be speculated that a reduction in the plasma levels of Vit C in cancer patients may be the result of an increased metabolic demand along with the reduction of Vit C transport caused by Dox.

Vit C exerts antioxidant effects and reduces oxidative/nitrosative stress *in vivo*. In this study, we observed a reduction in Dox mediated oxidative/nitrosative stress by Vit C in isolated cardiomyocytes as well as in rats. Vit C reduced the levels of lipid peroxidation, protein carbonyl and superoxide anion in cardiac tissue of Dox treated animals. Vit C has the ability to protect against lipid peroxidation by acting as a scavenger of ROS and by one electron reduction of lipid hydroperoxyl radicals via Vit C redox cycle (Traber and Stevens, 2011). Vit C was shown to reduce oxidation of LDL and hence reduced atherosclerosis and prevented endothelial dysfunction in arteries of patients with atherosclerosis (Kugiyama et al, 1998; Levine et al, 1996). Oral administration of Vit C (80 mg/kg), prevented isoproterenol induced changes in lipid peroxidation and endogenous antioxidant enzymes in adult rats (Yogeeta et al, 2006). Ascorbic acid effectively scavenges superoxide anion. This reduces availability of superoxide to interact with NO, thus protects NO bioavailability and prevents peroxynitrite formation (Jackson et al, 1998). Despite the role of SOD in scavenging superoxide anion, Vit C depletion in Gluo$^{-/-}$ mice resulted in enhanced superoxide generation in the brain (Kondo et al, 2008). Supplementation of Vit C increased serum SOD activity in diabetes mellitus patients (Kathore et al, 2014). Vit C supplementation upregulated the protein expression of antioxidant enzymes SOD, catalase and

GPx in patients with cardiovascular disease (Karajibani et al, 2010) as well as Dox treated isolated cardiomyocytes (Ludke et al, 2012b). Similarly, the antioxidant Probucol caused an upregulation of protein expression and enzyme activity of SOD, Catalase and GPX in cardiac tissue of Dox treated rats (Li et al, 2000; Li and Singal, 2000; Siveski-Iliskovic et al, 1994). Enhanced oxidative/nitrosative stress and inflammatory conditions also lead to peroxynitrite mediated nitration of SOD affecting the catalytic activity of the latter (Demicheli et al, 2007; Macmillan-Crow et al, 1996). Thus Vit C might also be protecting SOD activity by a reduction in superoxide and peroxynitrite production. In our *in vitro* study of Vit C treatment in Dox exposed cardiomyocytes, we saw a reduction in superoxide as well as peroxynitrite and hence reduced protein nitration and nitrosylation.

Vit C also plays a role in the function of eNOS by recycling the cofactor, BH4. Vit C increases the levels of BH4 (Baker et al, 2001; Huang et al, 2000) as well as protects BH4 from oxidation and thereby stabilizes eNOS for cellular synthesis of NO (Heller et al, 2001) in isolated cardiomyocytes as well as apoE deficient mice (d'Uscio et al, 2003). In this study, Vit C reduced Dox mediated monomerization of eNOS in isolated cardiomyocytes probably via protection of BH4 oxidation. eNOS derived NO is required for various cardiovascular functions including vascular relaxation. In various experimental models of hypertension, infusion of Vit C increased vasodilation as a result of increased NO bioavailability (Garry et al, 2009; Kupari et al, 2012; Maio et al, 2012). Additionally, in this study we observed Vit C mediated enhanced phosphorylation of eNOS at its activating site Ser1177 in Dox treated cardiomyocytes. Independent of its effect of stabilization of BH4, Vit C increases phosphorylation of eNOS Ser1177 via activation of kinases AMPK and Akt and reducing activation of phosphatases such as PP2A (Ladurner et al, 2012). Studies have demonstrated activation of PP2A under conditions

of oxidative stress (Han et al, 2010). Vit C by its antioxidant effects would reduce oxidative stress and thereby reduce activation of PP2A. Our present study has demonstrated Vit C mediated upregulation of phosphorylation of Akt.

Furthermore, we observed that Vit C downregulated protein expression of iNOS in isolated cardiomyocytes and cardiac tissue of Dox treated animals. Increased expression of iNOS is activated under pathological stimuli such as cytokines via NFκB (Nathan, 1997; Umar and van der Laarse, 2010). Vit C has shown to provide beneficial effect in reducing the levels of TNF-α and IL-1β production and inhibiting NFκB suggesting an anti-inflammatory action of Vit C (Bowie and O'Neil, 2000; El-Shitany & El-Desoky, 2016). Vit C supplementation (500 mg twice daily) in hypertensive and/ or diabetic obese patients had potential effect in reducing plasma CPR and IL-6 levels (Ellulu et al, 2015). Additionally, Vit C treatment in isoproterenol treated rats reduced the expression of iNOS (Ribeiro et al, 2009). In agreement with these observations, our study demonstrated that Vit C caused a reduction in Dox mediated increase in inflammatory cytokines such as TNF-α, IL-1β and IL-6 and NFκB. Thus Vit C treatment protected against Dox-induced activation of iNOS in this study. Reduced iNOS expression resulted in lowering NO levels and hence reduction in protein nitrosylation and nitration by Dox treatment *in vitro* as well as *in vivo*.

We observed Vit C increased the viability of Dox treated cardiomyocytes and also caused a reduction in the expression of pro-apoptotic proteins Bax, Bnip-3, Bak and Caspase-3 in the cardiac tissue of Dox treated animals. Several studies using different pathological models have highlighted the beneficial effect of Vit C in reducing cell death by reduction in apoptosis, necrosis and autophagy (El-Shitany and El-Desoky, 2016; Guaiquil et al, 2004; Ludke et al, 2012a; Moradi-Arzeloo et al, 2016). Although the exact mechanism involved in Vit C mediated

protection of apoptosis/autophagy is poorly defined, we believe reduction in the expression of apoptotic proteins and blunting of cell death pathways by Vit C is mediated by the anti-oxidant property of Vit C. This reduction in cell death was reflected as preservation of cardiac structure in Dox treated animals. Vit C maintained cardiac structure of Dox treated animals by reduction in vacuole formation as well as loss and disarray of myofibrils. Furthermore, these animals also demonstrated improved cardiac function.

The recommended dietary allowance of 60 mg/day of Vit C results in its plasma concentration of 24 µM. Thus the concentration of 25 µM used in our *in vitro* can be easily reached in patients. Furthermore, a dose of upto 2 g/day did not show any deleterious effects (Carr & Vissers, 2013). A dose of 25 mg/kg/day in rats was reported to result in plasma Vit C levels of 90 µM, which is in the physiological range of Vit C (Djurasevic et al, 2008[b]). Hence, the dose of 50 mg/kg/day used in our study can easily result in physiological levels of Vit C. Ascorbate concentration in the plasma of healthy unsupplemented individuals is around 30-60 µM (May, 2000). However the plasma concentrations reduce in several pathological conditions (Kim, 2013). As plasma concentrations are maintained by intestinal absorption, frequent oral intake of physiological doses may be an alternative to sustain higher plasma concentration of Vit C (Verrax and Calderon, 2009). Furthermore, as cancer patients have been reported to have low levels of Vit C (Goncalves et al, 2005; Mayland, 2005); supplementation of Vit C could provide additional health benefits. Moreover, high doses of Vit C have been observed to improve the quality of life in cancer patients (Takahashi et al, 2012; Yeom et al, 2007). Several studies have provided evidence confirming that antioxidants do not interfere with anti-tumor mechanism and efficacy of the chemotherapy drugs (Block et al, 2007; Iliskovic et al, 1999; Moss, 2006; Park et al, 2012; Shimpo et al 1991; Simone et al, 2007). Thus our *in vivo* study provides a basis for

design of further clinical trial to determine potential protective effect of Vit C in reducing Dox-mediated cardiotoxicity.

VII. CONCLUSIONS

In addition to oxidative stress, Dox-induced nitrosative stress was evident by an increase in reactive nitrogen species. The data also showed that this change was due to enhanced generation of both superoxide and NO. Furthermore, Dox caused an increase in total NOS activity and upregulated the protein expression of iNOS. Dox-mediated increase in peroxynitrite led to enhanced protein nitration and nitrosylation. An increase in the levels of TNF-α and decrease in IL-10 levels by Dox triggered the activation of iNOS. Additionally, Dox caused a downregulation of expression of Vit C transporter proteins. These Dox-mediated changes were prevented by Vit C (Figure 31).

Dox caused phosphorylation of eNOS at Thr495 thereby inhibiting eNOS. A differential phosphorylation of eNOS via an activation of Ser1177 and inhibition of Thr495 by Vit C resulted in the prevention of Dox-induced inactivation of eNOS. Dox-induced increase in the monomeric form of eNOS resulting in an inhibition of eNOS was also prevented by Vit C. These subcellular changes in cardiomyocytes, increased oxidative stress in cardiac tissue and reduced antioxidant enzyme activities as well as protein expression under Dox treatment. Akt, proteins involved in the cell survival, were reduced whereas stress induced pathways like p53 and NFκB were upregulated in cardiac tissue by Dox treatment. These changes may provide positive feedback leading to further increase in the generation of ROS and upregulation as well as activation of apoptotic proteins Bax, Caspase-3, Bnip-3 and Bak. Vit C reduced oxidative/nitrosative stress and improved antioxidant status and thereby reduced activation of apoptotic proteins. By preventing these Dox-mediated changes, Vit C reduced loss and disarray of myofibrils as well as vacuole formation in the cardiac tissue increased caused by Dox. These effects of Vit C were also reflected as an improved systolic (increased EF and FS) as well as

diastolic (reduction in IVRT and E/A) cardiac functions. Dox induced reduction in Vit C transporter proteins SVCT-2 and GLUT-4 may also contribute to the decreased Vit C entry and thus increase oxidative stress. Vit C supplementation increased the protein expression of SVCT-2 and GLUT-4. Overall through its anti-oxidant, anti-apoptotic and anti-inflammatory properties, Vit C improved the survival of Dox treated animals. This study therefore provides evidence for the beneficial effect of Vit C against Dox-induced cardiotoxicity. Furthermore mechanistic information on the role of Vit C in the mitigation of Dox-induced cardiotoxicity has set the stage for the design of a translational study.

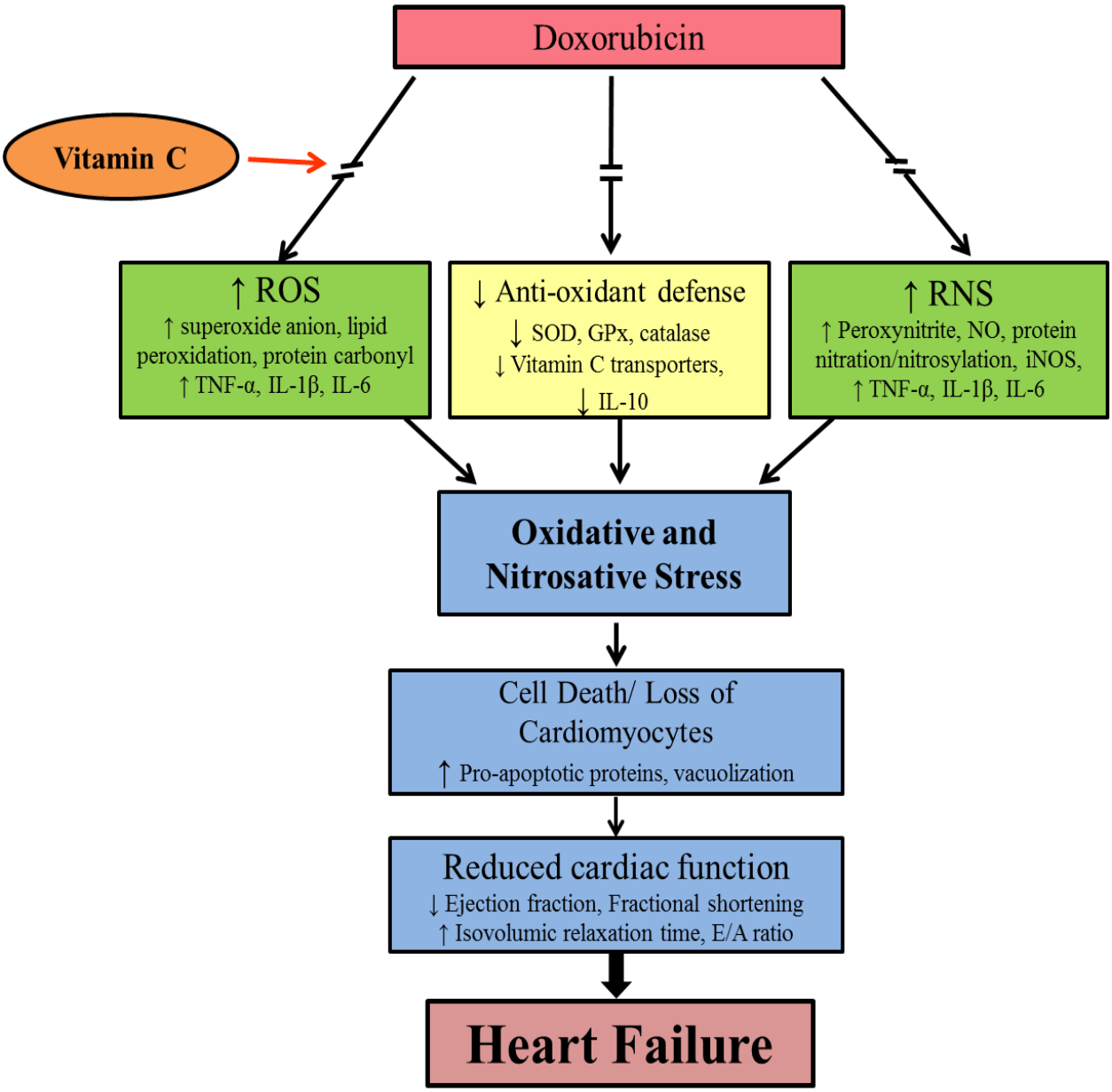

Figure 31: Schematic representation of Doxorubicin-induced subcellular changes leading to heart failure:
Doxorubicin (Dox) increases reactive oxygen species (ROS); reactive nitrogen species (RNS) and reduces anti-oxidant defense resulting in oxidative/nitrosative stress. This leads to various subcellular changes and activation of various cell death pathways and alteration in contractile function of cardiomyocytes resulting in compromised systolic and diastolic functions ultimately leading to heart failure. Vitamin C was protective against these Dox-mediated changes.

VIII. FUTURE DIRECTIONS

The present data obtained using isolated cardiomyocytes as well as in vivo animal experiments provide a strong basis for translational studies to determine the beneficial effect of Vit C in Dox treated patients. Our *in vitro* and *in vivo* studies demonstrated that Dox-mediated increase in nitrosative stress is via increased production of NO. Since many commercially available medications to support the heart function also act as NO donors, the role of these drugs in Dox treated patients need further investigation. Furthermore, future studies can be directed towards the development of exosome derived diagnostic markers as an indicator of Dox-induced cardiomyopathy. Exosomes are small microvesicles released from the cell and contain cellular lipids, proteins, different types of RNA from that cell. Exosomes can be isolated from the blood of Dox-treated patients and compared to a healthy individual to determine the diagnostic markers to identify the onset of cardiotoxicity in these patients.

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