Cytokine Imbalance in Heart Failure Subsequent to Myocardial Infarction in Rats

by

Kuljeet Kaur

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I dedicate this thesis to the memory of my Dad,

Mr. Amar Singh *B.E. (Electrical)*

A man for whom education was a measure for everything

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ABBREVIATIONS

Angiotensin converting enzyme (ACE)
Angiotensin I (Ang I)
Angiotensin II (Ang II)
Angiotensin Receptor Blockers (ARB)
C reactive protein (CRP)
Ceramide activated protein kinase (CAPK).
Copper/zinc superoxide dismutase (Cu/Zn-SOD)
Creatine kinase (CK)
Cytokine synthesis inhibitory factor (CSIF)
Dihydroflourescein diacetate probe (DCFDA)
Ejection fraction (EF)
Electron spin resonance (ESR)
End diastolic dimensions (EDD).
End systolic dimensions (ESD)
Enzyme-linked immunosorbent assay (ELISA)
Experimental Autoimmune Encephalomyelitis (EAE)
Extracellular-SOD (ECSOD)
Factors associated with death domain (FADD)
Factors associated with neutral sphingomyelinase activation (FAN)
Fractional shortening (FS)
Glutathione (GSH)
Glutathione peroxidase (GSHPx).
Granulocyte-macrophage colony stimulating factor (GM-CSF)
Hemeoxygenase (HO-1)
Hydrogen peroxide (H_2O_2).
Inhibitory κB (IκB)
Inhibitory κB kinase (IKK)
Interferons (IFN)
Interleukin-10 (IL-10)
Lactate dehydrogenase (LDH)
Left ventricular end diastolic pressure (LVEDP)
Left ventricular peak systolic pressure (LVPSP)
Leukotriene B4 (LTB4)
Lipopolysachride (LPS)
Macrophage inflammatory protein-1α (MIP-1α)
Malondialdehyde (MDA)
Manganese superoxide dismutase (MnSOD)
Membrane bound (mTNF-α)
Messenger RNA (mRNA)
Monocyte chemoattractant protein-1 (MCP-1)
Myocardial infarction (MI),
N-acetylcysteine (NAC)
New York Heart Association (NYHA)
NIK (NF-κb inducing kinase)

Phosphate buffer saline (PBS)
Polymorphonucleocyte (PMN)
Post myocardial infarction (PMI)
Reactive oxygen species (ROS).
Receptor activator of nuclear factor κB ligand (RANKL)
Receptor interacting protein (RIP).
Regulated on Activation Normal T cell Expressed and Secreted (RANTES)
Rennin angiotensin system (RAS)
Soluble TNF-α (sTNF-α)
Study of Left Ventricular Dysfunction (SOLVD)
Suppressors of cytokine synthesis (SOCS)
T helpers cells (TH)
The maximum rate of isovolumic pressure decay (-dP/dt)
The maximum rate of isovolumic pressure development (+dP/dt)
TNF like weak inducers of apoptosis (TWEAK)
TNF receptor associated death domain (TRADD)
TNF related apoptosis inducing agent (TRAIL)
TNF-R associated factors (TRAF)
TNF-α receptors (TNF-R)
Total radical-trapping antioxidant potential (TRAP)
Tumor necrosis factor-α (TNF-α)

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I. ABSTRACT

Heart failure is a progressive disease and a better understanding of the factors involved in its progression is needed to develop effective and specific therapies. Oxidative stress as well as inflammation is considered to be important factors in the pathogenesis of heart failure subsequent to myocardial infarction. It is now well documented that endogenous TNF-α play a central role in initiating and sustaining the inflammatory cytokine cascade. TNF-α is overexpressed in left ventricular dysfunction, cardiomyopathy, pulmonary edema and heart failure and is known to increase oxidative stress. In contrast, another cytokine, interleukin-10 (IL-10) is anti-inflammatory that down regulates the expression of several pro-inflammatory cytokines, including that of TNF-α. IL-10 has been shown to antagonize some of the deleterious effects of TNF-α. IL-10 is also known to inhibit the generation of reactive oxygen species (ROS). Considering the anti-TNF-α effects of IL-10, it is possible that the suggested role of TNF-α in the pathogenesis of heart failure may in fact be accentuated by a fall in the levels of IL-10.

In this study, I tested the possibility that i) A decrease in the levels of IL-10 along with an increase in TNF-α is involved in the pathogenesis of congestive heart failure subsequent to coronary artery ligation in rats. ii) An imbalance of these two cytokines in the pathogenesis of congestive heart failure leads to increased oxidative stress thus causing cardiac dysfunction, due to their disparate effects.

To test this hypothesis, congestive heart failure was induced in rats by occluding the left descending coronary artery. The cardiac function at 1, 4, 8 and 16 weeks post myocardial infarction (PMI) was assessed using indwelling catheter with a pressure sensitive tip transducer and as well as by echocardiography. RT-PCR was performed on frozen tissue samples to determine the mRNA levels of TNF-α and IL-10 and ELISA was performed to examine changes in the membrane bound and soluble protein fractions of these cytokines during the progression of heart failure. Losartan treatment (2 mg/ml in drinking water, daily) was given to modify the cardiac function to examine the validity of the relationship between these cytokine changes and cardiac function. In *in vitro* studies, isolated adult cardiac myocytes were used to test the interplay of these cytokines in regards to oxidative stress and cardiac cell injury. Myocytes were exposed for 4 hours to different concentrations of TNF-α and IL-10 (1-20 ng/ml) alone or in combination. Cell lysates were then analyzed for protein levels and mRNA levels for Copper/zinc superoxide dismutase (Cu/Zn-SOD), Manganese superoxide dismutase (MnSOD), Catalase (Cat) and Glutathione peroxidase (GSHPx). Oxidative stress was measured by quantifying the generation of reactive oxygen species by redox-sensitive fluorescence dye (H2DCFDA) as well as by the study of lipid peroxidation. Creatine kinase (CK) release in the culture medium was assayed to assess cell injury. As a positive control, cardiac myocytes were exposed to H_2O_2 to induce oxidative stress. All these parameters were also examined in these H_2O_2 exposed cardiac myocytes.

Cardiac function deteriorated with post ligation duration in PMI groups, with a severe failure seen at 16 W PMI. Echocardiographic studies revealed differences between

sham and respective PMI groups as early as 4 weeks after the coronary ligation. In the PMI groups fractional shortening, ejection fraction, left ventricular peak systolic pressure and (+)dP/dt and (-)dP/dt decreased significantly at all time points starting with 4W PMI. These changes were accompanied by an increase in left ventricular end diastolic pressure.

TNF-α protein fractions, both membrane bound and soluble, were significantly increased in 1 and 4W PMI groups and its levels were unchanged in 8 and 16 W PMI groups vs control groups. TNF-α mRNA was significantly increased in 4 and 8W PMI with no changes seen in 1 and 16 W PMI groups. Membrane bound IL-10 protein was significantly decreased in 4, 8 and 16W PMI groups and the soluble fraction of this protein showed no change. IL-10 mRNA was significantly reduced in all PMI groups. The ratio between membrane bound IL-10/TNF-α protein was significantly decreased at all time points in PMI groups and this correlated with the depressed cardiac function. Losartan treated, 4W PMI group, showed improved cardiac function as compared to the untreated 4W PMI group. Both membrane bound and soluble fractions of TNF-α and IL-10 proteins as well as their ratio were significantly improved towards normal values by losartan treatment. There was no effect of losartan treatment on TNF-α mRNA whereas IL-10 mRNA was significantly improved in losartan treated 4W PMI group.

After LPS stimulation, adult cardiac myocytes showed an increase in the release of TNF-α and IL-10 protein as well as in the expression of their mRNA. An exposure to H_2O_2 caused a significant decrease in the protein as well as mRNA content of all the antioxidant enzymes along with increased intracellular ROS production, lipid

peroxidation and cellular injury as measured by CK release. The redox ratio and glutathione activity was significantly decreased after H_2O_2 treatment. TNF-α treatment increased oxidative stress and cell injury at ≥ 10 ng/ml and also reduced both glutathione activity and redox ratio. IL-10 treatment alone had no effect on the protein levels of antioxidants but caused a dose dependent increase in the mRNA of all antioxidants with a significant change seen at 20 ng/ml. TNF-α induced decrease in MnSOD, Cat and GSHPx protein level was prevented by IL-10/TNF-α ratio of 1. Similarly, this ratio of 1 had an optimal effect on the mRNA levels for MnSOD and Cat. Combination treatment at a ratio of 1 also significantly decreased lipid peroxidation and intracellular ROS in these cardiac myocytes.

It is concluded that a decrease in IL-10 as well as the ratio of IL-10/TNF-α had a direct correlation with a decrease in function. This correlation held true even when the function in coronary ligated animals was improved by losartan treatment. A decrease in IL-10 and/or IL-10/TNF-α ratio caused an increase in oxidative stress in cardiac cells leading to cell injury. Thus in this model of heart failure, increased TNF-α, decrease in IL-10 and/or IL-10/TNF-α ratio caused an increase in the oxidative stress which may be one of the factors in the pathogenesis of heart failure.

II. INTRODUCTION

Despite intense research efforts, the death rate due to heart disease is still at an unacceptable level. A better understanding of the molecular mechanisms of myocyte death and/or dysfunction is necessary to develop specific and effective therapeutic strategies to prevent or manage heart failure. Number of factors including inflammation and an increase in oxidative stress has been implicated in the initiation and progression of heart failure subsequent to myocardial infarction. Cytokines as intracellular signaling molecules are thought to be involved both in inflammation and in oxidative stress.

Cytokines are classified into two major groups depending on their action i.e. inflammatory and anti-inflammatory. Tumor necrosis factor-α (TNF-α) is a key member of the inflammatory cytokine group and is expressed in a membrane bound form and/or soluble form. TNF-α mediates most of its effects through its receptors TNF-RI and TNF-RII. Endogenous TNF-α, is involved in the initiation and progression of inflammation and is overexpressed in inflammation related diseases such as rheumatoid arthritis, alcohol induced liver damage and inflammatory bowl disease. In the heart, all nucleated cells are capable of producing TNF-α. Both animal and human myocardium have also been shown to possess receptors for TNF-α. Overexpression of TNF-α has a variety of deleterious effects ranging from decreased contractility to increased apoptosis seen in left ventricular dysfunction, cardiomyopathy and heart failure. TNF-α is known to increase the generation of reactive oxygen species (ROS). Glutathione, an important substrate for the antioxidant glutathione peroxidase, has been shown to inhibit TNF-α induced cell

injury suggesting that some of the deleterious effects of TNF-α may be mediated through increased generation of ROS and oxidative stress.

Interleukin-10 (IL-10) belongs to the anti-inflammatory group of cytokines and is expressed by number of cells such as macrophages, monocytes and stimulated human skin cells, keratinocytes. This cytokine, both as membrane bound or soluble form is biologically active through binding to its receptors. IL-10 down-regulates the expression of several pro-inflammatory cytokines, including the synthesis of TNF-α. Therefore, it is also known as cytokine synthesis inhibitory factor. By inhibiting the synthesis of inflammatory cytokines and other mediators of inflammation, IL-10 affects the severity as well as duration of inflammation. In this regard, IL-10 knockout mice show exaggerated response to endotoxin-induced inflammation. IL-10 is known to reduce the generation of ROS and decreases oxidative stress. Exogenous administration of IL-10 has been shown to protect against the TNF-α mediated, oxidative stress-induced acute lung injury. In contrast, the use of IL-10 antibody resulted in augmented acute lung injury in the same model.

It is also known that endogenous antioxidant enzymes offer protection against oxidative stress. With age as well as with certain disease conditions, this inherent defense mechanism can be overwhelmed by increased oxidative stress resulting in cell injury. Number of studies from our laboratory as well as other laboratories has shown that during heart failure, there is increased generation of ROS, decreased redox ratio and/or antioxidants resulting in an increase in oxidative stress. This increase in oxidative stress

has been suggested to play a role in the pathogenesis of heart failure due to a variety of conditions.

These data raise the possibility that during the progression of heart failure an imbalance between these two opposing cytokines, i.e. IL-10 and TNF-α may play a crucial role in modifying oxidative stress and contribute to the progression of heart failure. The objective of my study was to characterize changes in both protein and mRNA for IL-10 as well as TNF-α during the development of heart failure subsequent to myocardial infarction in rats.

In this study on rat hearts, myocardial infarction was achieved by ligation of the left descending coronary artery. Functionally assessed animals were used at 1, 4, 8 and 16 weeks post myocardial infarction to analyze the relationship between changes in the IL-10, TNF-α and cardiac function during the progression of heart failure. Validity of the changes in IL-10 and TNF-α in relation to cardiac function was further tested by *in vivo* treatment of the animals with losartan to improve the cardiac function. In order to examine the significance of these changes on the pathogenesis of cardiac dysfunction, the interplay of IL-10 and TNF-α, increased in oxidative stress and cell injury was examined in isolated adult cardiac myocytes. For this purpose, cardiac myocytes were exposed to different concentrations of IL-10, TNF-α or their combination. These cells were analyzed for ROS generation, lipid peroxidation, antioxidant enzymes and creatine kinase release and LDH release.

The results from this study show a correlation between the decrease in IL-10 as well as IL-10/TNF-α ratio and depressed cardiac function. Furthermore, this decrease in IL-10/TNF-α ratio may have increased oxidative stress and have been the cause for depressed cardiac function.

III. LITERATURE REVIEW

1.0 HEART FAILURE

Heart failure is a clinical condition with signs of peripheral edema, breathlessness and fatigue after exercise or at rest depending on the severity of the disease. The major effect of heart failure is insufficient perfusion of vital organs and ultimately progressive failure of these organs follows the progression of heart disease. Although heart failure is a significant health issue, its molecular basis is still poorly understood.

Heart failure is generally classified as Diastolic heart failure or Systolic heart failure depending on the inability of heart either to relax or contract. Systolic heart failure is further classified as right heart failure and left heart failure on the basis which side of the heart is unable to contract properly. The most commonly used classification by the cardiologists is, the one provided by New York Heart Association (NYHA): Class I: Symptoms with more than ordinary activity; Class II: Symptoms with ordinary activity; Class III: Symptoms with minimal activity (Class IIIa: No Dyspnea at rest; Class IIIb: Dyspnea at rest) and Class IV: Symptoms at rest.

1.1 Risk Factors and mechanisms

The most common cause of heart failure is coronary artery disease (Teerlink et al., 1991). Patients with uncontrolled high blood pressure also commonly die of heart failure (Kannel et al., 1972). There is a direct correlation between the high blood pressure and the incidence of heart failure (Kannel et al., 1972; Levy et al., 1996). A better control

of hypertension with antihypertensive drugs has reduced incidences of hypertension associated with myocardial infarction and stroke (Moser and Hebert 1996). Furthermore, because of a better management of hypertensive patients, now coronary artery disease has become the number one cause of heart failure (Teerlink et al., 1991; Gheorghiade and Bonow 1998).

Diabetes mellitus is another major risk factor for developing heart failure (He et al., 2001) as it is associated with increased atherosclerosis (Ginsberg 2000) and/or hypertension (Reaven et al., 1996). Similarly, the incidence of heart diseases is increased in patients with chronic kidney disease and about half of the deaths associated with kidney disease are due to heart failure (Culleton et al., 1999). Pulmonary diseases, like pulmonary hypertension and chronic obstructive pulmonary diseases are also the risk factors for heart failure (Naeije 2005; Dart et al., 2003). Uncontrolled pulmonary hypertension leads to heart failure and death occurs within 5 years and airflow limitation is an independent risk factor for cardiovascular events (Adiutori 2000).

Certain anticancer drugs and other chemicals are also associated with heart damage. In these cardio-toxic drugs, anthracyclines are the most common contributor to the drug-induced heart failure (Singal et al., 2000). About 30% of the patients receiving high doses of anthracyclines develop heart failure (Rhoden et al., 1993). Other anticancer drugs such as cyclophosphamide and paclitaxel are also associated with heart failure (Manthorpe and Svensson 1996; Jekunen et al., 1994).

Increased alcohol consumption is also responsible for increased incidences of heart failure related morbidity and mortality (Urbano-Márquez et al., 1998). The increased levels of coxsackievirus B antibodies in patients with cardiomyopathy suggested that viral infections can be one of the causes of heart failure (Woodruff 1980). Congenital heart disease could be another reason for the development of heart failure (Nora et al., 1991).

In response to these factors or stresses, there are several myocardial as well as humoral adaptive changes. One of the compensatory mechanisms is heart hypertrophy. The activation of adrenergic system, rennin angiotensin system (RAS) and activation of the inflammatory system are some of the humoral responses (Paulus 2000; Yamazaki and Yazaki 1999; Cunningham and Gotlieb 2005).

Among many sub-cellular changes, an increase in oxidative stress and apoptosis has been reported during the progression of heart failure (Giordano 2005; Yaoita et al., 2000). Overstimulation of different neurohumoral systems has been shown to activate pro-inflammatory cytokines, increase oxidative stress and apoptosis (Murray et al., 2000). These physiological adaptations under chronic conditions may become pathological adaptations and can cause heart failure. This is the reason why most of the therapeutic approaches are to inhibit these systems by using adrenergic blockers, RAS blockers as well as anti-inflammatory approaches respectively (Davila et al., 2005; Frangogiannis et al., 2002).

In this literature review, I will focus on pro-inflammatory and anti-inflammatory

cytokines, oxidative stress and rennin angiotensin system.

2.0 INFLAMMATION AND THE HEART

2.1 General

Although inflammation is a normal reaction of the body to protect against the invading microorganisms, increased or uncontrolled inflammation plays a major role in the pathogenesis of various diseases (Matsumori 2003). In the rat model of myocardial infarction (MI), an activation of the complement-system was reported (Hill and ward 1971). A reduction in the infarct size in canine model of MI by corticosteroids (Libby et al., 1973) and subsequent protection against myocardial ischemia by complement depletion using cobra venom (Maroko et al., 1978) provided the initial evidence for the role of inflammation in extending myocardial injury.

In mice heart, overexpression of inflammatory protein, monocyte chemoattractant protein-1 (MCP-1), decreased myocardial contractility, increased hypertrophy and lead to the ventricular dilatation (Kolattukudy et al., 1998). On the contrary, macrophage inflammatory protein-1α (MIP-1α) knock-out mice were protected against viral myocarditis (Sasayama et al., 2000). P-selectin-deficient mice or blockade of L-selectin and P-selectin with specific monoclonal antibody or recombinant analog of P-selectin glycoprotein ligand-1 (PSGL-1) showed decreased myocardial injury (Palazzo et al., 1998; Ma et al., 1993; Hayward et al., 1999). Another protein β2 integrin (CD18), known to be involved in the neutrophil transmigration during inflammation, has been suggested to play a role in myocardial injury (Palazzo et al., 1998b). CD18 knockout mice were

better protected against myocardial ischemia (Palazzo et al., 1998b). Treatment of dogs with anti-CD18 antibodies has also been shown to reduce infarct size (Arai et al., 1996). Similarly, mast-cell deficient mice had better cardiac function as compared to the wild type after aortic banding. Furthermore the use of mast cell stabilizer to inhibit degranulation of mast cells had a protective effect in the wild type mice, supporting the role of mast cell in progression of heart failure (Hara et al., 2002).

Framingham Heart Study suggests that increased C-reactive protein (CRP) - a marker of inflammation, is a good indicator of the increased risk of congestive heart failure (CHF) (Vasan et al., 2003). Normal healthy individuals showing higher baseline value for CRP had increased risk for MI and stroke (Ridker, et al., 1997). Similar correlation between increased CRP and increased risk of MI and stroke was seen in healthy women (Ridker, et al., 2002). Increased CRP was related to the development of left ventricular hypertrophy (Conen et al., 2006). Furthermore, in patients with atherosclerosis and unstable angina, CRP was shown to be associated with increased risk of MI, sudden cardiac death, stable angina and development of arterial diseases (Bhatt and Topol 2002; Arroyo-Espliguero et al., 2004). Patients with class IV heart failure showed the highest CRP levels as compared to the patients with class III or II and this increase in CRP was associated with increased mortality and hospitalization (Alonso-Martinez et al., 2002; Berton et al., 2003).

Similarly in clinical studies, all types of infiltrating cells (T cells, B cells, macrophages, granulocytes) were increased in hearts with idiopathic dilated

cardiomyopathy compared with normal hearts (Holzinger et al., 1995). Other markers of inflammation like chemokines, MCP-1, MIP-1α and RANTES (Regulated on Activation Normal T cell Expressed and Secreted) were also shown to be elevated in patients of heart failure (Shioi et al., 1997). 5-lipoxygenase activity, as measured by increased arachidonic acid metabolite, in urine, was increased in angina patients (Carry et al., 1992) and this activity correlated with anginal pain (Takase et al., 1996). Modulation of inflammation by lipoxygenase inhibitors, leukotriene B4 (LTB4) antagonists and neutrophil antibodies reduced reperfusion induced injury in dogs (Shappell et al., 1990; Romson et al., 1983). Beneficial effects of physical training have been associated with reduced serum markers for inflammation in heart failure patients (Adamopoulos et al., 2001). Polymorphonucleocyte (PMN) depletion before cardiac surgery also reduced creatine kinase activity and improved cardiac index as compared to control group (Chiba et al., 1993).

Mechanical stretch of isolated endothelial cells without any injury or infection was able to induce chemoattractants (Okada et al., 1998). Moreover CRP was able to induce markers of atherosclerosis in isolated vascular cells, dose dependently (Devaraj et al., 2003). These experimental studies suggest that under the conditions of mechanical stretch, occurring due to pressure or volume overload during the progression of heart diseases can have inflammatory reaction in the absence of injury or infection.

In brief, there is a significant increase seen in inflammatory cells and expression of CRP as well as other markers of inflammation in heart failure both in animal and

humans. Cardio-protection due to a decrease in the expression or antagonism of these markers suggests that inflammation may have a causal role in the pathogenesis of heart failure. Since cytokines are one of the important proteins involved in the initiation and progression of inflammation, I examined the role of cytokines in the pathogenesis of heart failure.

2.2 Cytokines

The term cytokine came from words *cyto* meaning cell and *kinin,* - a general term used for hormones (Cohen et al., 1974). Cytokines are small soluble or membrane bound proteins with molecular weight ranging from 8 to 40 kDa (Joseph 2000). Interferons (IFN) were the first cytokines to be identified and described in 1957 (Isaacs and Lindenmann 1957). So far about 150 biologically active cytokines have been recognized and characterized (Cope Andrew 2003). Infact with the recent identification of IL-32, the numbering in the interleukin (IL) family has reached 32 (Kim et al., 2005). Virtually all nucleated cells can produce cytokines and are in turn affected by them. These proteins are bioactive at very low concentrations, roughly in the range of 1pg–1ng/mL and can act in autocrine, paracrine, juxtacrine or even in endocrine fashion (Joseph 2000).

Cytokine general classification

Cytokines are either pro-inflammatory for e.g. IL-1, TNF-α, IFN-γ, IL-12, IL-18 or anti-inflammatory e.g. IL-4, IL-10, IL-13, transforming growth factor-β. Based on their structure and function, cytokines have also been grouped as **Chemokines** (IL-8, IL-18, macrophage inflammatory protein-1 and β-Thromboglobulin); **IL-1 family** (IL-1α,

IL-1β, IL-1 δa, IL-1ε); **IL-6 family** [(IL-6, IL-11, Leukemia inhibitory factor (LIF), oncostatin M, Ciliary neurotrophic factor (CNTF), cardiotrophin-1, IL-27)]; **Interferons** (IFN-α, IFN-β, IFN-δ, IFN-γ, IFN-κ); **Hematopoietic factors** [(various colony stimulating factors like G-CSF, GM-CSF, M-CSF), SCF (stem cell factor) and various Interleukins (IL-3, IL-5, IL-6)]; **Growth factor** [(Epidermal growth factor (EGF), Transforming growth factor (TGF), Fibroblast growth factors (FGF), Vascular endothelial growth factor(VEGF) and Platelet-derived growth factor(PDGF)]; **Immunomodulators:** [IL-2 family (IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21), IL-10 family (IL-10, IL-19, IL-20, IL-22 (IL-TIF), IL-24 and IL-26)] and **TNF ligand superfamily** [(TNF-α, TNF-β, LT-β, CD27 ligand, CD30 ligand, CD40 ligand, TNF related apoptosis-inducing agent (TRAIL)] (Cope Andrew 2003).

Cytokine and mediation of Inflammation

Cytokines released from immune and non-immune cells are the major activators of leukocytes and other immune cells involved in inflammation (Carlos and Harlan 1994; Schall and Bacon 1994). Cytokines also upregulate adhesion molecules such as VCAM-1, ICAM-1, E-selectin, and L-selectin leading to migration of neutrophils, monocytes T-helper and T-cytotoxic cell to the site of tissue injury (Heremans et al., 1989; Sundy and Haynes 2000). Cytokines can also stimulate arachidonic acid metabolism in leukocytes and increase production of variety of lytic enzymes, reactive oxygen and nitrogen species (Das et al., 1990; Klebanoff et al., 1986). Pro-inflammatory cytokines are potent inducer of other mediators including IL-1, IL-6, and granulocyte-macrophage colony stimulating factor (GM-CSF) and chemokines (Zentella et al., 1991; Tracey and Cerami 1994; Schall

and Bacon 1994). This creates a loop of inflammation-induced injury and injury-induced inflammation.

In downstream signaling pathway, activation of nuclear factor-κB (NF-κB) plays an important role in inflammation. This transcription factor regulates the expression of a number of mediators including pro-inflammatory cytokines, adhesion molecules, chemokines and other (Baldwin 1996; Kishimoto et al., 1994). Inflammatory mediators such as TNF-α and IL-1 intern can also activate NF-κB (Nomura 2001; Baldwin 1996). On the other hand IL-10, an anti-inflammatory cytokine, decreases the activation of NF-κB and thus modulates inflammation (Weber et al., 2001). Thus the activation as well as inactivation of NF-κB is extensively involved in the process of inflammation.

Increased levels of inflammatory cytokines have been implicated in variety of inflammatory or non-inflammatory diseases. High levels of TNF-α, IL-6, IL-18 and IL-1 have been reported in synovial fluid and serum of rheumatoid arthritis patients which correlated with the severity of disease (Russo et al., 2002; Maeno et al., 2004). In clinical studies anti TNF-α, IL-1 and IL-6 therapy were shown to be effective in controlling the progression of rheumatoid arthritis (Cohen et al., 2004; Nishimoto et al., 2004). These and other cytokine are also involved in neurological diseases (Mennicken et al., 1999). Furthermore overexpression of IL-1 and TNF-α plays a significant role in the pathogenesis of inflammatory bowl diseases and cystic fibrosis (Blam et al., 2001; Moss et al., 2000).

In contrast, IL-10 is down regulated in various diseases. Clinical data suggest that subjects with low IL-10 production present an increased risk for stroke (van Exel et al., 2002). Furthermore, stroke patients displayed significantly lower levels of IL-10 in their serum (Perini et al. 2001). Mice over-expressing human IL-10 were resistant to the development of experimental autoimmune encephalomyelitis (EAE) (Cua et al., 1999). Bronchial, tracheal and epithelial cells from cystic fibrosis patients showed decrease IL-10 content (Chmiel et al., 2002). Furthermore, interleukin-10 can also reverse the cartilage degradation mediated by antigen-stimulated mononuclear cells from patients with rheumatoid arthritis (van Roon et al., 1996).

Thus, these reports suggest that overexpression of pro-inflammatory cytokines and decreased expression anti-inflammatory cytokines is involved in the progression of various inflammatory and non-inflammatory diseases. As inflammation has also been suggested as one of the factors in the pathogenesis of heart failure, it is important to study the role of these cytokines in cardiovascular diseases.

Cytokines and cardiovascular system

Patients with myocarditis, dilated cardiomyopathy and hypertrophic cardiomyopathy have shown increased levels of IL-1α, IL-1β, TNF-α, IL-6, IL-8 and IFN-γ in their serum (Matsumori et al., 1994; Shioi et al., 1997). Plasma levels of IL-6 and IL-18 were shown to be associated with increased mortality due to cardiac disease in elderly patients independent of other risk factors such as diabetes, smoking, age or sex (Harris et al., 1999; Blankenberg, et al., 2002). Wide variety of stimuli such as microbial

antigens, shear stress, hypoxia, hemodynamic overload, ischemia and oxidized LDL-cholesterol can cause increased generation of inflammatory cytokines in cardiac diseases (Okada et al., 1998; Baumgarten et al., 2002; Janabi et al., 2000). A sustained upregulation of these inflammatory cytokines can cause irreversible damage to the myocardium structure and function by increasing apoptosis, ROS generation and cardiac remodeling. There are number of good reviews, which discuss the role of inflammatory cytokines in cardiac diseases (Mann 2002; Torre-Amione 2005). In this section, I have focused on TNF-α and IL-10 as these two cytokines are the subject of my study.

2.2.1 Tumor Necrosis Factor-Alpha (TNF-α)

In the early 18th century, Deider (1725) pointed out that tumors of syphilitic patients were cured more often than others thus the connection was drawn between the incidence of cancer and microbial infection. He also noticed that prostitutes infected with syphilis had a lower frequency of cancer than the average population (Deider 1725). This historic report of Deider still holds true, as an inverse correlation between severe infections and melanoma has been reported recently (Kolmel et al., 1999).

Serum from mice challenged with bacterial endotoxin regressed tumor in cancer bearing mice. It was concluded that bacterial endotoxins produce tumor destroying factor which is responsible for the anti-tumor activities of these endotoxins (OMelley et al., 1962). This protein was also responsible for the necrosis of methylcholantheren induced transplantable sarcoma and was named as TNF-α (Carswell et al., 1975). TNF-α protein was purified and sequenced following the isolation and characterization of lymphotoxin-

α (LT- α or TNF-β), a related member of TNF family (Aggarwal et al., 1985). Historically, the sensitivity of some tumors to TNF-α explains the origin of its name. Moreover TNF-α is one of the most evolutionary conserved cytokines and is also found in invertebrates, suggesting the importance of this cytokine in the normal physiology.

Although we have known the beneficial effects of immunological response of the cytokines for nearly 275 years, the importance of different components of this system remains an enigma. Therefore, in the present study, an interplay of TNF-α and IL-10 in the pathogenesis of heart failure was studied.

TNF-α protein and receptors

TNF-α belongs to a family of structurally related cytokines. This super family contains more then 20 different ligands including TNF-α, TNF-β (LT-α), LT-β, TRAIL (TNF related apoptosis inducing agent), CD 27, CD30, CD40, TWEAK (TNF like weak inducers of apoptosis), VEGI (vascular endothelial cell growth inhibitor), RANKL (receptor activator of NF- κB ligand) and others (Wajant et al., 2001).

TNF-α protein: TNF-α protein is highly conserved in mammalian species. It is first synthesized as a trans-membrane precursor and is either embedded in the membrane or processed to be released in the soluble form via proteolytic cleavage (Hooper et al., 1997). Thus, TNF-α is expressed in various cells in two forms: membrane bound (mTNF-α, 26 kDa,) and soluble form (sTNF-α, 17 kDa,) (Spiegelman and Hotamisligil 1993). Previously, it was suggested that sTNF-α is the only active form but now the

importance of mTNF-α is also being increasingly recognized. Existing data indicates that mTNF-α is capable of exerting a variety of cellular responses such as apoptosis and inflammation (Grell et al., 1995; Gerspach et al., 2000). Furthermore, mTNF has been implicated in a number of diseases such as acute hepatitis, rheumatoid arthritis and neurological disorders (Kusters et al., 1997; Agostini et al., 1995; Georgopoulos et al., 1996; Akassoglou et al., 1997). A recent report by Borst and colleagues (2005) suggests that insulin resistance induced by high fat diet in rats is accompanied by increased levels of tissue expression of mTNF-α with no detectable changes in the circulating levels (Borst and Conover 2005). mTNF production is also significantly increased in different rodent models of obesity as well as in obese humans without any change in the soluble fraction (Xu et al., 2002).

TNF-α receptors: Two types of TNF-α receptors have been isolated (Loetscher et al., 1990; Smith et al., 1990). The first TNF-α receptor has a molecular weight of 55 kDa and is referred to as TNF-R55 or TNF-RI and binds sTNF-α. The second TNF-α receptor has molecular weight of 75 kDa and is referred as TNF-R75 or TNF-RII this receptor binds mTNF-α with higher affinity. For a long time, TNF-RI was considered as the principle mediator of TNF-α signaling because TNF-RII binds sTNF-α with a 20 fold lower binding affinity than TNF-RI (Grell et al., 1995). However, it is now known that TNF-RII is preferentially activated by mTNF-α and acts both in a paracrine and autocrine ways (Haas et al., 1999; Weiss, et al., 1997). Furthermore, these two receptors are independently regulated (Vassalli P, 1992).

TNF-α signaling

Both TNF-RI and TNF-RII contain an extracellular pre-ligand binding assembly domain (PLAD) that precomplexes receptors and facilitates their trimerization upon activation by ligand (Chan et al 2000). These two receptors possess sequences that are capable of binding intracellular adaptor proteins that link TNF-R stimulation to activation of many signaling processes. These TNF-R associated factors (TRAFs) and adaptors, transduce the TNF-α signaling within the target cells (Wajant et al., 2001).

TNF-RI contains a death domain which is critical in the death inducing activity of TNF-RI (Tartaglia et al., 1993). TRADD (TNF receptor associated death domain) is receptor activated and was the first of the several TNF-R associated proteins identified (Hsu et al., 1995). TRADD recruits FADD (factors associated with death domain) and RIP (receptor interacting protein). Activated FADD and RIP then activate apoptotic pathways and induce injury. TNF-RI also recruits RAIDD which then interacts with RIP and activates caspase 2. TNF-α, through the activation of FAN (factors associated with neutral sphingomyelinase activation) adaptor protein activates ceramide activated protein kinase (CAPK). The latter activates Raf kinase, which then leads to MAP kinase activation.

TNF-α signaling also activates certain lipases including phospholipase (PL) C and D. PL activation by TNF-RI activates a downstream acidic membrane bound sphingomyelinase activity which breakdowns sphingolipids and ceramide. Membrane bound neutral sphingomyelinase is activated through TNF-RI via FAN adaptor proteins.

Another lipase stimulated by TNF-RI is PLA2. PLA2 is responsible for the liberation of arachidonic acid which can be converted to prostaglandins and leukotriene stimulating eicosanoids sensing receptors. These eicosanoids are responsible for the generation of oxygen radicals. Activation of MAP kinase and PKC by TNF-RI can also lead to PLA2 activation (Chang et al., 1992). There is TNF-α stimulated PLA2 gene induction leading to increased expression of PLA2 protein and its activity. It has been shown that PLA2 protein and activity is crucial for TNF-α mediated cell death in many cell types (Hoeck et al., 1993).

TNF-RII does not contain death domain motif but still recruits adaptor proteins including TRAF2 (TNF-R associated factors). TRAF2 interacts with TNF-RII and also with NIK (NF-κB inducing kinase) directly. NIK phosphorylates inhibitor of κB (IκB) which activates NF-κB. This activation of NF-κB has positive feed back effect on the synthesis of TNF-α and other cytokines. Similarly, RIP and FADD can directly bind TNF-RII via TRAF 2 and can induce apoptosis. TNF-RII is able to induce apoptosis through so called ligand passing because of greater affinity and quick dissociation of TNF-α from TNF-RII, therefore, increasing TNF-α concentration in the vicinity of TNF-RI (Tartaglia et al 1993 b).

TNF-α in cardiac system

Earlier it was thought that activated macrophages were the only source for TNF-α release (Matthews 1978), but it is now known that virtually all nucleated cells in the body are capable of producing this widely active cytokine. Immunohistochemical studies have

confirmed the production of TNF-α by myocardial cells (Torre-Amione et al., 1996; Kapadia et al., 1995). There are several hypotheses with respect to the source of pro-inflammatory cytokines in heart failure: 1) The activation of immune system after myocardial injury may be responsible for the release of cytokines in the injured myocardium; and 2) Decreased cardiac output in heart failure causes reduced perfusion of systemic tissue leading to decreased removal of endotoxins and thus increased cytokine production (Rauchhaus et al., 2000). Pharmacokinetic studies in patients with metastatic cancer show that TNF-α has a half life of 14 to 18 min (Blick et al., 1987). This may suggest that elevated levels of TNF-α in heart failure represent its biosynthesis at the local level. Furthermore, the elevated myocardium levels may spillover into the plasma leading to secondary activation of immune system (Kapadia et al., 1998). However, intracardiac TNF-α, as measured in explanted hearts showed no correlation between serum and cardiac TNF-α protein and mRNA (Kapadia et al., 1995). Moreover, isolated cardiac myocytes and fibroblasts under different stimuli such as ischemia and endotoxins were able to release increased levels of TNF-α in culture medium (Yue et al., 1998; Giroir et al., 1992).

Although TNF-α protein and mRNA does not appear to be constitutively expressed in the unstressed heart (Torre-Amione 2005; Torre-Amione et al., 1996b), both mRNA and protein are rapidly synthesized in response to appropriate stimuli both in animals as well as humans (Giroir et al., 1992; Kapadia et al., 1995, 1997). In clinical studies, higher levels of TNF-α were shown to correspond with severity of heart failure. In this regard, in the SOLVD (Study of Left Ventricular Dysfunction) trial, a trend

towards increase in mortality with increase in the levels of TNF-α was seen (Torre-Amione 2005). Along with the increased level of TNF-α both receptors were also increased and predicted mortality in CHF patients (Rauchhaus et al., 2000b). TNF-α content as measured by immunohistochemistry, decreased after the use of left ventricular (LV) assist devices in patients with end stage heart failure, this fall in TNF-α level was associated with recovery of cardiac function (Torre-Amione et al., 1999).

In rat model of MI, gene expression of TNF-α rose progressively which correlated with LV end diastolic dimensions as well as with the interstitial fibrosis (Irwin et al., 1999). Isolated feline hearts showed increased TNF-α production after higher diastolic pressure whereas there was no change in TNF-α production at low LV diastolic pressure (Kapadia et al., 1997). The change in TNF-α protein and mRNA in this model was evident as early as 30 min suggesting that even increased wall stress can quickly induce cardiac TNF-α production (Kapadia et al., 1995). Increased expression of TNF-α was reported even after brief aortic banding in rabbits. Blockade of TNF-α signaling with TNF-α antibody was associated with improved cardiac function in rabbits (Stamm et al., 2001).

Acute administration of TNF-α lead to cardiac dysfunction in dogs, pigs, rats and guinea pigs (Heard et al.,1992; Pagani et al., 1992; Eichenholz et al.,1992). TNF-α infusion promoted LV dysfunction, pulmonary edema, cardiomyopathy, LV remodeling and induced apoptosis in dogs (Pagani et al., 1992; Heard et al., 1992). Chronic infusion of TNF-α into the peritoneal cavity of rats suggested that TNF-α can promote unfavorable LV remodeling (Bozkurt et al., 1998).

Transgenic mice with cardiac specific over-expression of TNF-α developed all signs of clinical heart failure. These mice exhibited pulmonary edema, peripheral edema, cardiac dilatation, decreased cardiac contractility and loss of weight, all indicators of sever CHF (Kubota et al., 1997). On the other hand, TNF-α knockout mice had fewer cardiac ruptures in contrast to the wild type mice, one week after MI ,and had reduced number of apoptotic myocytes (Sun et al., 2004).

Activation of latent collagenase MMP-1 plays a prominent role in remodeling of myocardium at the site of infarction (Cleutjens et al., 1995). TNF-α is capable of activating a family of metalloproteinase that are responsible for degradation of extracellular matrix (Rawdanowicz et al., 1994). One week after coronary ligation there was a correlation between the expressions of TNF-α and expression of type 1 and III collagen (Yue et al., 1998). Exogenous TNF-α in animal studies has been demonstrated to cause reduced histological staining for fibrillar collagen and thus disruption in myocardial extracellular matrix (Bozkurt et al., 1998; Li et al., 2000b). This degradation of extracellular matrix was progressive and was associated with LV dilatation in these rats. Transgenic mice over-expressing TNF-α, demonstrated increased soluble myocardial collagen and TNF-α antagonism decreased matrix metalloproteinase activation, decreased collagen denaturation, and thus reduced left ventricular remodeling (Li et al., 2000b). All these studies suggest an important role of TNF-α in the pathogenesis of heart failure.

Suppression of pro-inflammatory cytokines was suggested to be one of the

explanations for improved cardiac function in 102 idiopathic dilated cardiomyopathy patients after prednisone treatment (Parrillo et al 1989). Pentoxifylline, a platelet anti-aggregator, also inhibited TNF-α production and significantly lowered TNF-α blood level and improved LV ejection fraction in patients with idiopathic cardiomyopathy after 5 months treatment (Sliwa et al., 1998). Heart failure was significantly less common in antiTNF-α treated rheumatoid arthritis patients (Wolfe and Michaud 2004). TNF-α antibody (cV1q) treatment in TNF-α transgenic mice significantly improved basal fractional shortening, responsiveness to beta-adrenergic stimulation and decreased cardiac dilation (Kadokami et al., 2001). TNF-α antibody treatment prior to the left anterior descending artery ligation also decreased area of necrosis, number of circulating endothelial cells, and lipid peroxidation product malonaldehyde (Li et al., 1999). Etanercept, a soluble TNF-α receptor significantly improved left ventricular ejection fraction and left ventricular remodeling in patients with NYHA class III/IV heart failure (Bozkurt et al., 2001). However, despite the positive results from these and other studies, clinical results of two bigger trials were disappointing. The failure of these trials may have been due to the functional state of the heart in these subjects as well as due to the dose of the anti-TNF-α therapy used (Mann 2002).

2.2.2 Interleukin-10

IL-10 represents one of the most important immune regulating cytokines. It was discovered in 1989 as a cytokine synthesis inhibitory factor (CSIF). This factor, produced by type 2 T helper cells (TH_2), inhibited activation of cytokine production by type 1 T helpers cells (TH_1) (Fiorentino et al., 1989). Like many other cytokines CSIF, has

multiple biological effects on different cell types and is now commonly known as IL-10 (Moore et al., 1990). Name IL-10 is also used for B cell derived T cell growth factor and T cell growth inhibitory factor.

IL-10 protein and receptors

IL-10 is expressed as membrane bound and/or soluble protein (Capsoni et al., 1998; Moore et al., 2001). The soluble form of this protein consists of 160 amino acids and primarily exists in extracellular space as a homodimer with a molecular mass of 37 kDa. Mouse and human IL-10 share greater than 80% homology in DNA sequence (Howard and OGarra 1992).

The biological activities of IL-10 are dependent on two receptors: one that bind IL-10 with high affinity, (IL-10R1), and the other that binds IL-10 with low affinity (IL-10R2). Differential affinity of these receptors results in a sequential binding mechanism in which IL-10 first binds IL-10R1 and subsequently IL-10R2 (Moore et al., 2001).

IL-10 signaling

Only limited knowledge is available regarding the intracellular signaling transduction pathway for IL-10. The IL-10/IL-10R interaction activates the tyrosine kinase Jak1 and Tyk2 that are associated with IL-10R1 and IL-10R2, respectively (Moore et al., 2001). The receptor engagement and tyrosine phosphorylation activates the cytoplasmically localized inactive STAT 1, 3, 5, and results in the translocation and gene activation (Finbloom and Winestock 1995).

IL-10 controls the inflammatory processes by suppressing the expression of pro-inflammatory cytokines, chemokines and adhesion molecules (Moore et al., 2001; Walter 2002). Most of inflammatory proteins are transcriptionally controlled by NF-κB. It was suggested that IL-10 may exert its anti-inflammatory effect by inhibiting this transcription factor. Number of studies show that IL-10 blocks nuclear translocation of classic NF-κB p65/p50 heterodimer in monocytes and macrophages (Wang et al., 1995; Clarke et al., 1998). It has been shown that IL-10 inhibits NF-κB translocation through dual mechanisms: 1) It blocks NF-κB translocation by inhibiting IKK activity thus inhibiting degradation of IκB: and 2) IL-10 inhibits DNA binding of NF-κB already present in the nucleus with no increase in the nuclear levels of IκB (Schottelius et al., 1999). This inhibition of DNA binding could be due to selective induction of p50 nuclear translocation while blocking translocation of classical p65/p50 (Driessler et al., 2004). IL-10 can also inhibit the synthesis of IL-1, IL-6 and TNF-α proteins by promoting the degradation of their mRNA among other things. The degradation of mRNA by IL-10 is species dependent as IL-10 is unable to affect mRNA stability in human monocytes but is a major mechanism of down-regulation of cytokines in murine and porcine blood cells (Wang et al., 1995). IL-10 through the induction of suppressors of cytokine synthesis (SOCS3), probably via STAT3 dependent pathway, also suppresses the transcription of inflammatory mediators (Cassatella et al., 1994, Ito eta1999). There is evidence that SOCS3 plays a key role as mediator of the inhibitory effects of IL-10 on macrophage activation (Donnelly et al., 1999).

IL-10 is a pleiotropic cytokine that inhibits cell-mediated immunity while enhancing humoral immunity (Moore et al., 2001). TH_1 cells produce pro-inflammatory

cytokines and contribute to cell mediated immunity while the TH_2 associated cytokines augment humoral immunity. The cytokines produced by one type of helper cells regulate the other cell activity. TH_2 associated IL-10 has a variety of immunomodulatory properties involving inhibition of TH_1 cells, macrophage function and production of pro-inflammatory cytokines (Shanley et al., 1995; Furukawa et al., 1999).

IL-10 inhibits the synthesis of a number of cytokines such as IFN-γ, IL-2 and TNF-α. Data suggests that IL-10 is a neutral component of inflammation and serves to reduce both duration and magnitude of the process. IL-10 knockout mice and normal mice treated with IL-10 antibody manifest an exaggerated inflammatory reaction in response to endotoximea or peritonitis (van der Poll et al., 1995; Standiford et al., 1995). In IL-10 gene deficient mice, an overproduction of inflammatory cytokines and development of chronic inflammatory diseases have been shown (Kumar and Creery 2000).

IL-10 in cardiac system

There is not much data reporting IL-10 production by cardiac myocytes. However, IL-10 is known to be produced by different cells of immune system. T cell subsets, monocytes, macrophages and several other cells have been reported to produce IL-10 (Moore et al., 2001; Redondo et al., 1998). IL-10 promoter contains several transcription factor response elements (Platzer et al., 1994). Thus macrophages, the major source of IL-10, are stimulated to produce IL-10 by several endogenous and exogenous factors such as endotoxins, TNF-α, catecholamines, etc. (Platzer et al., 1995, 1999, 2000).

On the other hand systemic release of TNF-α also induces IL-10 via a negative feedback using NF-κB dependent pathway (Barsig et al., 1995; Meisel et al., 1996). Recent data suggest that p38 mitogen activated kinase pathway also regulates the human IL-10 promoter via the activation of sp1 transcription factor (Ma et al., 2001).

In my study, I conducted experiments to establish IL-10 production by isolated cardiac myocytes after exposing these cardiac myocytes to lipopolysachride.

IL-10 effects in cardiac system

Elevated serum levels of IL-10 were shown to be associated with a more favourable prognosis in patients with acute coronary syndromes (Heeschen et al., 2003). In dialysis patients, an IL-10 promoter polymorphism which is responsible for decreased IL-10 production was associated with increased cardiovascular mortality (Girndt et al., 2002). IL-10 deficient mice developed increased atherosclerotic lesions as compared to wild type mice and these lesions regressed with IL-10 treatment (Pinderski-Oslund et al., 1999; Mallat et al., 1999). Indeed, animal experiments have confirmed such an antiatherosclerotic effect of the over-expression of IL-10 in low-density lipoprotein receptor knockout mice (Vonder Thusen et al., 2001). In murine experimental model of heart failure, caused by encephalo myocarditis virus, the survival in mice treated with recombinant human IL-10 (rhIL-10) was significantly higher than the control group. rhIL-10 treatment in this model attenuated myocardial lesions and suppressed TNF-α and IL-2 production in heart (Nishio et al., 1999).

In ischemia and reperfusion model of cardiac injury, lymphocytes infiltrating the

ischemic and reperfused myocardium express IL-10 and showed a significant role in cardiac healing by modulating mononuclear cell phenotype and inducing TIMP-1 expression (Frangogiannis et al., 2000). Other investigations indicated that IL-10 knock out mice showed increased inflammation after ischemia reperfusion as indicated by enhanced neutrophil infiltration, plasma levels of TNF-α, nitrite/nitrate and increased tissue expression of intercellular adhesion molecule-1. These animals also exhibited higher cardiac injury as shown by increased infarct size and myocardial necrosis. Moreover IL-10 knockouts showed increased mortality after reperfusion (Yang et al., 2000). IL-10 has also been shown to suppress metalloproteinase and stimulate metalloproteinase inhibitors in macrophages to favor matrix preservation (Lacraz et al., 1995).

2.3 Interplay of TNF-α and IL-10

Inflammatory response represents a complex interrelated series of events modulated by numerous cytokines. TNF-α is central in initiating and sustaining the pro-inflammatory cytokine cascade (Ksontini et al., 1998). Both TNF-α and IL-10 are key regulators of TH_1/TH_2 balance which is critically skewed towards one-way or other depending upon the disease (Fearon and Locksley 1996; Mosmann and Sad 1996). IL-10 has been shown to inhibit the TNF-α release by endotoxin stimulated monocytes and neutrophils (Cassatella et al., 1994; Kasama et al., 1995; Wang et al., 1994). Pretreatment of mice with IL-10 attenuated the plasma TNF-α response and lethality associated with endotoximic shock (Howard et al., 1993; Gerard et al., 1993). Similarly IL-10 pretreatment, prior to skeletal muscle ischemia and reperfusion, in rat model reduced

plasma level of TNF-α during reperfusion (Engles et al., 1997).

IL-10 knockout mice are much more susceptible to ethanol hepatotoxicity and exhibit increased levels of TNF-α (Hill et al., 2002). Monocytes from alcoholic patients have been shown to produce less IL-10 after LPS stimulation than control monocytes and this decreased IL-10 production was associated with excessive TNF-α production in these monocytes (LeMoine et al., 1995). These patients also showed decreased IL-10/TNF-α in their plasma (Park et al., 2001).

Recent data suggests that IL-10 induces hemeoxygenase (HO-1), a heat shock protein, and plays an essential role in controlling tissue homeostasis in inflammation by inhibiting TNF-α synthesis and inducing anti-apoptotic processes (Lee and Chau 2002). An increase in IL-10 has been shown to prevent apoptotic death in B cells by induction of Bcl-2 protein (Byrne and Reen 2002; Levy and Brouet 1994) whereas TNF-α has been associated with increased apoptosis and cell injury (Gerspach et al., 2000).

These studies suggest that the interpretation of pathogenic role of TNF-α and IL-10 system cannot be exclusively based on the levels of any one of the cytokines. Thus, there is a need to study these cytokines in relation to each other. Furthermore, both the fractions i.e. membrane bound as well as soluble fraction of these proteins should be studied to obtain a more comprehensive picture. The transmembrane fraction of these cytokines might be a potential mechanism to restrict activities to specific local microenvironment and might be critical in the generation of systemic versus local

response.

3.0 OXIDATIVE STRESS

Free radical as well as antioxidants is an integral part of physiology. With age or in certain disease conditions, the inherent protection against increased generation of free radicals flux may be overwhelmed. This inability of the biological system to counterbalance the increased levels of free radicals leads to a physiological state called oxidative stress. Increased oxidative stress leads to cell injury through structural and functional changes in lipids, proteins, DNA and carbohydrates. Free radicals have been implicated in the development and progression of various diseases including heart diseases. The oxidative stress induced cell injury and death are important events in inflammation (Kaul et al 1993; Singal et al., 2000Kaneto et al., 2005, Niedowic and Daleke 2005, Sacks et al., 1978).

3.1 Reactive Oxygen Species (ROS)

ROS include free radicals such as superoxide anion, hydroxyl radicals and the non-radical hydrogen peroxide (H_2O_2). All redox reactions in the cell have the capability of generating ROS. In cardiac cells different membrane systems including sarcolema, mitochondria and sarcoplasmic reticulum are important source of ROS (Kaul et al., 1993; Lenaz et al., 2002). Immune cells through the action of lipoxygenases, cyclooxgenases, peroxidases nicotinamide adenine dinucleotide phosphate (NADPH) oxidases and xanthine oxidase also lead to production of ROS (Babior 1999; Meneshian and Bulkley 2002; Kaul et al 1993).

There are number of direct and indirect methods which can be used for the determination of ROS and the list includes, electron spin resonance (ESR) technique (Utsumi and Yamada 2003), reduction of ferricytochrome C, reduction of nitroblue tetrazolium (NBT), chemiluminescent detection of lucigenin, luminal and coelenterazine. Aminotriazole-mediated catalase inactivation is used to measure H_2O_2 level (Tarpey and Fridovich 2001; Griffiths et al., 2002). Cell permeable fluorescent probes are now being frequently used in the estimation of intracellular generation on ROS by flow cytometry or fluorescent microscopy (Halliwell and Whiteman 2004).

In biomolecules, protein, DNA and lipids are generally affected by increased oxidative stress and the effect of these ROS on these biomolecules is determined to evaluate the level of oxidative stress. Oxidative status of tryptophan, tyrosine, aliphatic thiol and carbonyl amino acids is determined by HPLC or spectrophotometry (Griffiths et al., 1999). ROS produce strand breaks and modify bases and cause DNA damage. Comet assay in isolated cells and HPLC in biological tissue samples are widely used to measure 8-oxoguanosine (8-oxo-Guo) levels to determine oxidative damage to DNA (Dizdaroglu 1991). Different types of lipid carrying lipoproteins and cell membrane lipids are highly susceptible to oxidative damage. ROS cause a chain reaction leading to generation of highly damaging lipid peroxides. Quantification of lipid peroxidation by measuring malondialdehyde (MDA) or conjugated dienes are some of the commonly used method (Thompson and Smith 1985; Ohishi et al., 1985).

3.2 Antioxidants

The evolution of aerobic respiration in eukaryotes lead to the expansion of cellular defense mechanisms through the development of several antioxidants (Benzie 2000). These antioxidants defenses against free radicals in eukaryotes includes enzymatic antioxidants such as superoxide dismutase (SOD), glutathione peroxidase (GSHPx), catalase and non-enzymatic antioxidants like Vit A, Vit C, Vit E, uric acid, albumin etc.

Superoxide dismutase (SOD) enzymes are responsible for accelerating the reduction of superoxide ions to hydrogen peroxide. There are two major types of SOD present in eukaryotic cells. Cu/Zn-SOD, is generally present in cell cytosol and is the first SOD to be identified in 1969 (McCord and Fridovich 1969). Mitochondrial matrix contains MnSOD which is responsible for 90% of SOD activity in cardiac cells (Fridovich 1995). Biochemically the activity of these two enzymes can be differentiated by using cyanide to inhibit Cu/Zn-SOD in the reaction mixture (Beauchamp and Fridovich 1973). In plasma, extracellular matrix, lymph, and cerebrospinal fluid, a different type of SOD has been identified and is called Extracellular-SOD (ECSOD) (Marklund 1994). Mammalian ECSOD is Cu/Zn bound and is a tetrameric glycosylated protein as compared to cytosolic Cu/Zn-SOD which is dimeric (Okado-Matsumoto and Fridovich, 2001; Petersen and Enghild, 2005). In yeast and bacteria there are reports suggesting the existence of iron (FeSOD) and nickel (NiSOD) bound SOD (Young et al., 1996; Fridovich 1995).

Glutathione peroxidase (GSHPx) was first discovered in 1957 as erythrocyte enzyme responsible for protecting hemoglobin from oxidative breakdown (Millis 1957). GSHPx is thought to be of more physiological importance because of its colocalization with SOD that is in cytosol, mitochondria and even in extracellular compartments. Glutathione peroxidase uses GSH as thiol substrate to catalyse the reduction of a variety of hydroperoxides ranging from H_2O_2 to lipid hydroperoxides (Jornot et al., 1998). GSHPx enzymes are either selenium dependent or selenium independent (Mates 2000; Singh and Shichi 1998).

Catalase was the first antioxidant to be identified in 1901as a H_2O_2 detoxifying compound from plant cells (Loew 1901). Catalase is an iron containing tetrameric protein which is a generally present in the peroxisomes and is involved in the detoxification of H_2O_2 to water (Chio and Tappel 1981). GSHPx and catalase differ in their affinity for H_2O_2. i.e. low concentration of H_2O_2 is generally reduced by GSHPx where as catalase is more active at higher concentration of H_2O_2 (Kaul et al., 1993).

There are number of nonenzymatic compounds (α-tocopherol, ascorbic acid, carotenoids, GSH, uric acid, ubiquinol) exist in the body which play a significant role in decreasing the levels of ROS both in intracellular and extracellular compartments. These agents are either synthesized in the body or are integral part of our diet (Kaul et al 1993).

α-Tocopherol is a lipid soluble, most active, structural isoform of Vit E (tocopherol). α-Tocopherol efficiently reduces free radicals generated during lipid

peroxidation, acts as chain reaction breaker and protects lipoproteins and membranes (Esterbauer et al., 1991; Kagan et al., 1990). It reacts with hydroxy radicals and superoxides as well leading to a tocopheroxyl radical which is stable and can be further reduced by other antioxidants like ascorbic acid, glutathione or ubiquinol (Pascoe et al., 1987; Niki et al., 1985).

Vit C (ascorbic acid) is a water-soluble, non-enzymatic antioxidant. Vit C has to be included in human diet as human body is unable to synthesize and it is an integral part of the normal protection mechanism. Major function of Vit C is to regenerate α - tocopherol (Janisch et al., 2005). However, Vit C also has the capability to reduce peroxyl, hydroxyl, superoxide radicals and metal ions (Sies and Stahl 1995). This antioxidant is the most effective water-soluble antioxidant in protecting plasma lipid peroxidation (Frei 1991; Frei et al., 1990; Jialal et al., 1990).

Because of long chain of conjugated double bonds, carotenes are excellent scavengers for reactive free radicals and protect against lipid peroxidation by reducing ROS. Carotenes also inhibit xanthine oxidase induced lipid peroxidation (Krinsky 1982), thus acting as powerful nonenzymatic antioxidants.

Low-molecular-weight thiol, reduced glutathione (GSH), is the most abundant thiol present in almost all cells and is another very important component of the antioxidant protection system. GSH quickly reduces free radicals, generally hydroxyl and carbon radicals, by donating a hydrogen atom. Purine metabolism end product, uric

acid, also possess strong antioxidant properties, probably by sequestering transition metals such as iron and copper (Davies et al., 1986) which are responsible for the generation of ROS.

Ubiquinol, reduced form of Ubiquinone, an electron transport chain protein, is a strong antioxidant as well. Ubiquinol has been suggested to regenerate Vit E and protect against lipid peroxidation (Frei et al., 1990; Ernster et al., 1992). Number of other proteins such as transferrine, ferritin, albumin, ceruloplasmin and bilirubin, present in plasma, can bind metal ions and reduce the production of ROS through the Haber-Weiss or Fenton reaction (Hellman and Gitlin 2002; Trenam et al., 1992).

Oxidative stress being the imbalance between the generation and detoxification of free radicals, a number of analytical methods have been developed to estimate the antioxidant capacity of a biological system (Abuja and Albertini 2001). The total radical-trapping antioxidant potential (TRAP) method, measures the time taken by a biological sample to reduce artificially generated radicals (Wayner et al., 1985). Another method, ferric reducing ability of plasma (FRAP), measures the ability of a sample to reduce ferric ion complex (Benzie and Strain 1999). FRAP method is more direct and can be easily automated. In recent years the fluorescence decay of B- or R-phycoerythrin (PE) in presence of antioxidants is also being used as a measure of oxygen radical absorbance capacity (ORAC) of a biological sample (Cao and Prior 1999). All these methods are capable of determining the total antioxidant capacity of tissues and/or body fluids.

Commercial kits are now available for measuring the activities of superoxide

dismutase (Spitz and Oberley 1989), glutathione peroxidase (Flohé and Günzler 1984) and catalase (Aebi 1984). Non-enzymatic antioxidants like Vit E, Vit C, uric acid, carotenoides and /or ubiquinol (Q10) can be measured by high performance liquid chromatography (HPLC), gas chromatography (GC) or with mass spectrometry (MS) (Abidi 2000; Finckh et al., 1995).

GSH is oxidized to its disulfide form (GSSG, oxidized glutathione) when acting as antioxidant. Redox ratio, which is the ratio of concentration of GSH/GSSG, is regularly used as a measure of oxidative stress (Asensi, et al., 1999).

3.3 Oxidative stress in cardiovascular diseases

During normal physiology of cell differentiation, arrest of growth, immunity, apoptosis and defense against microorganisms, low levels of ROS play a significant positive role (Ghosh and Myers 1998, Bae et al., 1997). On the other hand increased generation of ROS has been suggested to be responsible for a number of diseases including cardiac diseases (Kaul et al., 1993; Kaneto et al., 2005, Niedowic and Daleke 2005).

Evidence from our lab suggests that long term treatment of rats with adriamycin decreased GSHPx activity and increased lipid peroxidation (Siveski-Iliskovic et al., 1994). Adriamycin treatment also decreased redox ratio and albumin level (Iliskovic N et al., 1998). There was a decrease in cardiac antioxidant protein and mRNA levels both after short and long-term treatments with adriamycin (Li et al., 2000, 2002). The changes

in oxidative stress and cardiac function after adriamycin treatment were prevented by an antioxidant probucol (Iliskovic et al., 1998, Li et al., 2000) suggesting that cardiac injury is associated with increase in ROS generation and a decrease in the antioxidant reserve.

Heart failure subsequent to coronary artery ligation was also associated with a decrease in the myocardial antioxidant enzymes activities, mRNA, protein level, redox ratio and an increase in lipid hydroperoxides (Khaper et al., 2003; Khaper and Singal 2001). Myocardial infarction induced heart failure also leads to reduced levels of non enzymatic antioxidants. Vit E supplementation improved cardiac function in these animals (Palace et al., 1999). Similar results were obtained in monocrotoline induced right heart failure in rats (Pichardo et al., 1999). Increase in lipid peroxidation and decrease in cardiac GSHPx, SOD and catalase are also associated with experimentally induced hypertension and aortic banding induced CHF (Bello-Klein et al., 2001; Dhalla et al., 1996).

Direct assessment of hydroxyl radicals by ESR documented increased production of ROS from mitochondria of myocytes obtained from a canine rapid pacing model of heart failure (Ide et al., 2000). In the same model of heart failure, xanthine oxidase activity was increased which probably lead to xanthine oxidase induced ROS generation (Ekelund et al., 1999). Increased generation of ROS in cardiac tissue regulates MMP/TIMP balance and causes overexpression of MMP2 and MMP9 and these MMPS are involved in cardiac remodeling and in the transition to CHF (Spinale 2002). Overexpression of GSHPx in TG mice was associated with decreased MMP 9 and

preservation of systolic function (Shiomi et al., 2004) and detoxification of ROS by the administration of dimethylthiourea (DMTU) was associated with decreased LV dilation and improved cardiac contractility in coronary artery ligated mice (Kinugawa et al., 2000).

ROS also reduces the capability of myocytes to generate ATP by decreasing mitochondrial respiration leading to contractile dysfunction (Xie et al., 1998). In isolated system of cardiac myocytes, exogenous H_2O_2 increased DNA laddering, nuclear condensation, increased p38 and JNK activation and apoptosis thus leading to cardiac injury (Kwon et al., 2003, von Harsdorf et al., 1999). Antioxidant treatment improved contractility and reduced oxidative stress-induced cardiac damage in experimental animals (Xie et al., 1998; von Harsdorf et al., 1999). Overexpression of ECSOD preserved post ischemic myocardial function in isolated murine hearts (Chen, et al., 1994) whereas Cu/Zn-SOD knockout mice showed increased infarct size after coronary ligation (Yoshida et al., 2000). Similarly MnSOD knockout mice developed severe cardiomyopathy (Lebovitz et al., 1996). In another report overexpression of GSHPx protected against myocardial injury where as GSHPx knockout mice were more susceptible to myocardial damage (Yoshida et al., 1996, 1997). In an isolated system of neonatal cardiac myocytes, SOD inhibition increased DNA laddering and apoptosis (Siwik et al., 2002) suggesting the protective effects of antioxidants in cardiac system.

Similar to animal studies, number of clinical studies have also reported increased generation of ROS in heart failure patients. Direct measurement of ROS by ESR method

suggested increased production of free radicals during the pathogenesis of heart failure (Ellis et al 2000). Lipid peroxidation as measured by breath pentane, plasma TBARS and urinary 15-F2t-isoprostane levels are also increased in heart failure patients as compared to control subjects (McMurray et al., 1993; Singh et al., 1995). Studies also showed an increase in oxidative stress at the cardiac level with the progression of heart failure. Levels of isoprostane in pericardial fluid and 4-hydroxy-2-nonenal (HNE)-modified protein in myocardium was increased in heart failure patients (Mallat et al., 1998; Nakamura et al., 2002) and these isoprostane levels in pericardial fluid correlated with LV impairment (Mallat et al., 1998).

Along with increased ROS levels, heart failure patients also present lower levels of non enzymatic antioxidants such Vit C, Vit E and selenium in their plasma (de Lorgeril et al., 2002; Torun et al 1995). Antioxidant enzyme activity also decreased with the progression of heart failure and these changes in oxidative stress correlated with reduced ejection fraction in these patients (Ghatak et al., 1996).

Vitamin C treatment not only improved endothelial function but also reduced plasma levels of circulating apoptotic cells and suppressed the proapoptotic activity of the serum of CHF on endothelial cells (Hornig et al., 1998; Rossig et al., 2001). Vit C treatment also reduced neutrophil superoxide generation in heart failure patients (Ellis et al., 2000). Coenzyme Q 10, another non enzymatic antioxidant, significantly improved hemodynamic function in NYHA functional class III/IV heart failure patients (Sacher et al., 1997). Increased plasma levels of Vit E were associated with decreased risk of heart

diseases (Gey et al., 1991).

Although some of these studies showed improvement in cardiac function with antioxidant therapy (Stephens et al., 1996; Keith et al., 2001) other studies failed to show any benefit of these antioxidant treatments to prevent cardiovascular disease (Miller et al., 2005; Lonn et al., 2005; Yusuf et al., 2000). The failure of antioxidant therapy in clinical settings could be due to a number of factors such as timing, bioavailability of the antioxidant at the tissue level, pharmacological properties of synthetic antioxidants and the difference in the oxidative stress status of patients and the effects of number of uncontrolled factors such as diet and exercise on oxidative stress.

3.4 Oxidative stress and Cytokines

N-acetylcysteine (NAC) and GSH treatment in tumor bearing mice significantly decreased tumor necrosis and host survival after TNF-α treatment (Obrador et al., 1997). In tumor bearing mice treated with recombinant human TNF-α (rhTNF-α), it was found that mitochondrial GSSG content rises as compared to control (Obrador et al., 1997). Tumor cells, most sensitive to TNF-α mediated cytotoxicity appear to be the ones with limited capacity for ROS scavenging (Zimmerman et al., 1989). rhTNF-α induced cell death is much higher when GSH content was lower (Estrela et al., 1995). Recombinant mouse TNF-α (rmTNF-α) induced cytotoxicity in L929 cells can be partially prevented by antioxidants, iron chelators or inhibition of the mitochondrial electron transport complex I and II (Schulze-Osthoff et al., 1992). Another pathway, whereby TNF-α induces hypertrophy in neonatal rat cardiac myocytes, via the activation of ROS has also

been suggested (Nakamura et al., 1998). Exogenous TNF-α, increased ROS production within neonatal cardiac myocytes after 1 hr as measured by DCFDA (Suematsu et al., 2003). Pretreatment of endothelial cells with the TNF-α reduced the intracellular concentration of GSH and increased the concentration of GSSG (Ishii et al., 1992 Marcho et al., 1991). Similarly antioxidants such as butylated hydroxyanisole and catalase treatment inhibited TNF-α induced cardiac hypertrophy in cultured cardiac myocytes (Nakamura K et al., 1998). Taken together these studies suggest that TNF-α increases oxidative stress in number of cells including cardiac cells.

On the other hand IL-10 has emerged as an anti-inflammatory cytokine that inhibits the secretion of pro-inflammatory cytokines by monocytes and/or macrophages along with a lowering of ROS production (Pezzilli et al., 1997). rhIL-10 was able to block the release of ROS (Bogdan et al., 1991) and antagonized pro-inflammatory cytokine induced oxidative burst (Gougerot-Podicalo et al., 1996). IL-10 has been shown to inhibit PMN-dependent phagocytosis and killing of the E. coli *in vitro* by inhibiting the production of superoxide (Laichalk et al., 1996). IL-10 pretreatment in lipopolysachride (LPS) treated cells inhibited both NF-κB activation and IκB degradation and both of these processes were also inhibited by catalase (Dokka et al., 2001). ESR studies further confirm the formation of OH° in LPS-treated cells. Addition of IL-10 in these cells inhibited the IκB degradation and generation of OH° in response to LPS (Kelly et al., 2001). It is suggested that IL-10 along with effecting the production of inflammatory cytokines also inhibits cytokine-induced oxidative stress.

In a recent study, using human fibroblasts, decreased expression of NADPH

oxidase 4 (NOX4) was associated with decreased phosphorylation of smad2/smad 3 in absence of TGF-β (Cucoranu et al., 2005). Phosphorylation of these two proteins is significantly involved in TGF-β induced hypertrophy. Furthermore, it was suggested that increased generation of ROS can have direct effect on the cytokine signaling pathway in the absence of ligands. Similar to this report, H_2O_2 has been shown to be involved in direct activation of MAPK pathway and increased NF-κB binding. Both these signaling pathways are integeral part of proinflamatory cytokine signaling thus highlighting that overexpressed ROS can have direct deleterious effects in the absence of cytokine proteins (Abe et al., 2000; Schmidt et al., 1995)

Since inhibition of rennin-angiotensin system (RAS) has been shown to reduce mortality and improve prognosis both in MI animals and humans. Correlation of TNF-α and IL-10 with heart function was further tested by examining these cytokines (mRNA and protein) in losartan treated 4 weeks post MI rats with respect to heart function

4.0 RENIN ANGIOTENSIN SYSTEM

RAS is an important hormonal system in the cardio renal homeostasis. The pathophysiological role of RAS has been extensively studied in CHF and myocardial ischemia. Elevated plasma and cardiac levels of different components of RAS are observed in cardiac patients and in different animal models of heart failure (Michorowski and Ceremuzynski, 1983, Hebert et al., 2003; Pagliaro and Penna 2005). Cardiac cells are under the influence of both circulating and local cardiac production of angiotensin I (Ang I) and angiotensin II (Ang II) (Danser et al., 1994; Neri Serneri et al., 1996).

Although angiotensin converting enzyme (ACE) is mainly responsible for the

conversion of Ang I to Ang II, there are some other pathways responsible for the production of Ang II (Fig A). These alternative pathways include cathepsin G, which converts angiotensinogen directly to Ang II (Tonnesen et al., 1982). In 1990, Urata and group discovered that in a human heart, a specific chymase was responsible for the conversion Ang I to Ang II (Urata et al., 1990; Kokkonen et al., 1998). In recent years, the discovery of another enzyme, angiotensin-converting enzyme-related carboxypeptidase (ACE2), involved in the metabolism of angiotensin has complicated this picture even more (Donoghue et al., 2000). This newly discovered enzyme, ACE2, has been associated with further hydrolysis of Ang II into Ang(1–7) (Danilczyk and Penninger 2006).

Among various components of RAS, Ang II is responsible for most of cardiovascular and renal effects of this system. There are 4 major angiotensin receptors identified: AT_1, AT_2, AT_3 and AT_4. First two of these, i.e. AT_1 and AT_2 have been isolated and cloned and have high affinity for Ang II. AT_1 receptors are further subdivided into AT_{1A} and AT_{1B} (Humphrey and Barnard, 1998). AT_3 has been shown to be activated by Ang III (Ang 2-8) and AT_4 can be activated Ang IV (Ang 3-8) but both these receptors have not been cloned yet (de Gasparo et al., 2000).

AT receptors belong to the family of seven transmembrane domain, G protein-coupled receptor (GPCR). AT_1 mediated signaling is responsible for most of the effects of Ang II (Timmermans PB et al., 1993). Ang II involved vasoconstriction is linked to increased calcium through PLC and PLD activation and opening of calcium channels and

negatively regulated adenyl cyclase through G protein mediated mechanisms (de Gasparo et al., 2000; Inagami T et al., 1994). Ang II induced hypertrophy, has been linked to the activation of tyrosin kinase, FGF, PDGF, MAPK and even to the activation of JAK/STAT pathway (Mascareno and Siddiqui 2000; Nakashima et al., 2006). Hypertrophic effects of Ang II are unrelated to its hypertensive effects because Ang II induced fibrosis by infusion or transgenic expression is not effected by blood pressure changes. Mice over expressing AT_1developed hypertrophy in the absence of hypertension or tachycardia (Mervaaia E, et al., 2000; Paradis et al., 2000).

AT_2 receptors on the other hand are believed to have opposite effects to AT_1 activation i.e. activation of AT_2 can cause vasodilatation and antigrowth and anti hypertrophic effects (Munzenmaier and Greene 1996; Hein et al., 1995; Tea et al., 2000).

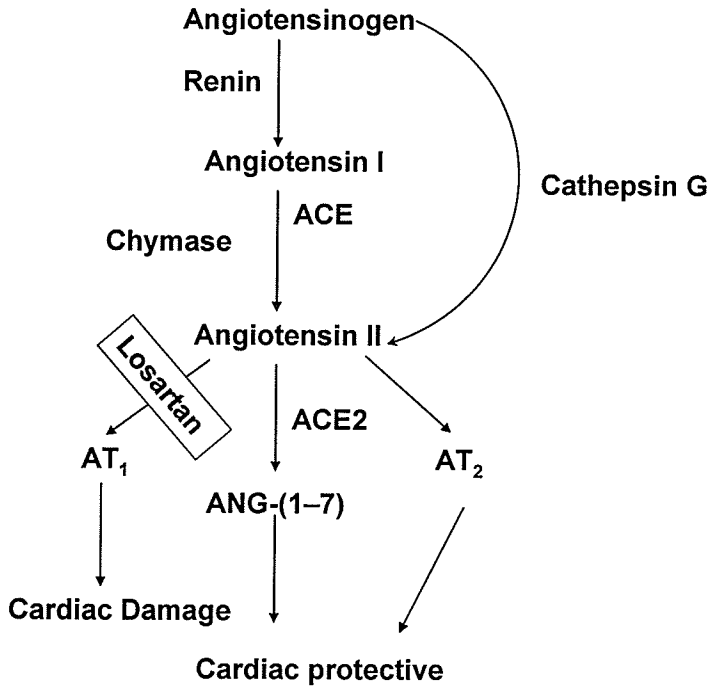


Figure A: Formation of Angiotensin II and its cardiac effects.

Though clinical use of ACE inhibitors have been shown to improve prognosis in hypertensive and heart failure patients (Demers et al., 2005; Staffileno 2005; Scow et al., 2003). Because of the effects of ACE on bradykinin metabolism and existence of other pathways for angiotensin production, Angiotensin Receptor Blockers (ARB), offer a more selective and specific inhibition of Ang II mediated cardiac effects.

Losartan, an AT_1 antagonist, is the first ARB developed and is in regular clinical use as an antihypertensive agent (Johnston, 1995). Losartan has been documented to have multiple beneficial effects in cardiac system both in animals and humans (Dickstein 2001; Eisenberg and Gioia 2006). In clinical studies losartan was better tolerated then captopril, an inhibitor of ACE (Pitt 1997). Contrary to an editorial which suggested that blockers of AT_1 can lead to increased infarction through overactivation of AT_2, which are thought to have antigrowth and vasodilator effects (Verma and Strauss 2004), a systematic analysis of 19 clinical trials with 31569 patients suggested that treatment with ARB is not associated with increased risk of myocardial infarction (McDonald et al., 2005).

The benefits of losartan over ACE inhibitors include complete blockade of Ang II effects regardless of the pathway by which Ang II is formed (Timmermans and Smith, 1994). Lack of any effect on bradykinin metabolism reduced cough symptoms and hypotension (Israili and Hall 1992). Another beneficial effect of losartan over ACE inhibitors could be through more generation of Ang (1-7) from Ang II by ACE2 mediated pathway. In this regard, losartan treated rats increased production of Ang(1–7) (Ferrario

et al., 2005). Similar findings were observed in the aorta of spontaneously hypertensive rats medicated with olmesartan (Igase et al., 2005). Ang (1–7) has been shown to have protective effects on cardiovascular and renal system (Burrell et al., 2004). Ang (1-7) overexpression was also protective against isoproterenol induced hypertrophy and reperfusion damage (Santos et al., 2004). All these data suggest that losartan is a better choice for cardiac function improvement.

In this comprehensive review of the topics related to my thesis, several gaps in our knowledge with respect to inflammation and anti-inflammatory cytokines and heart failure have been identified. In my study I have focused on the changes in TNF-α and IL-10 with respect to cardiac function in heart failure rats. Furthermore the interplay of these two cytokines was also investigated in regards to oxidative stress in cardiac myocytes.

IV. HYPOTHESIS

Increased TNF-α and decreased IL-10 contributes to the progression of congestive heart failure (CHF) post MI. Thus the ratio of IL-10/TNF-α is of greater importance in the pathogenesis of CHF subsequent to MI. It is further hypothesized that the interaction between these two cytokines takes place at the level of oxidative stress.

V. APPROACH AND OBJECTIVES

MI will be surgically induced in rats by ligating the coronary artery. Clinically, echocardiographically and hemodynamically assessed animals after 1 (non failure), 4 (mild failure), 8 (moderate failure), and 16 (severe failure) weeks of myocardial infarction will be used to achieve the following specific aims:

- Analyze TNF-α and IL-10 proteins and mRNA levels at different stages of heart failure.
- Determine whether the balance between IL-10 and TNF-α is perturbed with progression of heart failure.
- Progression of heart failure will be modulated by inhibition of RAS with losartan. Correlation of TNF-α and IL-10 with heart function will be further tested by examining these cytokines in losartan treated, 4 weeks post MI rats.

In order to further study the cause and effect relationship, effects of exogenous TNF-α and IL-10 on oxidative stress will be studied in isolated adult rat cardiac myocytes to achieve the following specific

OBJECTIVES

- Examine the effect of TNF-α and IL-10 on isolated cardiac myocytes in relation to changes in ROS generation, lipid peroxidation and antioxidant enzymes to examine oxidative stress.
- To test how the two cytokines modulate cardiac myocyte injury.
- Determine the effect of different combinations of IL-10 and TNF-α treatment in regards to oxidative stress and cell injury.

VI. METHODS

In the present study, I had used two-prong approach to study the significance of changes in cytokines during the progression of heart failure: A) an *In vivo* study of TNF-α and IL-10 in heart failure and B) an *in vitro* study of the effects of TNF-α and IL-10 on oxidative stress and cell injury in isolated cardiac myocytes.

1.0 *In Vivo* Studies

1.1 Animal Model

All experimental protocols including the animal studies were approved by the University of Manitoba Animal Care Committee, following the guidelines established by the Canadian Council on Animal Care. Male Sprague-Dawley rats weighing 150±10 g were maintained on standard rat chow and water *ad libitum*. Myocardial infarction (MI) was produced by occlusion of the left anterior descending coronary artery as reported earlier (Khaper and Singal, 2001). The animals were anesthetized with 2% isoflurane and the skin was then incised along the left sternal border. The third and fourth ribs were cut just to the left side of the sternum with the subsequent insertion of retractor. The pericardial sac was perforated and the heart was exteriorized through the longitudinal space. The left coronary artery was ligated with a 6-0 silk thread. After ligation, the heart was gently placed back in the chest. Excess air was drawn using a syringe after which the chest was closed with a purse string suture. The rats were maintained on positive pressure ventilation, delivering 2% isofurane. The entire surgical procedure was carried out in sterile conditions. Sham control animals were similarly handled, except that the suture around the coronary artery was not tied, and the silk thread was passed

only through the muscle as described previously (Khaper and Singal, 2001). Following the surgery, the animals were allowed to recover in post-surgery recovery room. These animals were monitored on a regular basis for their food intake, water intake, body weight, general behavior and mortality.

1.2 Study Groups

Sham and Post-MI animals were divided into ten groups as follows:

- 1-week sham control (without coronary ligation and without drug) (n=6)
- 1-week PMI (with coronary ligation but no drug treatment) (n=6)
- 4-week sham control (without coronary ligation and without drug) (n=6)
- 4-Week PMI (with coronary ligation and without drug) (n=6)
- 8-week sham control (without coronary ligation and without drug) (n=6)
- 8week PMI (with coronary ligation but no drug treatment) (n=6)
- 16-week sham control (without coronary ligation and without drug) (n=6)
- 16-week PMI (with coronary ligation but no drug treatment) (n=6)
- 4-week sham + losartan (without coronary ligation and losartan was given) (n=6)
- 4-week PMI + losartan (coronary-ligated group with the losartan treatment) (n=6)

The treatment with losartan (2 mg/ml, in drinking water, daily) was started on the day of surgery and was continued for 4 weeks. Similar dosage of losartan has been used in other studies (Milavetz et al. 1996). Daily average water consumption in drug treated control and experimental animals was about 30 ml.

1.3 Function assessment

For the study of heart function, two different methods were adopted *vis a vis* echocardiography and hemodynamic function as follows:

1.3.1 Echocardiography

Transthoracic echocardiography was performed on all animals at the end of study period. Animals were sedated with 2% isoflurane. Imaging of the heart was done using a 12 MHz (s12) ultrasound probe and a Sonos 5500 echographer (Agilent Technologies, Andover, MA). For M-mode recordings, the parasternal short-axis view was used to image the heart in 2D at the level of the papillary muscles. Left ventricular fractional shortening (FS) and ejection fraction (EF) were analyzed using an analysis program on the ultrasound machine.

1.3.2 Hemodynamic Measurements:

Rats were anesthetized with an intraperitoneal injection (i.p) of ketamine (90 mg/kg) and xylazine (10 mg/kg). A miniature pressure transducer catheter (Millar Micro Tip, model PR 249, Houston, TX) was inserted into the right carotid artery and then advanced into the left ventricle. The catheter was secured with a silk ligature around the artery. Using specific software (Acknowledge for Windows,Biopac Systems Inc, Goleta, CA), left ventricular end diastolic pressure (LVEDP), left ventricular peak systolic (LVPSP), the maximum rate of isovolumic pressure development pressure (+dP/dt) and the maximum rate of isovolumic pressure decay (-dP/dt) were recorded (Khaper and Singal, 2001). After these assessments, the rats were sacrificed and the hearts were

removed and processed according to the protocols described.

1.4 Estimation of Myocardial TNF-α and IL-10 proteins

After functional assessments, hearts were removed and washed with phosphate buffer saline (PBS). Viable ventricular tissue was flash frozen in liquid nitrogen immediately after washing. Frozen tissue (0.5-1.0 g) was homogenized in PBS buffer containing protease inhibitors (PMSF 1.49 mmol/L, leupeptin 0.48mmol/L and aprotinin 0.31μmol/L) for ~ 45 seconds with a PT-3000 homogenizer (Brinkmann Instruments, Inc.). Myocardial homogenate was centrifuged at 20,000g for 25 minutes at 4°C. The supernatant containing soluble fraction of cytokines was stored at -70°C until further analysis. The cell pellet, containing membrane fraction, was solublized in PBS with protease inhibitors and 1% w/v Triton X-100 by incubating at 4°C for one hour. The solublized fraction was centrifuged at 20,000g at 4°C for 20 minutes to collect supernatant which contained the membrane bound fraction of TNF-α and IL-10 (Torre-Amione, etal., 1996). Quantitative expression for membrane bound and cytosolic fraction of TNF-α and IL-10 protein was detected by the Enzyme-linked immunosorbent assay (ELISA) method (Jiang etal., 2002) using a commercially available kit (R&D systems). Briefly, a 50μl (50-80μg protein) sample of the protein was added to the assay buffer with appropriate standard and the assays were performed in duplicate according to the manufacturer's protocol. Spectrophotometeric readings were taken at a wavelength of 490 nm with a micro titer plate reader. Final results were expressed as picograms of cytokine per milligram of protein from cytosolic or membrane bound fraction.

1.5 Estimation of Myocardial TNF-α and IL-10 mRNA

RNA was isolated from the non-infarcted myocardial tissue using TRI REAGENT (Molecular Research Centre Inc, Cincinnati, Ohio). Approximately 50 mg of tissue sample was homogenized in 1ml of TRI Reagent and was stored at room temperature. After 5 minutes, 0.2 ml chloroform was added and samples were shaken vigorously for 15 seconds. The resulting mixture was kept at room temperature for 10 minutes and centrifuged at 12,000g for 15 minutes at 4°C. Following centrifugation, the mixture separated into a lower red phenol-chloroform phase, interphase and the colorless upper aqueous phase. Aqueous phase was transferred to a fresh tube. RNA was precipitated from the aqueous phase by mixing it with 0.5 ml of isopropanol. After 10 minutes at room temperature, aqueous phase with isopropanol was centrifuge at 12,000g for 8 minutes at 4°C. RNA precipitate formed a gel-like white pellet on the side or bottom of the tube. RNA pellet was washed with 75% ethanol and subsequently centrifuged at 7,500g for 5 minutes at 4°C. RNA was dissolved in nuclease free water, containing a trace amount of EDTA. The resulting mRNA was then treated with DNase at 37°C for 30 minutes to remove residual DNA. DNase in the reaction mixture was then treated with an inactivating agent for 2 minutes. After the isolation of total mRNA, its purity was assessed spectrophotometrically using the ratio of the absorbance at 260 and 280 nm.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) was performed on total mRNA (4μg) which was subjected to first strand synthesis of cDNA using a commercially available kit (Ambion). Briefly, 20μl of reaction mixture containing 2μl of RT buffer, 4μl of dNTP mix, 1μl of RNase inhibitor and 1μl of Reverse Transcriptase

was incubated at 44°C for one hour and then at 92°C for 10 minutes. The reaction mixture was stored at -20°C until ready to proceed to the PCR step.

The cDNA (5µl) was subjected to PCR, with β Actin as an internal standard, The PCR mixture contained 10 µl of 10 X PCR buffer, 2 µl of 25 mM $MgCl_2$, 2 µl of 10mM dNTP mix, 2.5U of thermostable DNA polymerase and 100 pM of each sense and antisense primers (Table 1). Each sample was subjected to 30 seconds denaturation at 94°C followed by 35 cycles of 60 seconds at 94°C followed by annealing at 55°C for 60 seconds and then for 120 seconds at 72°C. The final extension was done at 72°C for 10 minutes. The final product of PCR was stored at 4°C and 10 µl of each PCR product was analyzed on 2% agrose gel by electrophoresis using Vistra Green (Amersham) to visualize that cDNA bands. The intensity of each band was photographed and quantified using a Molecular Dynamics STORM scanning system (Amersham Biosciences Corp., PQ, Canada).

Table 1: Primers for TNF-α and IL-10

Gene	Oligonucleotides Sequences for Primers	Product (bp)
β Actin	5'-TGG AGA AGA GCT ATG AGC TGC-3' Sense 5'-TCC ACA CAG AGT ACT TGC GC-3' AntiScense	315
IL-10	5'-GCT CAG CAC TGC TAT GTT GC-3' Sense 5'-TTC ATG GCC TTG TAG ACA CC-3' AntiScense	469
TNF-α	5'-AGT CTT CCA GCT GGA GAA GG-3' Sense 5'-GCC ACT ACT TCA GCA TCT CG-3' AntiScense	318

2.0 *In Vitro* Studies

2.1 Adult Cardiac Myocytes Isolation

Ventricular myocytes were isolated from the normal adult male Sprague Dawley rats using a procedure described earlier (Piper etal 1982). Briefly, rats (250-300g) were injected with sodium heparin, after one-hour thoracotomy was performed, and hearts were cannulated *in situ*, perfused and rapidly excised. After the excision, hearts were mounted on a modified Langendorff perfusion apparatus, which allowed switching between a single pass and recirculating perfusion at a temperature of 37^0C. The perfusate consisted of calcium free 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). The perfusion was then switched to recirculating mode with the same buffer (modified Krebs buffer) that now contained 25 μM calcium, 0.1% w/v collagenase and 0.25% w/v bovine serum albumin for 20 minutes.

The hearts were removed, cut into small pieces and disaggregated in same buffer. Desegregation was achieved by gentle passing of the suspension through pipettes with progressively smaller tip diameters. The suspension was filtered using a nylon mesh (200μm) and re-suspended in Medium 199 containing $CaCl_2$ (1.8mM). After sedimentation (10 minutes), the cells were re-suspended in a serum-free Medium 199 (Sigma-Aldrich, Oakville, Ontario, Canada). Myocytes ($1x10^5$ per dish) were plated on 4% serum-coated polystyrene tissue culture dishes and incubated in serum-free culture medium 199 supplemented with antibiotics (streptomycin/penicillin 100 μg/ml) at 37^0 C under a 5% CO_2-95 % air atmosphere. After one to three hours of plating, the culture

medium was changed to remove unattached dead cells and the viable myocytes were incubated overnight.

2.2 Cell Treatment

After the initial incubation period of 24 hrs quiescent cardiac myocytes were treated for 4 hours as follows:

Table 2: Cardiac myocytes treatment groups

Group	The culture medium M199 was supplemented with *
C	Control, cells in the culture medium only
H	H_2O_2, 100μM
TNF-α	Tumor Necrosis Factor-α, 1, 10, 20 ng/ml
IL-10	Interleukin-10, 1, 10, 20 ng/ml
IL-10/TNF-α	IL-10/TNF-α in the ratio of 0.1, 1.0, and 2.0 TNF-α (10ng/ml) + IL-10 (1, 10 or 20ng/ml)
LPS	Lipopolysaccharide 10 μg/ml

*** In each group, the treatment duration was 4 hours**

2.3 TNF-α, IL-10 protein and mRNA in adult myocytes

2.3.1 TNF-α and IL-10 protein

ELISA estimations were done on 50 μl of cell free culture medium from LPS and control treated cardiac myocytes using a commercially available kit (R&D systems). Final results were expressed as pg of cytokine/ml of supernatant.

2.3.2 Estimation of TNF-α and IL-10 mRNA

RNA was isolated from LPS and control treated cardiac myocytes using mRNA isolation kit (from Ambion). Briefly, myocyte pallet was lysed in solution containing guanidinium thiocyanate. The lysate was then applied to a silica based filter, which binds mRNA. Following washing of the filter, the mRNA was eluted in nuclease free water containing a trace amount of EDTA. The mRNA was then treated with DNase at 37°C for 30 minutes to remove residual DNA. Then DNase was treated with an inactivating agent for 2 minutes. After the isolation of total mRNA, the purity was assessed spectrophotometrically using the ratio of the absorbance at 260 and 280 nm. RT-PCR was performed as described earlier for animal tissue samples.

2.4 Antioxidant enzymes

2.4.1 Estimation of Antioxidant enzyme Protein

Control, H_2O_2, TNF-α, IL-10 and cytokine combination treated isolated cardiac myocytes in different treatment groups (Table 2) were washed with PBS. Cells were then lifted from the dish bottom using a cell scraper and then suspended in PBS and centrifuged at 1000 rpm for 10 minutes. Supernatant was discarded and the pallet was re-suspended in PBS containing Protease inhibitor cocktail for mammalian tissues (Sigma-Aldrich, AEBSF, 100; Aprotinin, 0.08; Leupeptin, 2.2; Bestatin, 4.0; Pepstatin A, 1.5 and E-64, 144 in mM). The lysate was homogenized using a Polytron homogenizer and protein fraction was stored at –75°C.

The protein samples (20μg) were then subjected to one-dimensional sodium

dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in a discontinuous system (Laemmli 1970). The 5% gel was used for protein stacking phase while 15 % gel was used for the separation analysis of isolated proteins. The separated proteins were electrophoretically transferred to PVDF membrane using a transfer buffer, which consisted of 20mM Tris, 150 mM glycine, 20% methanol and 0.02% SDS. The nonspecific binding sites were blocked by overnight incubation at 4°C with 5% nonfat milk in TBST (Tris-buffered saline containing 0.1% Tween 20).

The PVDF membranes were processed for immunodetection using rabbit anti-rat GSHPx antibody, rabbit anti-bovine Cu/Zn-SOD antibody, rabbit anti-human MnSOD antibodies and rabbit anti-bovine catalase antibody (United State Biologicals, Swampscott, Massachusetts). Primary antibodies were detected using a goat anti-rabbit IgG horseradish peroxidase conjugate secondary antibody (Bio-Rad, Hercules, CA, USA). Molecular weights of the separated proteins were determined using a standard (Bio-Rad, Hercules, CA, USA) and biotinlated (Cell Signaling Technology inc., Beverly, MA, USA) protein ladder molecular weight markers. Detection of membrane-bound proteins was performed using the BM Chemiluminiscence (POD) Western blotting system (Roche Diagnostics GmbH, Manheim, Germany). The bands were visualized with Flour S-MultiImager MAX system (Bio-Rad, Hercules, CA, USA) and quantified by image analysis software (Quantity One, Bio-Rad, Hercules, CA, USA).

2.4.2 Estimation of antioxidant enzymes mRNA

RNA was isolated from Control, H_2O_2, TNF-α, IL-10 and cytokine combination

treated cardiac myocytes (Table 2) mentioned earlier using RNA isolation kit (Ambion). This isolated RNA was subjected to cDNA synthesis as was done for heart tissue. The primer's sequences for enzymes were obtained from Agardh etal 2002 and were ordered from Invitrogen.

The cDNA (5µl) was subjected to PCR, with β Actin as an internal standard. The PCR mixture contained 10µl of 10X PCR buffer, 2µl of 25mM $MgCl_2$, 2µl of 10mM dNTP mix, 2.5U of thermostable DNA polymerase and 100 pM of each sense and antisence primers (Table 3). Each sample was subjected to 30 seconds denaturation at 94°C followed by 35 cycles of 45 seconds at 94°C followed by annealing at 62°C for 30 second and then for 60 seconds at 72°C. The final extension was done at 70°C for 5 minutes. The final product of PCR was stored at 4°C until further analysis. The PCR products were analyzed by electrophoresis in 2% agarose gels using Vistra Green (Amersham) to visualize that cDNA bands. The intensity of each band was photographed and quantified using a Molecular Dynamics STORM scanning system(Amersham Biosciences Corp., PQ, Canada).

Table 3: Primers for Antioxidant enzyme

Gene	Oligonucleotide sequences of Primers	Product (bp)
Cu/Zn-SOD	5'-CAGGATTAACTGAAGGCGAGCATG-3' sense 5'-CAATCACACCACAAGCCAAGCGGC-3' Antisense	342
MnSOD	5'-CGTCACCGAGGAGAAGTACCACGA-3' sense 5'-CAGCCTGAACCTTGGACTCCCACA-3' Antisense	252
Catalase	5'-CACTCACCGCCACCGCCTGGGACC-3' sense 5'-TTTTCCCTTGGCAGCTATGTGAGA-3' Antisense	484
GSH-Px	5'-GTTCGGACATCAGGAGAATGGCAA-3' sense 5'-GGTTGCTAGGCTGCTTGGACAGCA-3' Antisense	368
β Actin	5'-TGG AGA AGA GCT ATG AGC TGC-3' sense 5'-TCC ACA CAG AGT ACT TGC GC-3' Antisense	315

2.5 Glutathione Peroxidase activity

Myocytes pellet was homogenized in 500µl of 75 mM phosphate buffer (pH 7.0). Homogenate was centrifuged at 20,000g for 30 minutes and the supernatant containing total cytosolic GSHPx was collected. GSHPx activity was measured in a 1ml cuvette containing 800 µl of 75mmol/L phosphate buffer, pH 7.0, 17 µl of 60 mmol/L glutathione, 33 µl glutathione reductase solution (30 U/ml), 17 µl of 0.12 mol/l NaN_3, 33 µl of 15 mmol/L Na_2 EDTA, 33.3 µl of 3.0 mmol/L NADPH, and 33.3 µl of cytosolic fraction. At the end, 33.3 µl of 7.5 mmol/L H_2O_2 was added to start the conversion of reduced nicotinamide adenine dinucleotide phosphate (NADPH) to oxidized nicotinamide adenine dinucleotide phosphate (NADH). GSHPx activity was monitored by a continuous recording of the change of absorance at 340 nm at one-minute intervals

for 5 minutes. GSHPx activity was expressed as nanomoles of reduced NADPH oxidized to NADP per minute per milligram protein, using a molar extinction coefficient for NADPH at 350 nm of 6.22 X 10^6 (Paglia and Valentine, 1967).

2.6 Redox ratio (GSH/GSSG)

To measure the redox ratio in the myocytes Glutathione reductase/DTNB (5,5' – dithiobis-[2-nitrobenzoic acid]) recycling assay was used to measure concentrations of total glutathione (GSSG + GSH) and oxidized glutathione (GSSG) as descried early (Anderson, 1985). Myocyte cell pallet was homogenized in 5% sulfosalicylic acid to prevent oxidation of GSH to GSSG during sample preparation. The homogenate was centrifuged for 10 minutes at 10000*g*. Supernatant was stored at 4°C until assayed. Total glutathione was measured by the rate of TNB formation at 412 nm. For GSSG assay sulfosalicylic acid supernatant was treatment with 2-vinylpyridine and triethanolamine. The solution was vigorously mixed, and the final pH of the solution was adjusted to between 6 and 7. After 60 minutes, the derivatized samples were assayed as described above in the DTNB–GSSG reductase-recycling assay. GSH values were calculated as the difference between total (GSSG+GSH) and GSSG concentrations. Values are reported as a ratio of GSH over GSSG equivalents.

2.7 Lipid hydroperoxides

This assay was done with a commercially available kit (LPO-CC assay, Kamiya Biomed Co., Seattle, WA, USA). Control, H_2O_2, TNF-α, IL-10 and cytokine combination treated isolated cardiac myocytes were washed with PBS. Myocytes were homogenized

in double distilled water and homogenate was suspended in chloroform:methanol which was then clarified with 0.6 ml of 0.9% saline. After centrifugation at 30, 000 g for 5 minutes, 1 ml of the bottom chloroform layer was completely dried under vacuum in a rotary evaporator and reconstituted in 100µl of isopropanol. An aliquot of (45 µl) this solution was used in this assay. The reaction produces methylene blue, which was quantified by spectrophotometry at 675 nm (Khaper and Singal 2001).

2.8 Endogenous production of reactive oxygen species (ROS)

Oxidative stress was quantified using a previously described method (Swift and Sarvazyan 2000). The endogenous production of ROS was quantified utilizing a 5-(6)-chloromethyl-2'7'-dihydroflourescein diacetate probe (CM-H2 DCFDA) (Molecular Probes, Eugene, Oregon, USA). Control, H_2O_2, TNF-α, IL-10 and cytokine combination treated isolated cardiac myocytes were washed with PBS. Cell loading with fluorescent probes was achieved by incubating the cells with 10 µM solution of CM-H2 DCFDA in PBS for 30 minutes. Incubation was performed in a humidified chamber, protected from light, at 37°C. Fluorescent images were taken using the Olympus BX 51 fluorescent microscope using U-MNIBA2 mirror unit. Fluorescence intensity was measured using a digital imaging processing software (Image Pro Plus).

2.9 Myocardial injury by cytokine treatment

Cellular damage was evaluated by measurement of the release of creatine kinase (CK) and lactate dehydrogenase (LDH) in the culture medium after H_2O_2, TNF-α, IL-10 and cytokine combination treatment. A spectrophotometric enzyme activity assay was

performed for LDH with a UV-Rate assay kit (Stanbio Laboratory, Boerne, TX). LDH specially catalyzes the oxidation of lactate to pyruvate with the subsequent reduction of NAD to NADH. The rate at which NADH forms is proportional to LDH activity. At the end of the experiments, NADH absorbance increase per minute at 340 nm (ΔA/min) of the supernatant was determined from each experimental group. Similarly, a spectrophotometric enzyme activity assay was performed for CK with a UV-Rate assay kit (Stanbio Laboratory, Boerne, TX). CK specifically catalyzes the transphosphorylation of ADP to ATP. Through a series of coupled enzymatic reactions, NADH is produced at a rate directly proportional to the CK activity. The method determines NADH absorbance increase per minute at 340 nm

3.0 Statistics and Protein analysis

Data is expressed as the mean +/-SEM. For a statistical analysis of the data, groups were compared by one-way ANOVA and Bonferroni's test was used to identify differences between groups. Value of $p<0.05$ was considered significant. Total protein concentration was determined using Bradford method (Bradford 1976) using bovine serum albumin as a standard.

VII. RESULTS

1.0 *In Vivo* Studies

1.1 General

Sham control and post-myocardial infarction (PMI) rats were monitored throughout the 16-week post surgery duration. Mortality in the coronary artery-ligated animals during or immediately after the surgery was about 20%. Another 13% of the animals died within 24 hrs following the surgery. There was a steady increase in the body weight of animals ranging from 210.0 ± 42.30 g at 1W PMI to 658.7 ± 19.7 g at 16W PMI. There was no significant difference in the body-weight gain between the PMI and their respective sham control groups (Fig 1). Animals in the 16W PMI group appeared lethargic, with clear signs of heart failure indicated by dyspnea and cyanosis of the peripheral extremities.

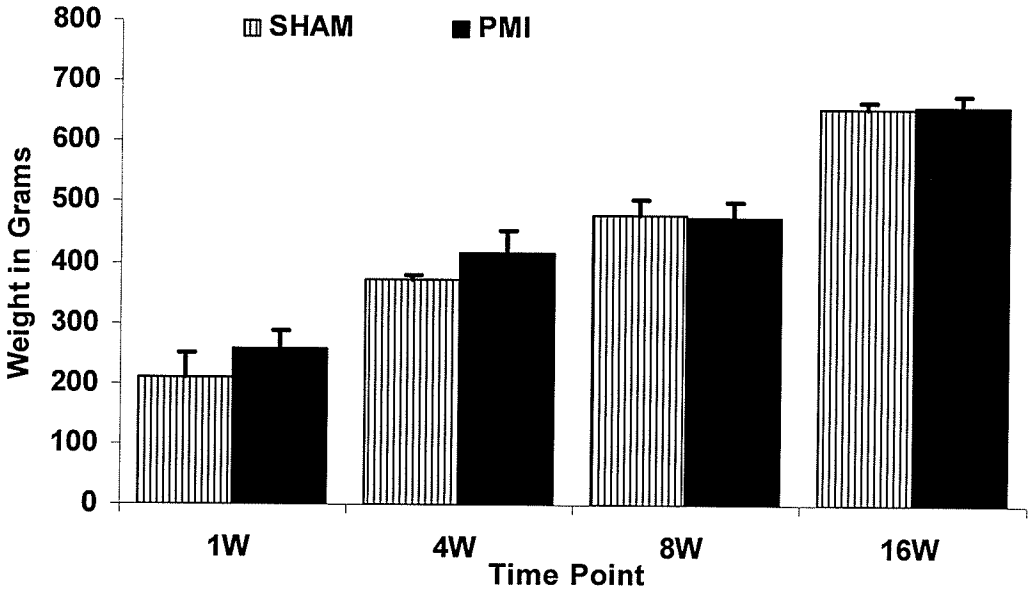


Fig 1: Body weight of sham and PMI animals after 1, 4, 8 and 16 weeks of coronay ligation. PMI (post-myocardial infarction), Coronary ligation rats; Sham, Sham operated rats. Data are presentated as mean ± SEM of 4 animals.

1.2 Function Assessment:

1.2.1 Echocardiographic studies

Echocardiographic data revealed differences between sham and respective PMI groups as early as 4 weeks after the coronary ligation (Figs 2 and 7). Fractional shortening (FS) in sham group at one week post surgery was 39.5% ± 2.2 and ejection fraction (EF) was 81.03% ± 6.4 (Tables 4-6; Figs 2 and 7). These values did not change with age in any of the sham groups (Tables 4).

In the PMI groups, both FS and EF decreased significantly at all time points starting with 4W PMI group (Table 5 and 6; Fig 2 and 7).

1.2.2 Hemodynamic studies

Hemodynamic data on the Left ventricular peak systolic pressure (LVPSP); Left ventricular end-diastolic pressure(LVEDP); maximal rate of increase of LV systolic pressure (+dP/dt); maximal rate of decline of LV systolic pressure (-dP/dt) in sham control and PMI groups are given in Tables 4-6. There were no significant differences in these values among different sham group animals.

The LVPSP was significantly decreased at 4, 8 and 16 week in PMI groups. The LVEDP was significantly increased at 1, 4, 8 and 16 week in the PMI groups. Both +dP/dt and -dP/dt were significantly depressed at all time points after 4 weeks in all the PMI groups (Tables 4-6).

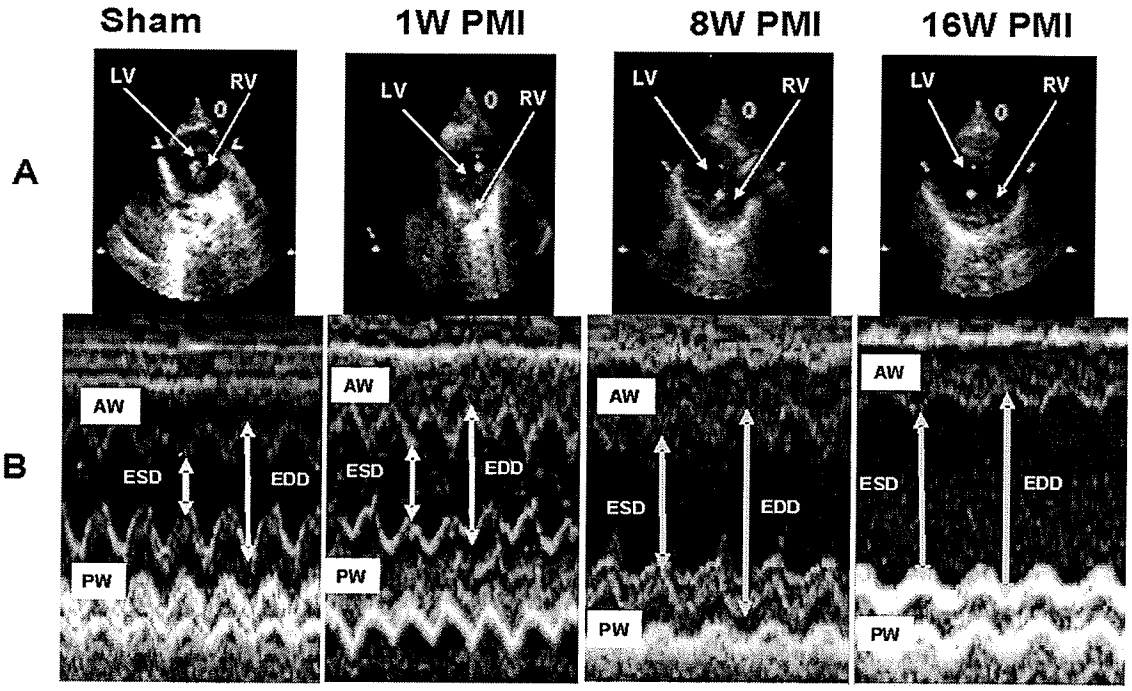


Fig 2 (A and B): Echocardiography images of rat heart at various time points after myocardial infarction. A, 2-D echocardiography at parasternal Short-Axis Plane; B, Representative M-mode Short-Axis of the left ventricle; AW, anterior wall; PW, posterior wall; ESD, end systolic dimensions; EDD, end diastolic dimensions. 1W PMI, one week post myocardial infarction; 8W PMI, eight week post myocardial infarction; 16W PMI, sixteen week post myocardial infarction; LV, left ventricular and RV, right ventricular.

Table 4: Cardiac function in Sham Control Rats

	1W	4W	8 W	16W
Weight	210 ± 42.3	370.5 ± 8.4	478.8 ± 25.3	656.43 ± 12.6
EF [%]	81.0 ± 6.4	73.1 ± 3.3	70.7 ± 4.6	79.3 ± 5.5
FS [%]	45.3 ± 2.4	39.5 ± 2.2	39.8 ± 1.2	43.8 ± 3.5
LVPSP [mmHg]	109.8 ± 5.3	117.9 ± 4.2	104.1 ± 6.9	109.4 ± 7.3
LVEDP [mm Hg]	2.99 ± 0.57	3.33 ± 0.49	3.88 ± 1.14	3.94 ± 1.14
+dP/dt [mmHg/s]	9898 ± 500	9494 ± 422	9676 ± 596.3	9911 ± 843
-dP/dt [mmHg/s]	10365 ± 1000	10303 ± 602	9252 ± 548	9600 ± 666

Data are mean ± SEM of 4-6 rats. 1W, 1 week post surgery sham; 4W, 4 week post surgery sham; 8W, 8 week post surgery sham; 16W, 16 week post surgery sham. EF, Ejection fraction; FS, Fractional shortening; LVPSP, Left ventricular peak systolic pressure; LVEDP, Left ventricular end-diastolic pressure; +dP/dt , maximal rate of increase of LV systolic pressure; -dP/dt , maximal rate of decline of LV systolic pressure.

Table 5: Cardiac function in Sham Control (Sham) and Post Myocardial infarction (PMI) Rats

Parameter	Sham	1 W PMI	8 W PMI	16 W PMI
EF [%]	79.3 ± 5.5	77.0 ± 5.3	54.7 ±4.2 *	29 ± 2.8 **
FS [%]	43.8 ± 3.5	44.2 ± 2.3	26.9 ± 2.4 **	33.5 ± 2.3 *
LVPSP [mmHg]	109.4 ± 7.3	92.9 ± 8.2	72.93 ± 7.4 *	66.5 ± 5.5 **
LVEDP [mm Hg]	3.94 ± 1.14	8.56 ± 0.98 **	12.4 ± 2.98 *	20.8 ± 1.66 **
+dP/dt [mmHg/s]	9911 ± 843	8736 ± 659	5550 ± 992 *	5804 ± 381 *
-dP/dt [mmHg/s]	9600 ± 666	9956 ± 892	6432 ± 786 *	5827 ± 615 *

Data are mean ± SEM of 4-6 rats. Significantly different (* $p< 0.05$; ** $p<0.005$) vs. Sham operated rats; 1W PMI, 1 week post myocardial infarction; 8W PMI, 8 week post myocardial infarction; 16W PMI, 16 week post myocardial infarction. EF, Ejection fraction; FS, Fractional shortening; LVPSP, Left ventricular peak systolic pressure; LVEDP, Left ventricular end-diastolic pressure; +dP/dt , maximal rate of increase of LV systolic pressure; -dP/dt , maximal rate of decline of LV systolic pressure. 1 week, 8 week and 16 week sham control were not different from each other and the data shown is from the 16 week sham only.

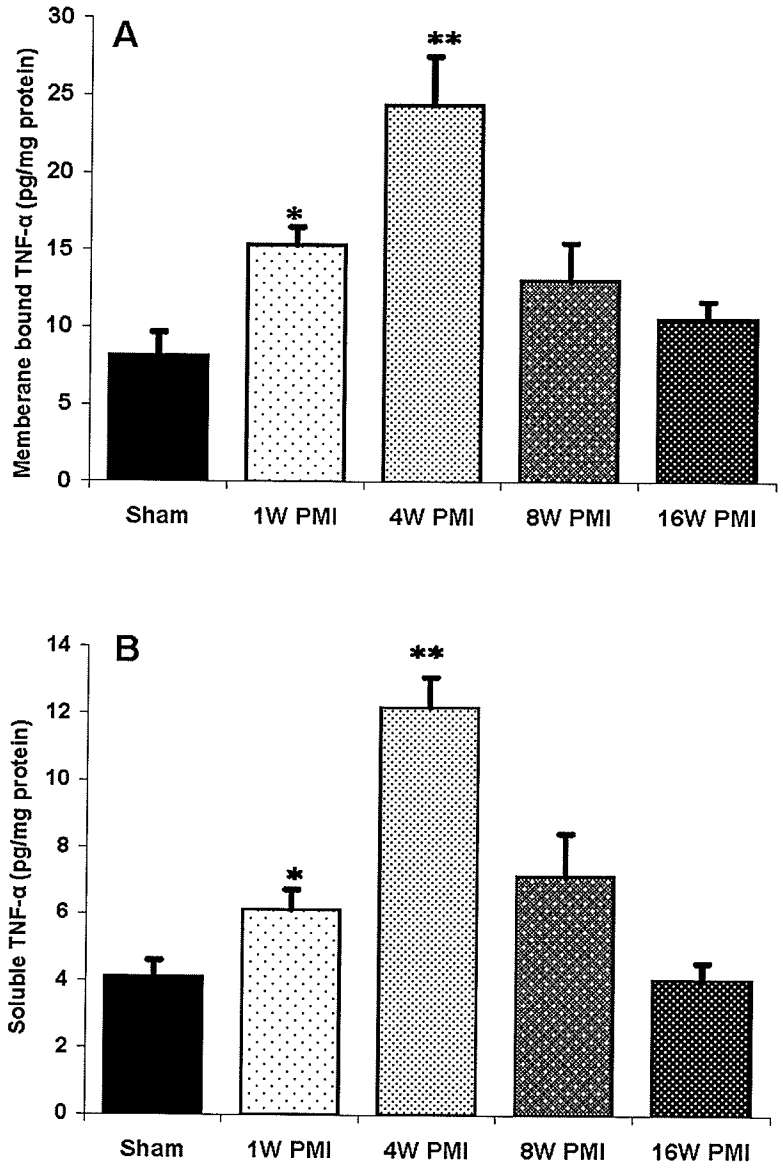
1.3 Myocardial TNF-α and IL-10 protein levels

TNF-α and IL-10 protein levels were determined from the membrane as well as soluble fractions isolated from the tissue homogenate as described in the method section.

1.3.1 Membrane bound and soluble TNF-α protein levels

Control baseline value for the membrane bound TNF-α was 8.17 ± 1.46 pg/mg protein (Fig 3A). Membrane bound TNF-α was significantly increased ($p < 0.008$) by 88% in 1W PMI and by 198% in 4W PMI ($p < 0.003$) group as compared to the sham control group. These values for TNF-α levels in 8 and 16W PMI groups were higher by 59% and 29% respectively but the differences were statistically insignificant with $p > 0.05$ (Fig 3A).

Soluble TNF-α in the sham control group was 4.11 ±0.50 pg/mg protein (Fig 3B). This value was significantly increased ($p < 0.042$) by 49% in 1W PMI and by 197 % in 4W PMI ($p < 0.001$) group as compared to sham control group (Fig 3B). Though there was 74 % increase in the soluble TNF-α levels at 8W PMI, this difference was not significant because of higher SEM ($p > 0.06$). In 16W PMI group, there was no change in the soluble TNF-α levels as compared to the sham control group (Fig 3B).



Figs 3 (A and B): Myocardial TNF-α, membrane bound (A) and soluble (B) protein expression at different time points after myocardial infarction in rat hearts by ELISA. Data are mean ±SEM of 4 hearts. Significantly different (* $p< 0.05$; ** $p<0.005$) vs. sham operated rats. Sham groups were not different from each other so that data from sham groups was pooled. 1 week post myocardial infarction; 4W PMI, four week post myocardial infarction; 8W PMI, 8 week post myocardial infarction and 16W PMI, 16 week post myocardial infarction.

1.3.2 Membrane bound and soluble IL-10 protein levels

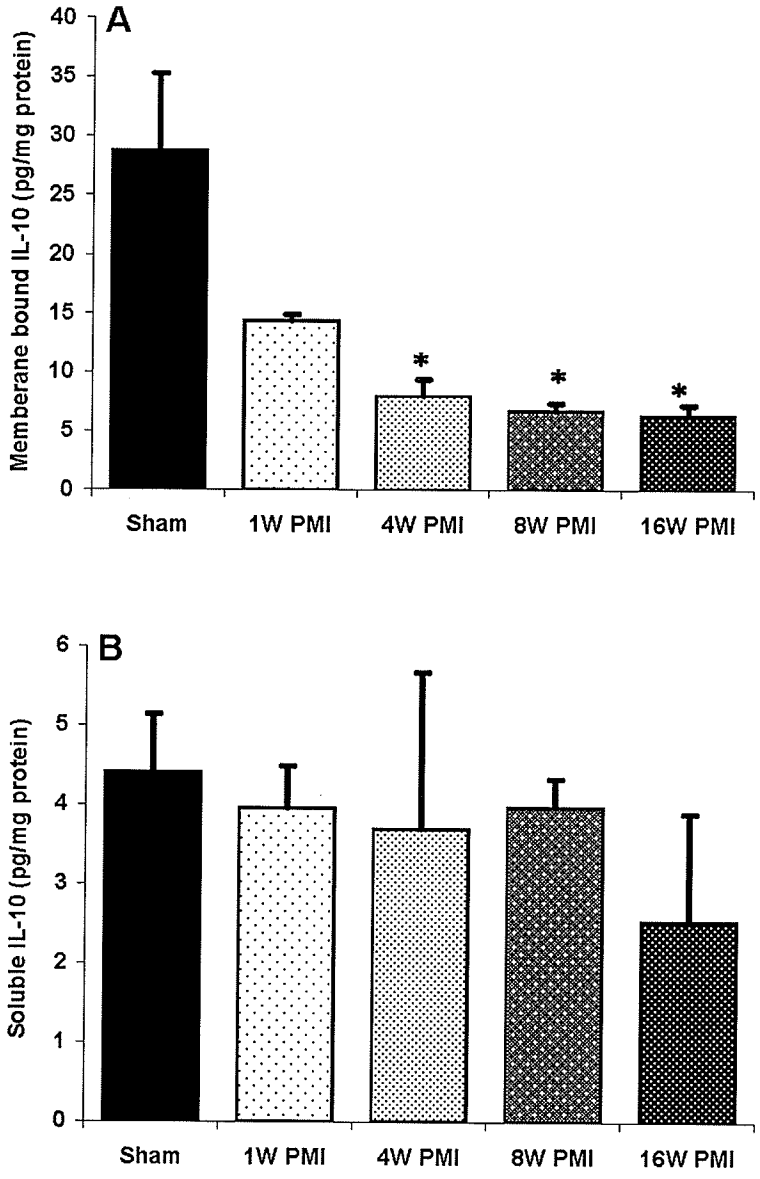
Membrane bound IL-10 in the sham control group was 28.74 ± 6.51 pg/mg protein. Although membrane bound IL-10 was decreased by about 50% in 1W PMI but this decrease was insignificant (p= 0.07) as compared to sham control group (Fig 4A). Membrane bound IL-10 in 4, 8 and 16W PMI groups was significantly decreased to 27%, 23% and to 21% of sham control values respectively.

Baseline value for soluble IL-10 protein levels in sham control group was 4.40 ± 0.74 pg/mg protein (Fig 4B). There was no significant difference in soluble IL-10 protein levels between different PMI groups and the baseline value.

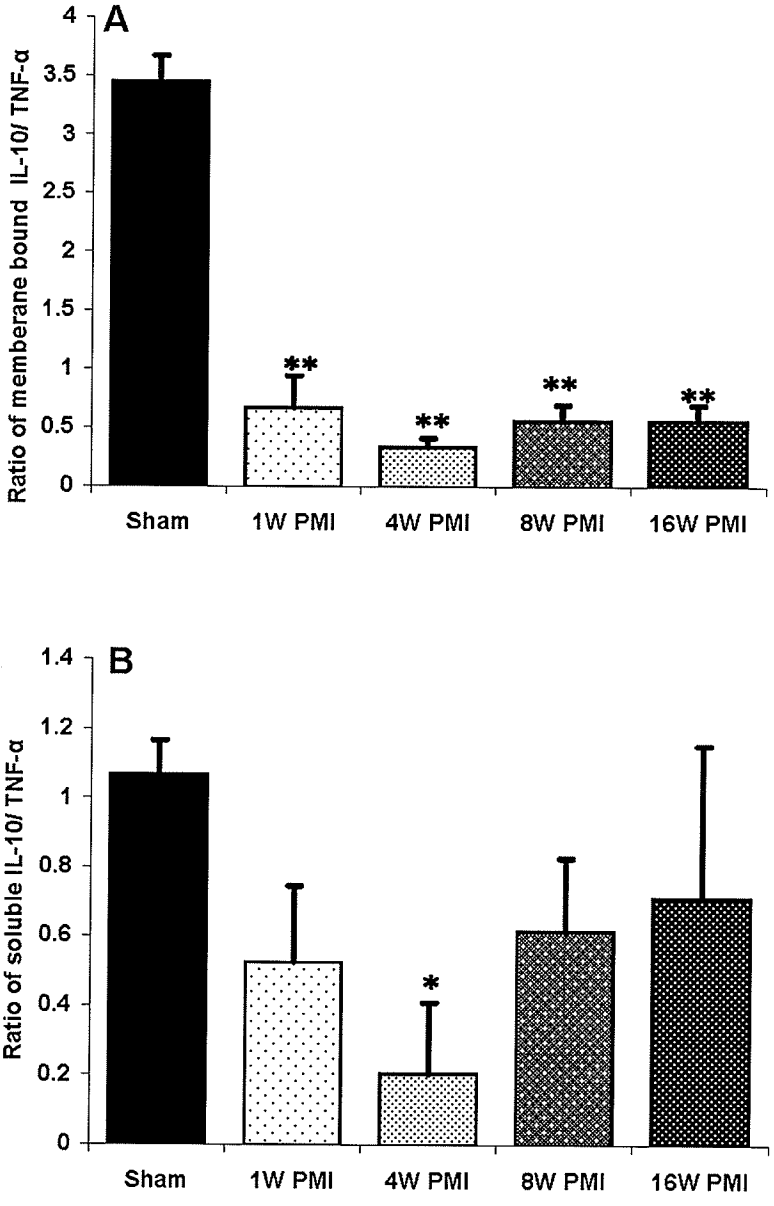
1.3.3 Membrane bound and soluble IL-10/TNF-αprotein level ratio

The control value for ratio between membrane bound IL-10 and TNF-α protein was 3.45 ± 0.21. This value was significantly decreased at all time points in PMI groups as compared to sham control group. 4W PMI group showed a maximum decrease to 0.34 ± 0.08 (Fig 5A).

The baseline value for this ratio for the soluble fraction was 1.07 ± 0.10. This ratio of IL-10 and TNF-α for the soluble fraction was significantly decreased to 19.33% only at 4W PMI (Fig 5B).



Figs 4 (A and B): Myocardial IL-10, membrane bound (A) and soluble (B) protein expression at different time points after myocardial infarction in rat hearts by ELISA. Data are mean ±SEM of 4 hearts. Significantly different * $p< 0.05$ vs. sham operated rats. 1 week post myocardial infarction; 4W PMI, four week post myocardial infarction; 8W PMI, 8 week post myocardial infarction and 16W PMI, 16 week post myocardial infarction.



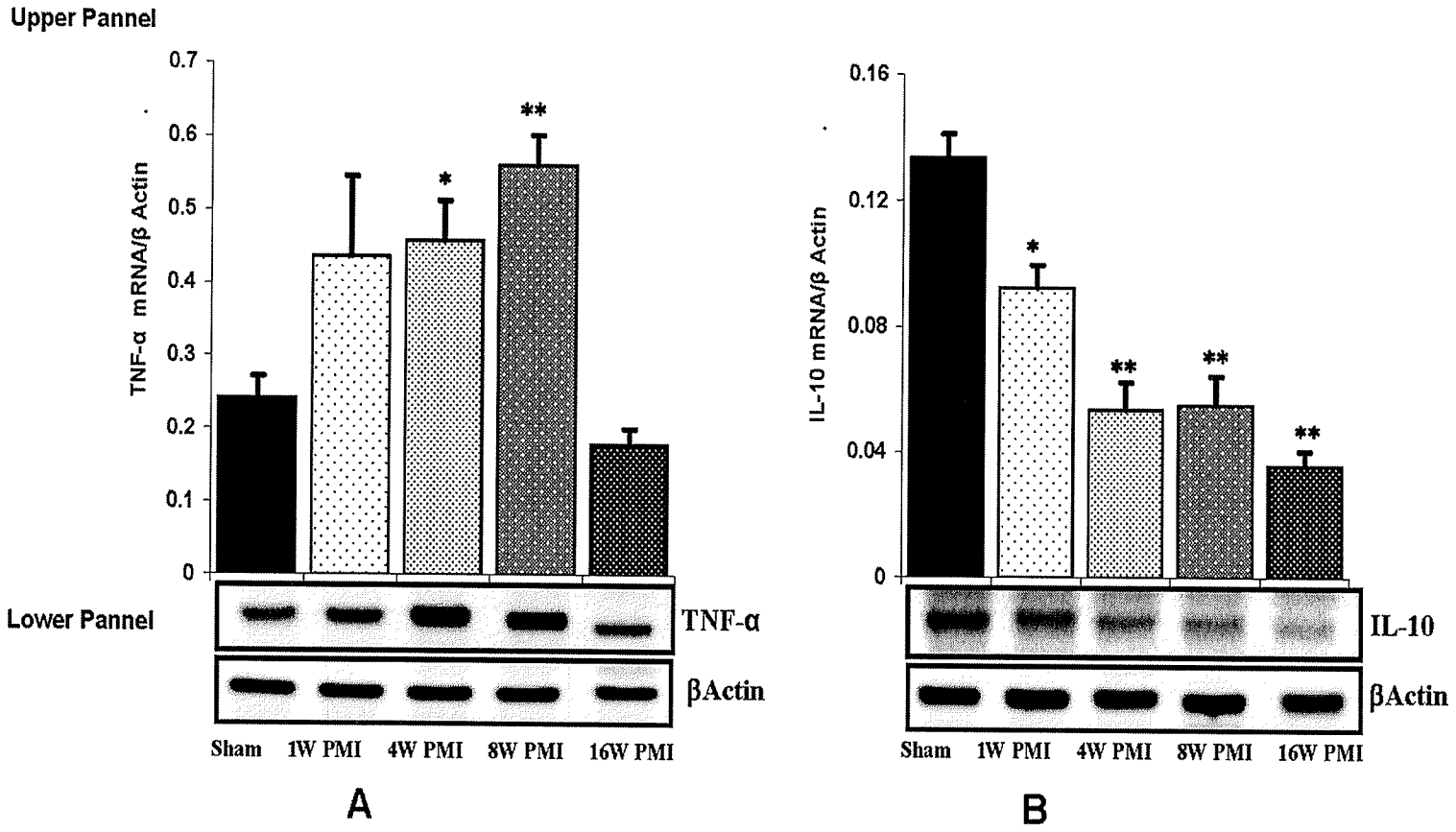
Figs 5 (A and B): Ratios of IL-10 to TNF-α of membrane bound (A) and soluble (B) protein expression at different time points after myocardial infarction in rat heart by ELISA. Data are mean ±SEM of 4 hearts. Significantly different (* $p<0.05$; ** $p<0.005$) vs. Sham operated rats. 1 week post myocardial infarction; 4W PMI, four week post myocardial infarction; 8W PMI, 8 week post myocardial infarction and 16W PMI, 16 week post myocardial infarction.

1.4 Myocardial TNF-α and IL-10 mRNA levels

Myocardial TNF-α and IL-10 mRNA was isolated from the sham Control and different PMI groups as described in the Methods section and the results (Gel images and densitometric data) are shown in figures 6A and 6B.

TNF-α mRNA was significantly increased ($p < 0.01$) by 89% in 4W PMI and by 131% in 8W PMI ($p < 0.001$) group as compared to sham control group. At 1W PMI and 16W PMI, TNF-α mRNA was not significantly different as compared to sham control group (Fig 6A).

IL-10 mRNA was significantly reduced in all PMI groups as compared to sham control group and the maximum decrease was seen in the 16W PMI group (Fig 6B).



Figs 6 (A and B): Myocardial expression of TNF-α mRNA (A) and IL-10 mRNA (B) at different time points after myocardial infarction in rat heart by RT-PCR. Upper panel: Densitometric analysis of mRNA signal. Data are mean ±SEM of 4 hearts. Significantly different (* $p<0.05$; ** $p<0.005$) vs. Sham operated rats. Lower Panel: Representative RT-PCR product gel images for mRNA. 1 week post myocardial infarction; 4W PMI, four week post myocardial infarction; 8W PMI, 8 week post myocardial infarction and 16W PMI, 16 week post myocardial infarction.

1.5 Changes in myocardial function and cytokines by Losartan

Losartan treatment of patients with heart failure subsequent to MI has been reported to improve function as well as prognosis (Pitt et al., 1997; Rump, 1998). Losartan treatment has also been shown to improve cardiac function in rats subsequent to MI (Khaper and Singal 2001). In my study, the effects of Losartan on the changes in cytokines in relation to function were also examined. For this purpose, animals were put on Losartan in drinking water immediately after the surgery and the treatment was continued up to 4 W PMI as described in the methods.

1.5.1 General appearance and myocardial function

Losartan treatment did not have any apparent effect on the general appearance and weight gain in the sham control and PMI group. There was no significant difference in the body weight between Losartan treated sham and PMI groups.

Losartan treatment improved fractional shortening (FS) and ejection fraction (EF) in the 4W PMI group to the point that there was no significant difference in Losartan-treated sham and PMI groups (Fig 7 and Table 6). The data on LVPSP; LVEDP; +dP/dt and -dP/dt in untreated and Losartan treated 4W PMI rats are also given in Table 6. In 4W PMI without Losartan, the LVEDP was increased by 331%. In the Losartan treatment group, this increase was only 107%. The LVPSP, +dP/dt and -dP/dt were close to normal in Losartan treated 4W PMI group (Tables 6).

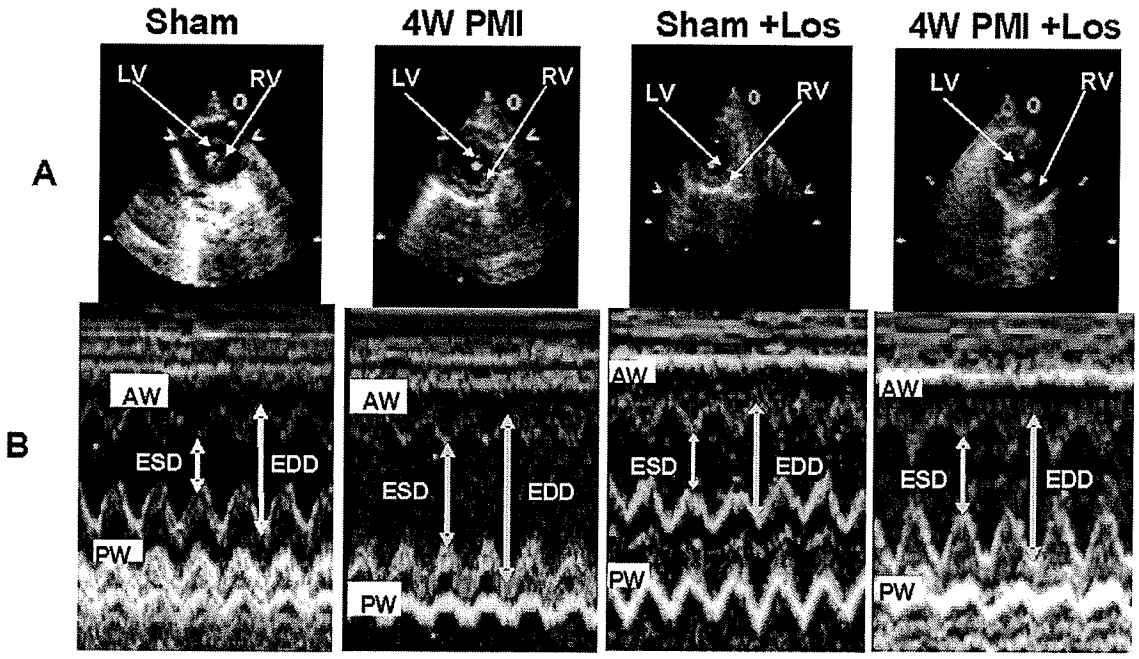


Fig 7 (A and B): Echocardiography images of rat heart at 4W MI and Sham group with or without losartan treatment. A, 2-D echocardiography at parasternal Short-Axis Plane; B, Representative M-mode Short-Axis of the left ventricle; AW, anterior wall; PW, posterior wall; ESD, end systolic dimensions; EDD, end diastolic dimensions. 4W PMI, Four-week post myocardial infarction; 4W PMI + Los, losartan treated Four-week post myocardial infarction group.

Table 6: Effects of losartan treatment on Cardiac function in 4 week Sham Control (Sham) and 4 week Post Myocardial infarction (PMI) Rats

Parameter	4 Week		4 Week + Losartan	
	Sham	PMI	Sham	PMI
EF [%]	73.1 ± 3.31	49.5 ± 6.39 *	76.3 ± 4.20	62.3 ± 5.23
FS [%]	39.5 ± 2.15	26.9 ± 3.87 *	42.3 ± 3.26	39.5 ± 3.3 ψ
LVPSP [mmHg]	117.9± 4.24	87.2 ± 9.05 *	108.6 ± 5.69	96.5 ± 8.9
LVEDP [mm Hg]	3.33± 0.49	14.3 ± 1.22 **	3.65 ± 1.2	7.56 ± 0.98 #ψ
+dP/dt [mmHg/s]	9494± 422.87	6801 ± 844*	8959 ± 562	7894 ± 759
-dP/dt [mmHg/s]	10303 ± 602.81	7357 ± 774*	9897 ± 759	8265 ± 689

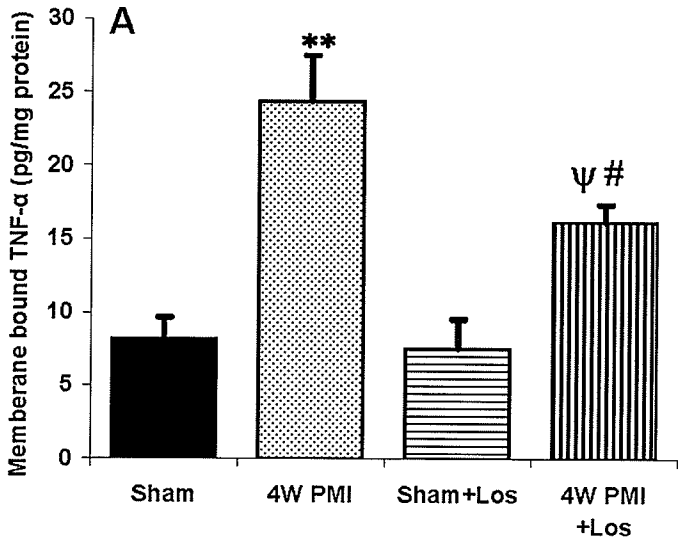
Data are mean ± SEM of 4-6 rats. Significantly different # $p< 0.05$ vs. Sham + Losartan; (* $p< 0.05$; ** $p<0.005$) vs. Sham operated rats and ψ $p< 0.05$ vs. 4W PMI. Los, losartan treated group. Units and symbols are same as in Table 4.

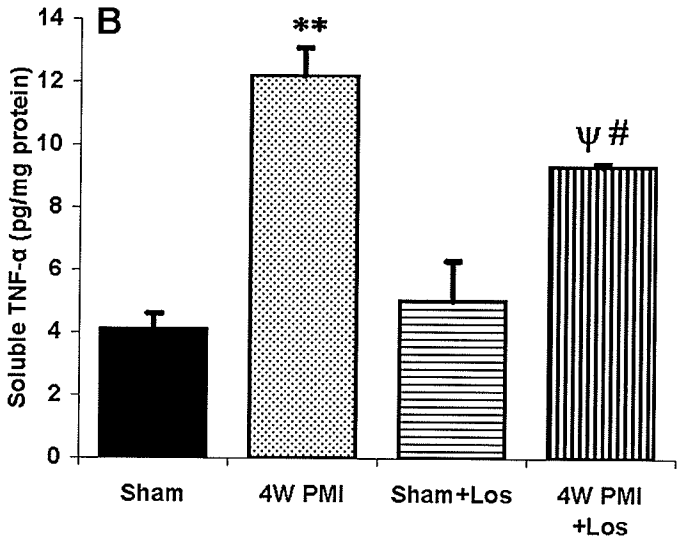
1.5.2 Myocardial TNF-α and IL-10 protein

There was no effect of Losartan on the sham animals in regards to both membrane bound and soluble TNF-α protein fraction. Both membrane bound and soluble fractions of TNF-α protein were significantly decreased ($p< 0.049$) in losartan treated PMI group as compared to untreated 4W PMI group (Figs 8A and 8B). However, these values in the Losartan treated PMI were significantly higher then Losartan treated sham group.

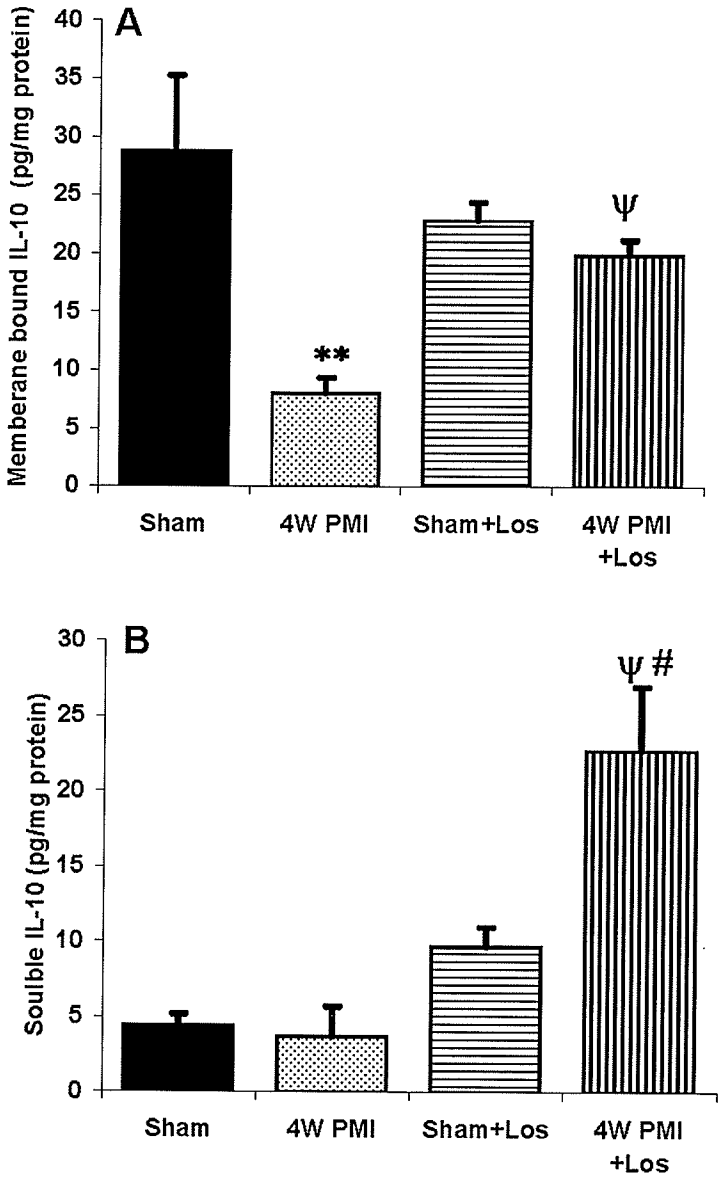
Levels of membrane bound and soluble IL-10 were significantly improved in Losartan treated 4W PMI group as compared to untreated 4W PMI group (Fig 9A and 9B). Membrane bound IL-10 levels in Losartan treated PMI and sham group were not different (Fig 9A). Soluble IL-10 was increased by Losartan treatment in both the sham group and 4W PMI group. However, this increase was significant only in the PMI group (Fig 9B).

Values for the ratio of membrane bound and soluble fractions of IL-10 and TNF-α protein levels are shown in Fig 10A and Fig 10B. Losartan treatment caused a significant improvement in the ratio of both membrane bound and soluble fractions in the 4W PMI group (Fig10A and 10B).The ratio of membrane bound IL-10 and TNF-α protein levels in the Losartan treated 4W PMI was significantly less as compared to Losartan treated sham (Fig 10A).

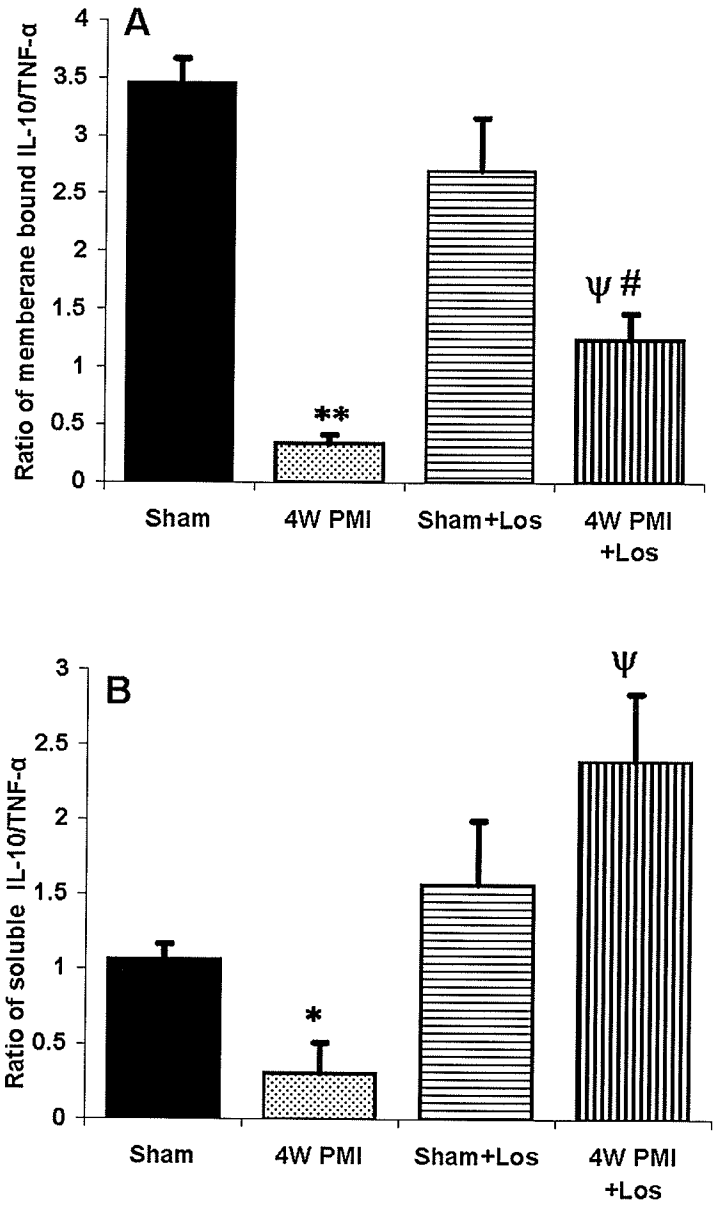




Figs 8 (A and B): Effects of losartan treatment on myocardial TNF-α, membrane bound (A) and soluble (B) protein fractions at 4W PMI in rat heart by ELISA. Data are mean ±SEM of 4 hearts. Significantly different # $p<0.05$ vs. Sham + Losartan; ** $p<0.005$ vs. Sham operated rats and ψ $p<0.05$ vs. 4W PMI. 4W PMI, Four-week post myocardial infarction; 4W PMI + Los, losartan treated four-week post myocardial infarction group.



Figs 9 (A and B): Effects of losartan treatment on myocardial IL-10, membrane bound (A) and soluble (B) protein expression at 4W PMI in rat heart by ELISA. Data are mean ±SEM of 4 hearts. Significantly different # $p<0.05$ vs. Sham + Losartan; ** $p<0.005$ vs. Sham operated rats and ψ $p<0.05$ vs. 4W PMI. 4W PMI, Four-week post myocardial infarction; 4W PMI + Los, losartan treated four-week post myocardial infarction group.

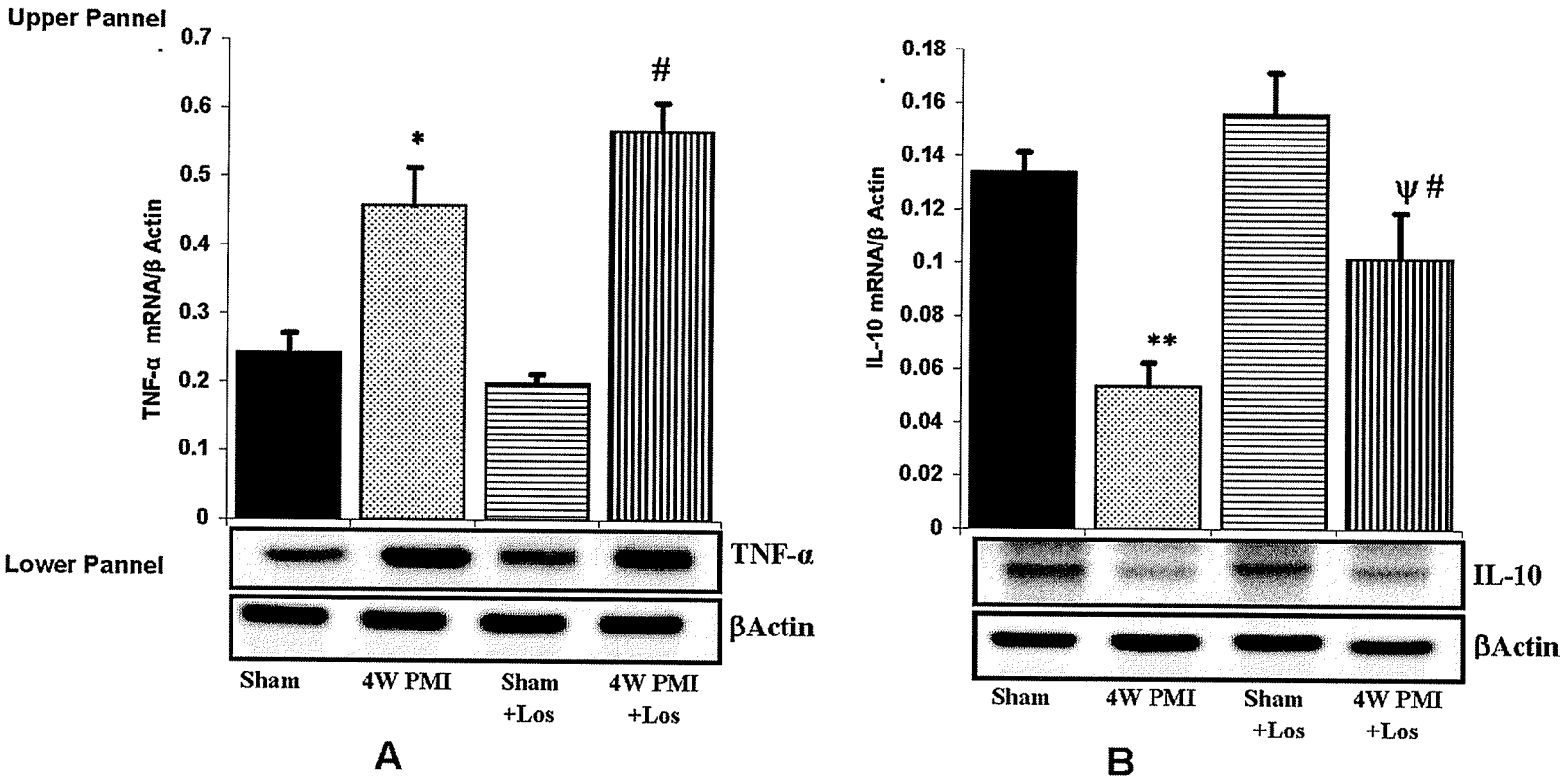


Figs 10 (A and B): Effects of losartan treatment on ratio of IL-10 and TNF-α, membrane bound (A) and soluble (B) protein expression at 4W PMI in rat by ELISA. Data are mean ±SEM of 4 hearts. Significantly different # $p<0.05$ vs. Sham + Losartan; (* $p< 0.05$; ** $p<0.005$) vs. Sham operated rats and ψ $p< 0.05$ vs. 4W PMI. 4W PMI, Four-week post myocardial infarction; 4W PMI + Los, losartan treated four-week post myocardial infarction group.

1.5.3 Myocardial TNF-α and IL-10 mRNA

TNF-α mRNA was significantly increased by 185.8% in Losartan treated 4W PMI as compared to Losartan treated sham group. There was no effect of Losartan treatment on TNF-α mRNA in the sham control group. The difference in TNF-α mRNA levels of treated and untreated 4W PMI was not significant either (Fig 11A).

There was no significant difference in the levels of IL-10 mRNA between treated and untreated sham groups. IL-10 mRNA in the losartan treated 4W PMI group was significantly improved as compared to untreated group (Fig 11B). However, this value in treated 4W PMI group was still less then the Losartan treated sham animals.



Figs 11 (A and B): Effects of losartan treatment on cardiac expression of TNF-α (A) and IL-10 (B) mRNA at 4W PMI in rat by RT-PCR. Upper panel: Densitometric analysis of mRNA signal. Data are mean ±SEM of 4 hearts. Significantly different # $p<0.005$ vs. Sham + Losartan; (* $p< 0.05$; ** $p<0.005$) vs. Sham operated rats; and ψ $p< 0.05$ vs. 4W PMI. Lower Panel: Representative RT-PCR product gel images for mRNA. 4W PMI, Four-week post myocardial infarction; 4W PMI + Los, losartan treated four-week post myocardial infarction group.

2.0 *In Vitro* Studies

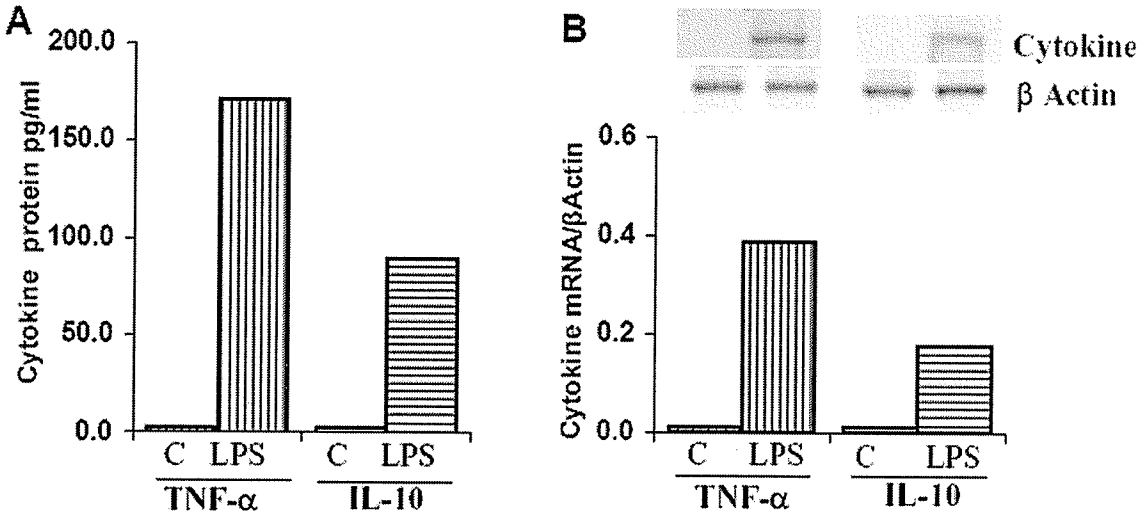
There have been multiple studies indicating that cardiac myocytes can produce TNF-α (Comstock etal 1998; Wright etal 2002). However, there is no parallel data to show, if the myocytes are able to produce IL-10. We did a preliminary study to test whether cardiac myocytes can be stimulated to produce IL-10. In this regard, we also tested the possibility of stimulating myocytes to produce TNF-α as well. Cardiac myocytes were challenged with lipopolysaccharide (10 μg/ml) for 4 hours to test the production of TNF-α and IL-10.

Myocytes were also exposed for 4 hours to TNF-α, IL-10 (1, 10 and 20 ng/ml) or combination of TNF-α and IL-10 in different ratios. In order to further examine the role of oxidative stress, myocytes were also treated with H_2O_2 (100 μM) for 4 hours as a positive control. Cell lysates were then analyzed for protein and mRNA levels for Copper/zinc superoxide dismutase (Cu/Zn-SOD), Manganese superoxide dismutase (MnSOD), Catalase and Glutathione peroxidase (GSHPx), generation of ROS and lipid peroxidation. Creatine Kinase (CK) and lactate dehydrogenase (LDH) release was studies to determine cell injury. In some experiments, Redox Ratio (GSH/GSSG) and GSHPx enzyme activity were also studied.

2.1 LPS treatment; TNF-α and IL-10, protein and mRNA

Protein levels for TNF-α and IL-10 as well as their mRNA were analyzed from untreated and LPS treated myocytes. TNF-α and IL-10 protein as well as their mRNA were undetectable from control myocytes. After LPS treatment, 171.0 pg/ml of TNF-α

and 89.4 pg/ml of IL-10 protein was detected in culture medium (Fig 12A). Similarly, mRNA expression was increased for both TNF-α and IL-10 after LPS treatment (Fig 12B).



Figs 12 (A and B): Effect of lipopolysaccharide treatments of cardiomyocytes for 4 hr, on TNF-α, IL-10 (A) Protein by ELISA (therefore no bands for protein) and mRNA (B) by RT-PCR. 12B upper panel; representative RT-PCR bands for TNF-α and IL-10 mRNA with β Actin band. 12B lower panel; densitometric data. Control (C); lipopolysaccharide 10 μg/ml (LPS). Samples were pooled from 3 different myocyte preparations.

2.2Antioxidant enzyme proteins and mRNA

2.2.1 Cu/Zn-SOD

Cu/Zn-SOD protein levels showed no change with TNF-α, IL-10 or combination treatment, whereas H_2O_2 treatment caused a 78 % decrease (Fig 13A). Cu/Zn-SOD mRNA showed no significant change at any concentration of TNF-α treatment alone, whereas IL-10 caused an increase at 10 and 20ng/ml but the change was significant only at 20ng/ml. Cu/Zn-SOD mRNA was significantly increased only at IL-10/TNF-α ratio of 1 as compared to control and this value was significantly more as compared to levels at 10ng/ml of TNF-α. H_2O_2 treatment decreased Cu/Zn-SOD mRNA by about 35% (Fig 13B).

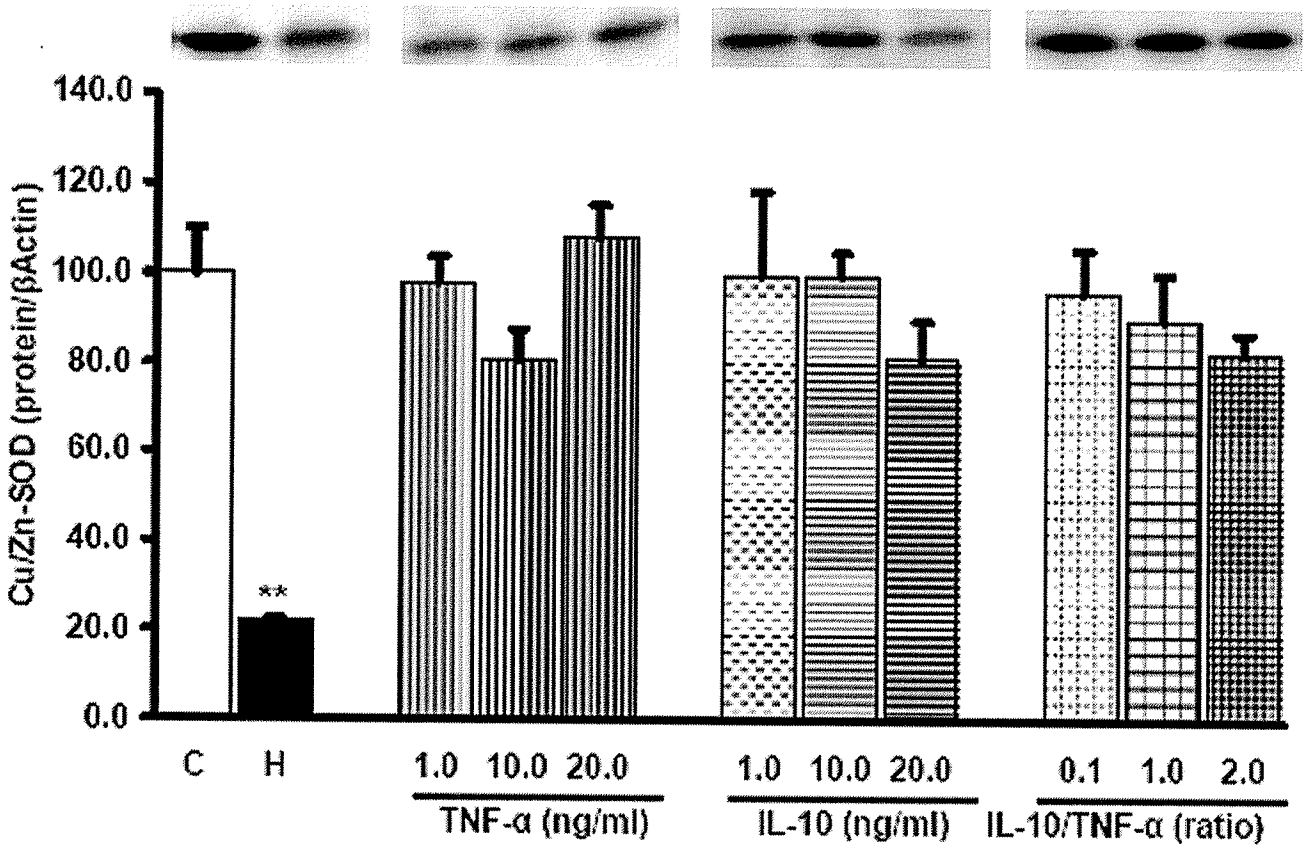


Fig 13A: Effects of H_2O_2, TNF-α, IL-10 and IL-10/TNF-α combination treatments on Cu/Zn-SOD (copper/zinc-superoxide dismutase) protein levels. For combination treatment, the TNF-α concentration was kept at 10ng/ml, whereas IL-10 was used in three different concentrations (1, 10 and 20 ng/ml). Upper panel; representative protein bands for Cu/Zn-SOD. Lower panel; densitometric data. Data are Mean ± SEM from 5 experiments. Significantly different, **$p<0.005$ vs Cont. Control values were taken as 100%. Control (C); H_2O_2 100 μM (H)

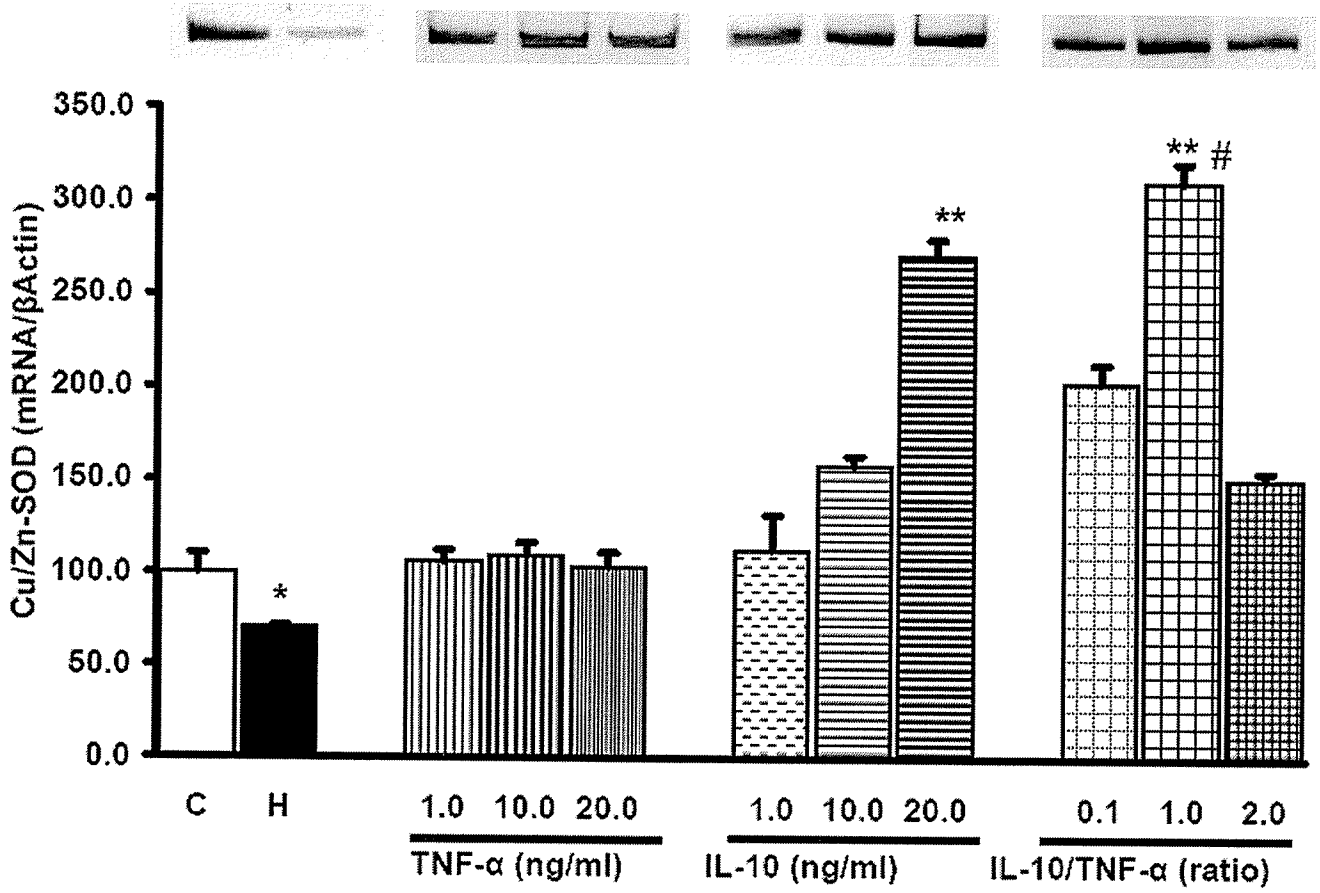


Fig 13B: Effects of H_2O_2, TNF-α, IL-10 and IL-10/TNF-α combination treatments on Cu/Zn-SOD (copper/zinc-superoxide dismutase) mRNA levels. For combination treatment, the TNF-α concentration was kept at 10ng/ml, whereas IL-10 was used in three different concentrations (1, 10 and 20 ng/ml). Upper panel; representative RT-PCR bands for Cu/Zn-SOD mRNA. Lower panel; densitometric data. Data are Mean ± SEM from 5 experiments. Significantly different, **$p<0.005$ vs Cont. Control values were taken as 100%. Control (C); H_2O_2 100 μM (H)

2.2.2 MnSOD:

There was no change in MnSOD protein levels after 1ng/ml of TNF-α but at 10 and 20ng/ml the protein was significantly decreased by about 50% or more. There was no change in the protein levels due to IL- 10 treatment at any of the concentrations used. MnSOD protein levels were significantly reduced only at a ratio of 2 of IL-10/TNF-α combination as compared to control. At a ratio of 1, the combination treatment significantly improved MnSOD protein levels ($p < 0.041$) as compared to levels with 10ng/ml of TNF-α alone. MnSOD protein was significantly decreased by about 90% of the control levels after H_2O_2 treatment (Fig 14A).

MnSOD mRNA was also significantly decreased with 10ng/ml and 20ng/ml concentration of TNF-α. IL-10 at levels of 20ng/ml significantly increased MnSOD mRNA where as lower concentrations showed no effect. In combination treatment, MnSOD mRNA was significantly increased both at a ratio of 0.1 and 1.0 as compared to control as well as to 10ng/ml of TNF-α alone. H_2O_2 treatment caused a maximum decrease (≈ 95%) in MnSOD mRNA levels as compared to levels (Fig 14B).

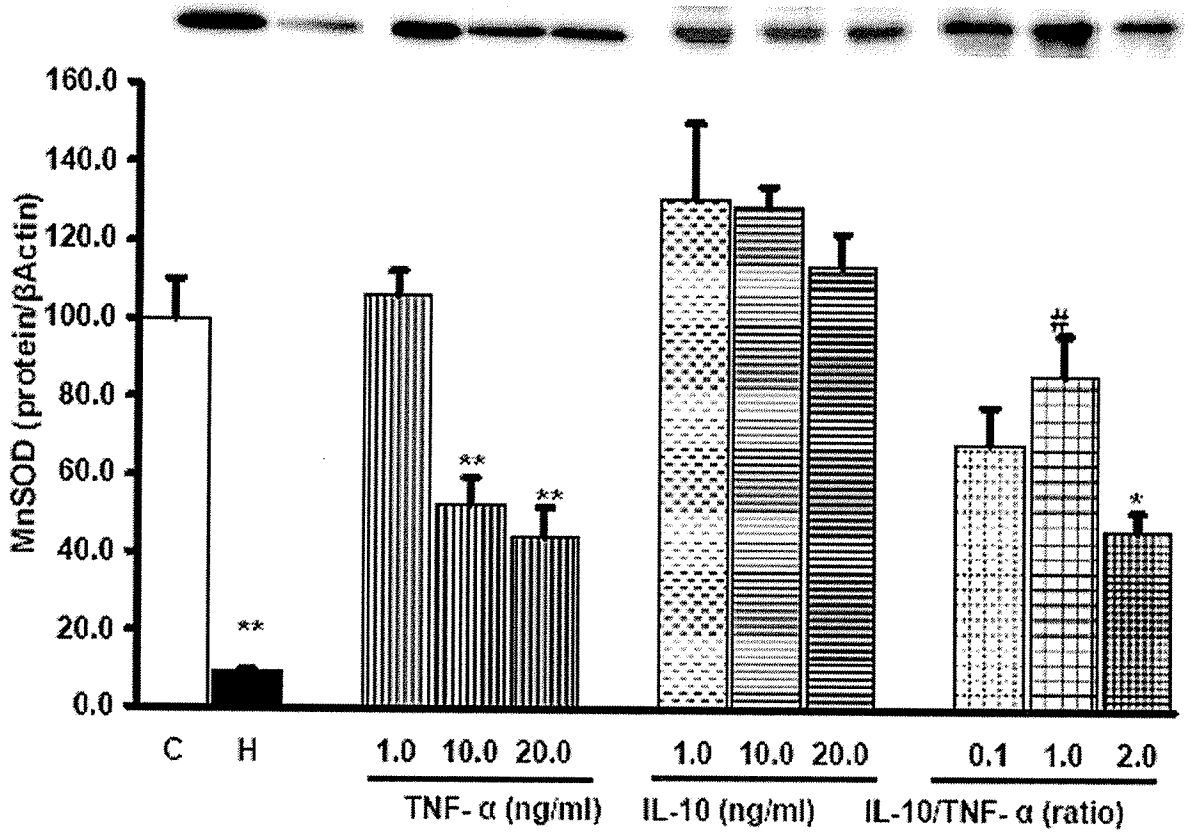


Fig 14A: Effects of H_2O_2, TNF-α, IL-10 and IL-10/TNF-α combination treatments on MnSOD (Manganese Superoxide Dismutase) protein levels. For combination treatment, the TNF-α concentration was kept at 10ng/ml, whereas IL-10 was used in three different concentrations (1, 10 and 20 ng/ml).Upper panel; representative protein bands for MnSOD. Lower panel; densitometric data. Data are Mean ± SEM from 5 experiments. Significantly different (*$p< 0.05$, **$p<0.005$) vs Cont; (# $p< 0.05$) vs 10ng/ml TNF-α. Control values were taken as 100%. Control (C); H_2O_2 100 μM (H).

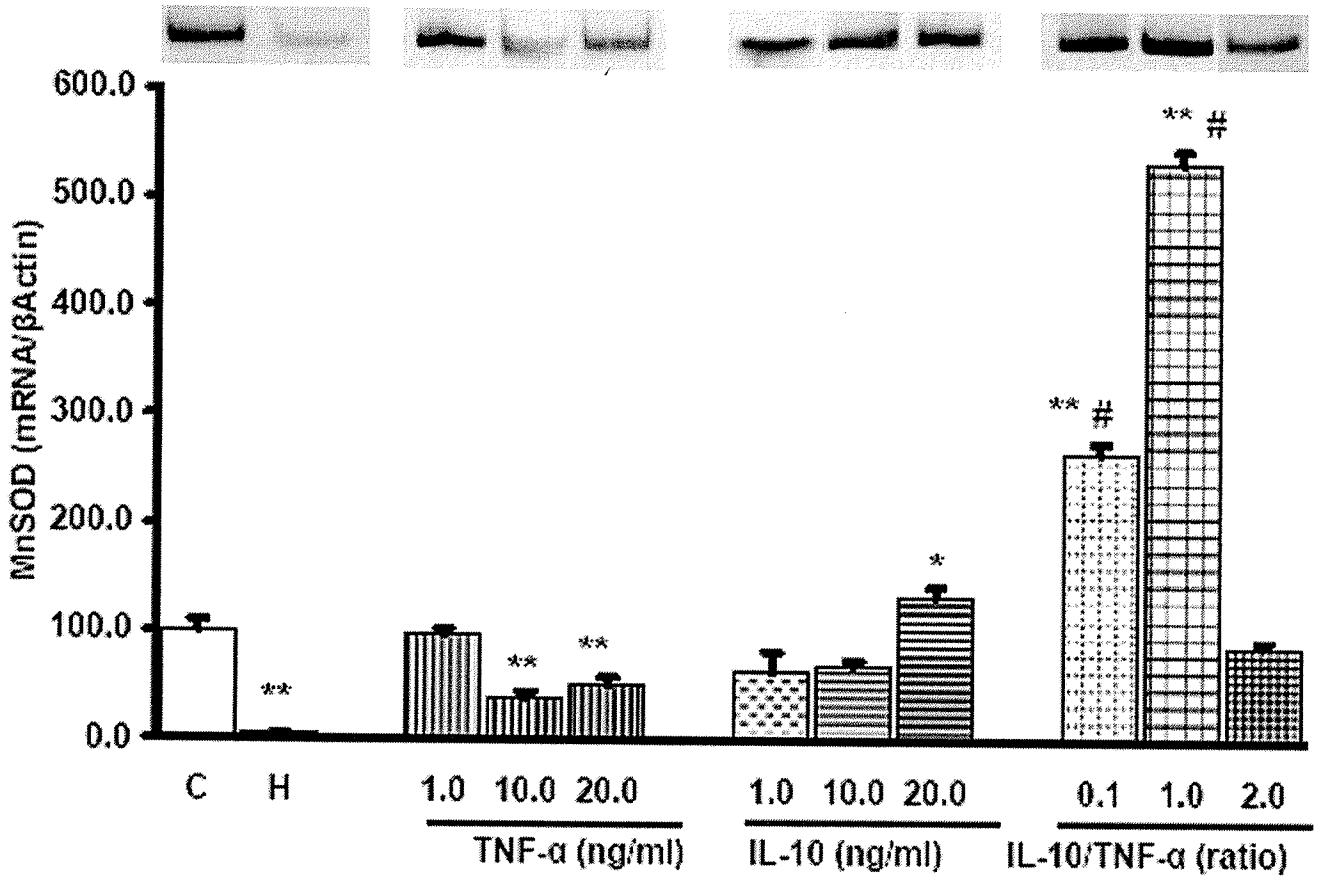


Fig 14B: Effects of H_2O_2, TNF-α, IL-10 and IL-10/TNF-α combination treatments on MnSOD (Manganese Superoxide Dismutase) mRNA levels. For combination treatment, the TNF-α concentration was kept at 10ng/ml, whereas IL-10 was used in three different concentrations (1, 10 and 20 ng/ml).Upper panel; representative RT-PCR bands for MnSOD mRNA. Lower panel; densitometric data. Data are Mean ± SEM from 5 experiments. Significantly different (*$p< 0.05$, **$p<0.005$) vs Cont; (# $p< 0.05$) vs 10ng/ml TNF-α. Control values were taken as 100%. Control (C); H_2O_2 100 μM (H).

2.2.3 Catalase

Catalase protein was significantly decreased with 10 and 20ng/ml concentration of TNF-α and showed no change with IL-10 treatment. There was no significant difference in catalase protein levels as compared to control with any combination treatment. However, catalase protein levels were significantly increased ($p< 0.006$) at 10/10ng/ml as compared to levels with 10ng/ml of TNF-α. H_2O_2 treatment caused a 66% decreased in Catalase protein (Fig 15A).

Catalase mRNA was significantly decreased by TNF-α at 20ng/ml concentration where as IL-10 at 20ng/ml increased catalase mRNA levels. Catalase mRNA was significantly increased at 10/10ng/ml combination concentration as compared to control as well as to 10ng/ml. H_2O_2 treatment decreased catalase mRNA by 48% (Fig 15B).

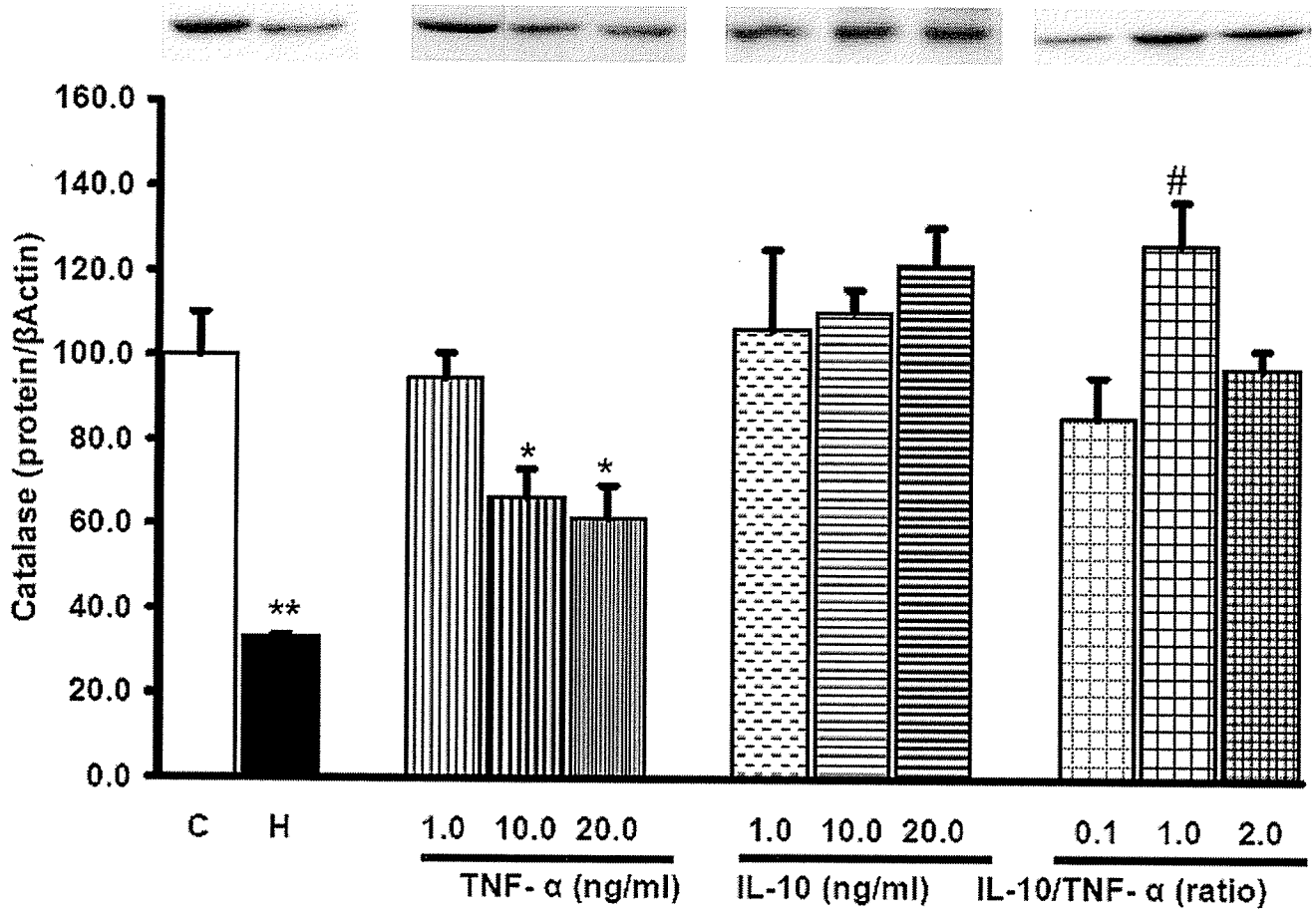


Fig 15A: Effects of H_2O_2, TNF-α, IL-10 and IL-10/TNF-α combination treatments on Catalase protein levels. For combination treatment, the TNF-α concentration was kept at 10ng/ml, whereas IL-10 was used in three different concentrations (1, 10 and 20 ng/ml). Upper panel; representative protein bands for Catalase. Lower panel; densitometric data. Data are Mean ± SEM from 5 experiments. Significantly different (*$p< 0.05$, **$p<0.005$) vs Cont; (# $p< 0.05$) vs 10ng/ml TNF-α. Control values were taken as 100%. Control (C); H_2O_2 100 μM (H)

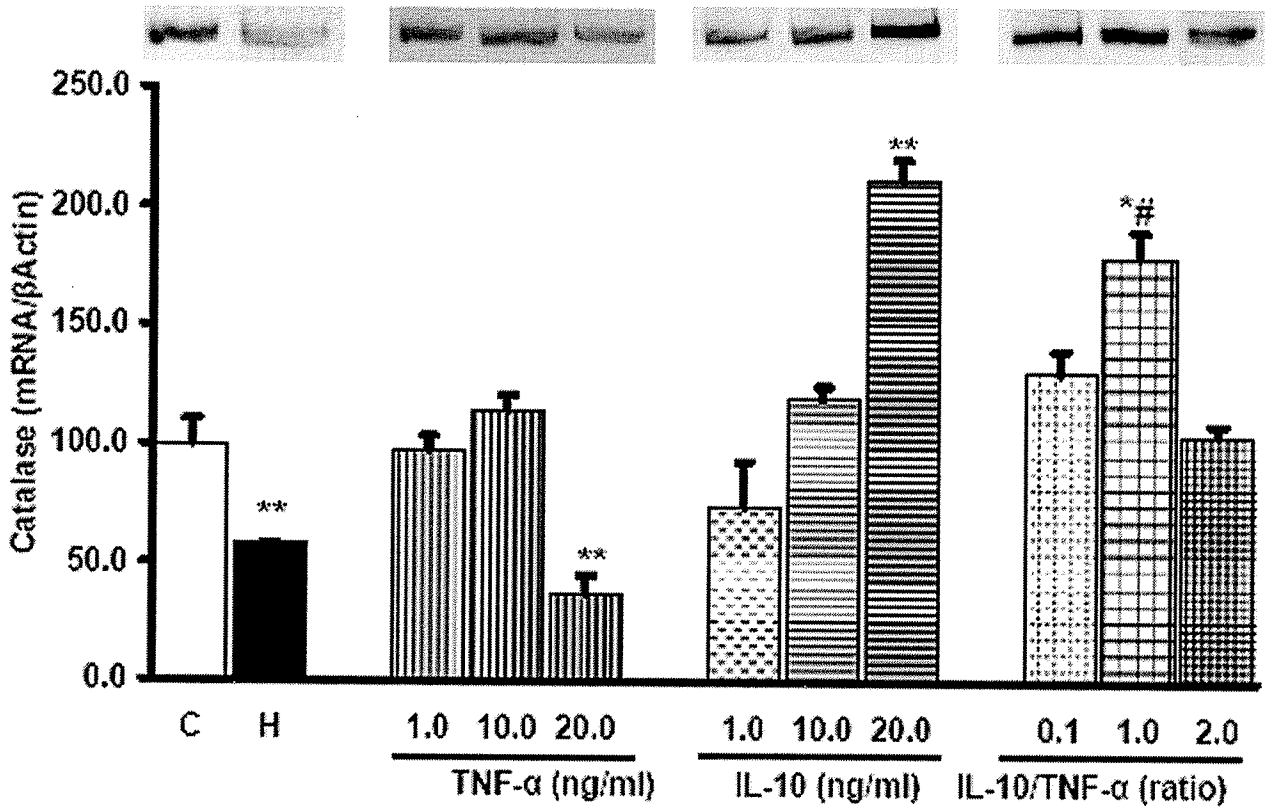


Fig 15B: Effects of H_2O_2, TNF-α, IL-10 and IL-10/TNF-α combination treatments on Catalase mRNA levels. For combination treatment, the TNF-α concentration was kept at 10ng/ml, whereas IL-10 was used in three different concentrations (1, 10 and 20 ng/ml). Upper panel; representative RT-PCR bands for Catalase mRNA. Lower panel; densitometric data. Data are Mean ± SEM from 5 experiments. Significantly different (*$p< 0.05$, **$p<0.005$) vs Cont; (# $p< 0.05$) vs. 10ng/ml TNF-α. Control values were taken as 100%. Control (C); H_2O_2 100 μM (H).

2.2.4 GSHPx

GSHPx protein was significantly decreased with 10ng/ml of TNF-α, but was unchanged with 1 and 20ng/ml of TNF-α. The protein level did not change with any of the IL-10 concentrations. There was no significant difference in GSHPx protein levels as compared to control with any combination treatment. However GSHPx protein levels were increased in all combination groups as compared to levels with 10ng/ml of TNF-α with the highest increase at 10/10ng/ml. H_2O_2 treatment caused a 92% decrease in levels GSHPx protein (Fig 16A).

GSHPx mRNA levels were significantly increased by TNF-α at 10ng/ml and by IL-10 at 20ng/ml concentration. There was no significant difference in GSHPx mRNA with any of the combination treatments. H_2O_2 treatment caused a 78 % decrease in GSHPx mRNA levels (Fig 16B).

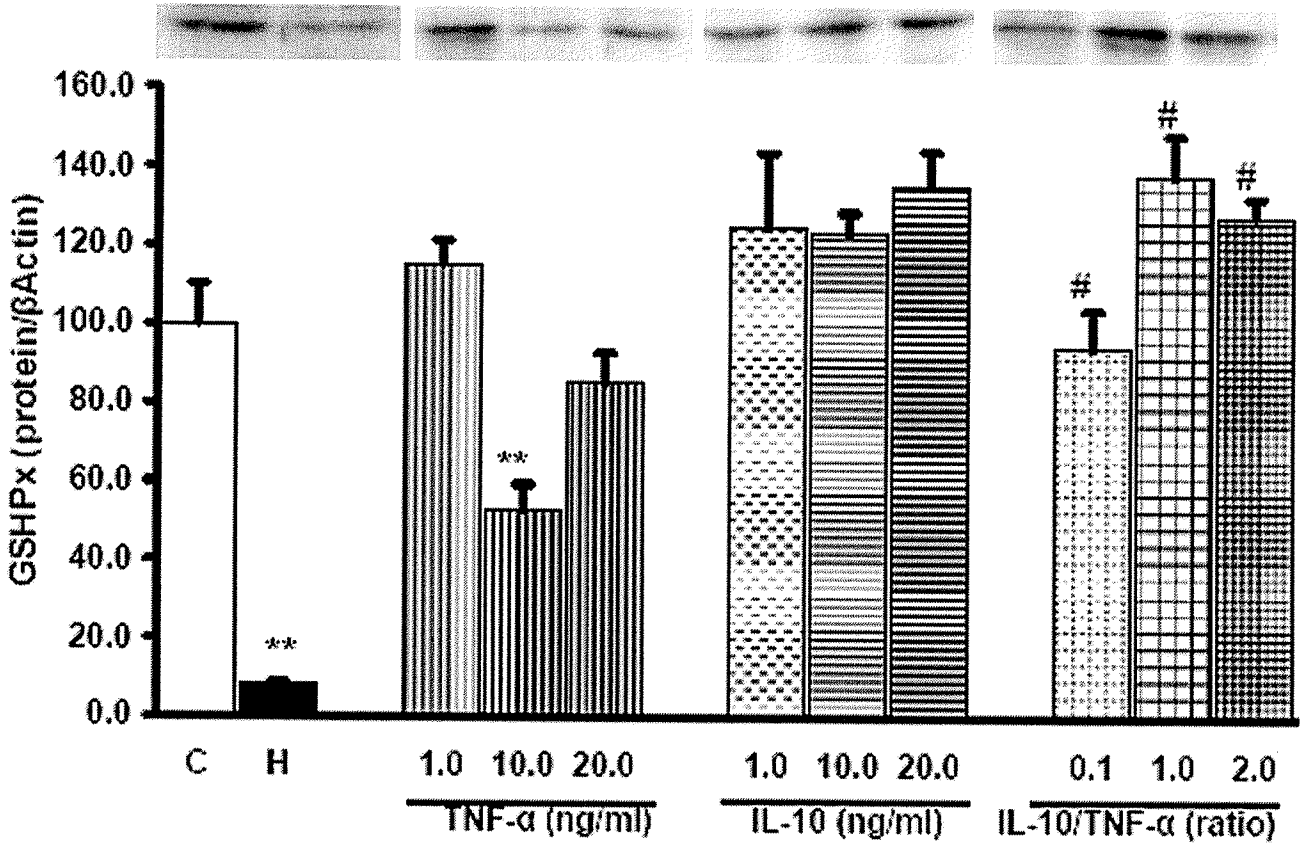


Fig 16A: Effects of H_2O_2, TNF-α, IL-10 and IL-10/TNF-α combination treatments on GSHPx (Glutathione peroxidase) protein levels. For combination treatment, the TNF-α concentration was kept at 10ng/ml, whereas IL-10 was used in three different concentrations (1, 10 and 20 ng/ml). Upper panel; representative protein bands for GSHPx. Lower panel; densitometric data. Data are Mean ± SEM from 5 experiments. Significantly different (*p< 0.05, **p<0.005) vs Cont; (# p< 0.05) vs 10ng/ml TNF-α. Control values were taken as 100%. Control (C); H_2O_2 100 μM (H).

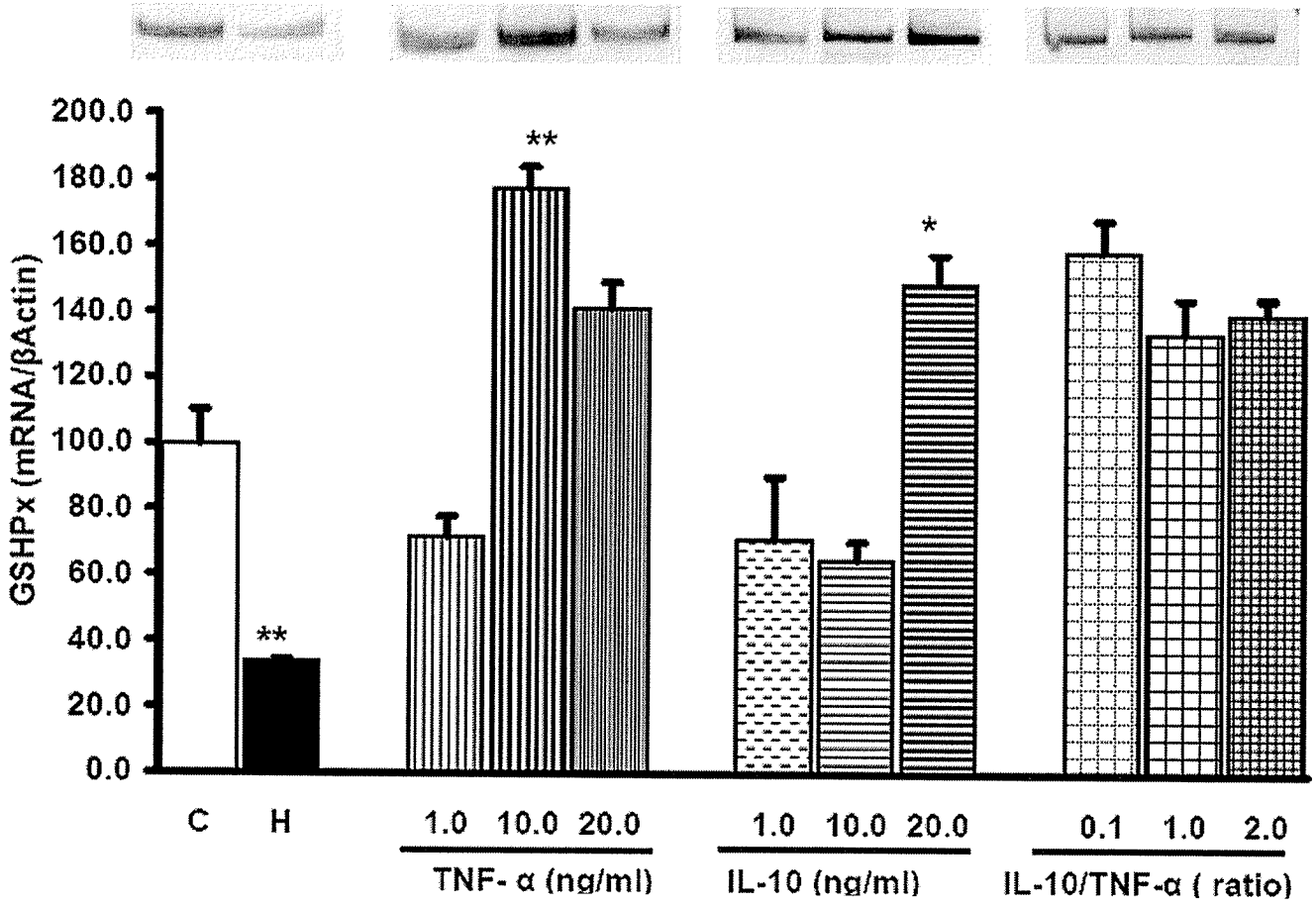


Fig 16B: Effects of H_2O_2, TNF-α, IL-10 and IL-10/TNF-α combination treatments on GSHPx (Glutathione peroxidase) mRNA levels. For combination treatment, the TNF-α concentration was kept at 10ng/ml, whereas IL-10 was used in three different concentrations (1, 10 and 20 ng/ml).Upper panel, representative RT-PCR bands for GSHPx mRNA. Lower panel, densitometric data. Data are Mean ± SEM from 5 experiments. Significantly different (*$p< 0.05$; **$p<0.005$) vs Cont; (# $p< 0.05$) vs 10ng/ml TNF-α. Control values were taken as 100%. Control (C); H_2O_2 100 μM (H).

For GSHPx alone, I studied the enzyme activity and the data are shown in Fig 17. Baseline value for glutathione activity was 54.95 nmole/mg protein. After 1ng/ml of TNF-α, there was no change in the glutathione activity whereas this activity was significantly decreased by more then 20% by TNF-α at 10 ng/ml and 20 ng/ml concentrations. H_2O_2 treatment significantly decreased this activity by 40% as compared to control (Fig 17).

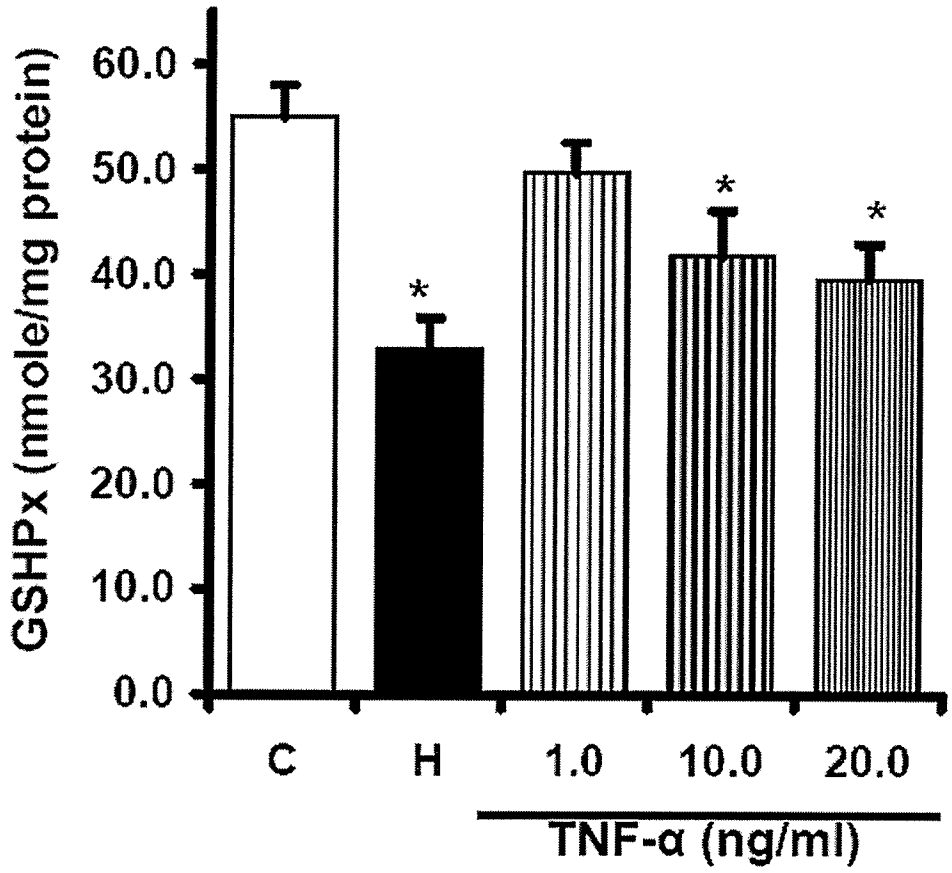


Fig 17: Effects of H_2O_2 and TNF-α treatments on GSHPx (Glutathione peroxidase) enzyme activity. GSHPx activity is expressed as nM of reduced NADPH oxidized to NADH per minute per milligram protein. TNF-α was used in three different concentrations (1, 10 and 20 ng/ml). Data are Mean± SEM from 5 experiments. Significantly different (*$p < 0.05$) vs Cont. Control (C); H_2O_2 100 μM (H)

2.3Oxidative stress studies

2.3.1 Reactive oxygen species

The production of intracellular reactive oxygen species (ROS), as measured by green fluorescent intensity, was significantly increased by TNF-α at 10ng/ml (53%) and at 20ng/ml (56%) concentrations (Fig 18). With the three concentrations of IL-10, intracellular ROS levels ranged from 87% to 101% of control levels and these changes in intracellular ROS levels were no different from the control myocytes (Fig 18). The production of intracellular ROS was significantly increased (142%) in cardiac myocytes after H2O2 treatment (Fig 18).

Intracellular ROS levels were significantly increased (35%) at 1:10 ng/ml combination treatment concentration compared to control (Fig 18). There was no significant difference in intracellular ROS levels at 10:10 ng/ml or 20:10 ng/ml combination treatment as compared to control (Fig 18).

At 1:10 ng/ml combination there was no significant difference in intracellular ROS levels as compared to 10ng/ml TNF-α. At 10:10 ng/ml and 20:10 ng/ml combination concentrations intracellular ROS levels were significantly decreased to 65.6% and 71.8% when compared to levels with 10ng/ml TNF-α alone (Fig 18).

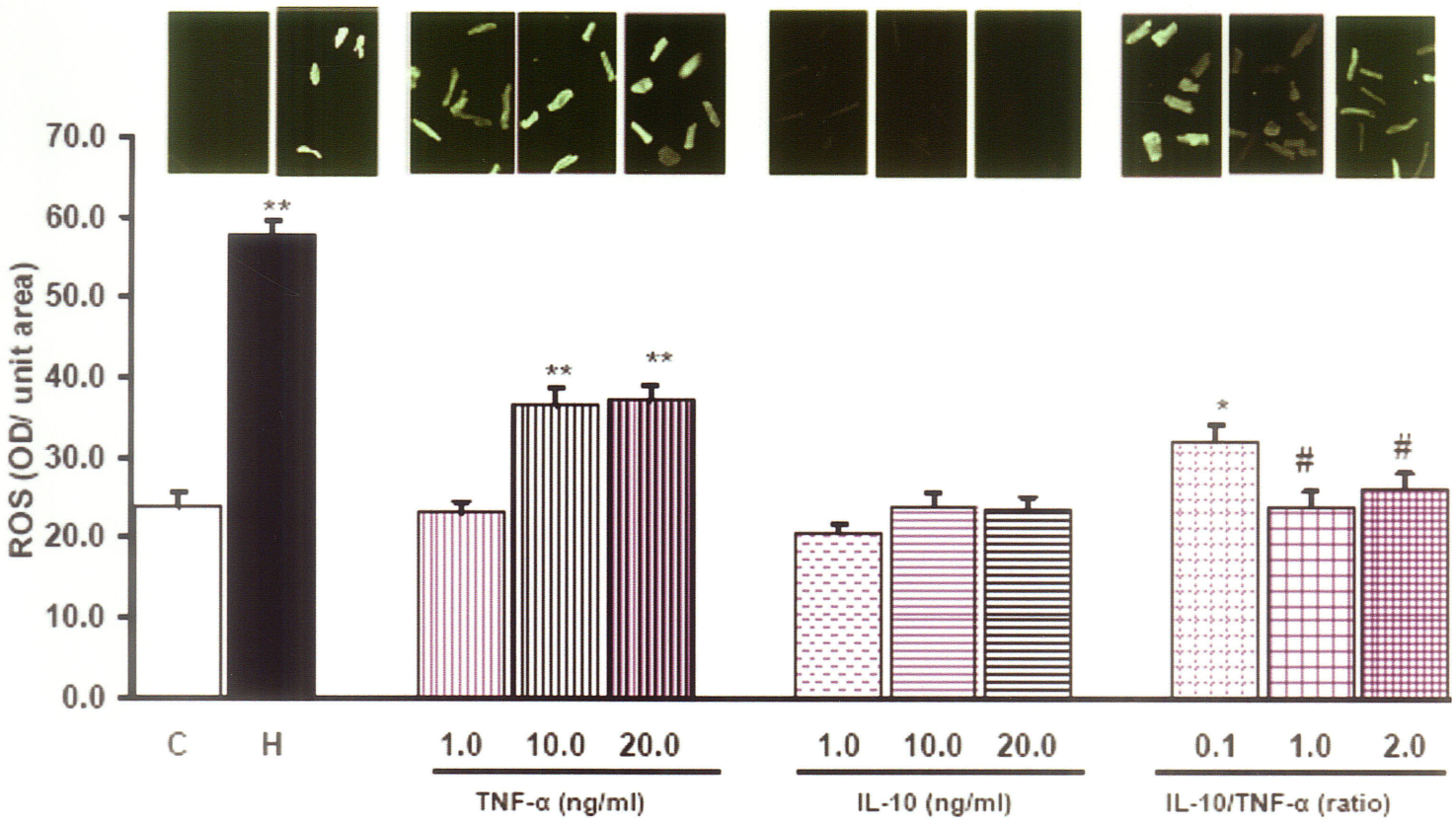


Fig. 18: Effects of H_2O_2, TNF-α, IL-10 and IL-10/TNF-α combination treatments on reactive oxygen species (ROS) generation. Upper panel; representative fluorescent microscope images for myocytes. Lower panel; fluorescent intensity data from 6 different experiments expressed as DCF fluorescence for ROS/ unit area. Data are Mean ± SEM. Significantly different (*$p< 0.05$; **$p<0.005$) vs Cont; (# $p< 0.05$) vs. 10ng/ml TNF-α. Combination treatment and symbols are the same as in Fig 13A.

2.3.2 Lipid hydroperoxides

There was no significant change in lipid hydroperoxides (LPO) after 1ng/ml of TNF-α treatment. LPO was significantly increased by >200% at 10ng/ml and 20ng/ml of TNF-α treatment (Fig 19). With the three concentrations of IL-10, LPO levels ranged from 95.9% to 142.3% of control levels and these values were not significantly different as compared to control. H_2O_2 treatment significantly increased LPO by 352% as compared to control levels, which was 2.74 ± 0.65 nm/mg protein (Fig 19).

There was no significant difference in LPO levels as compared to control at 10:10 ng/ml combination treatment. LPO levels were significantly increased by 224% and 163% at 1:10 ng/ml and 20:10 ng/ml combination treatment concentrations, as compared to control. As compared to 10ng/ml of TNF-α alone, LPO levels were significantly decreased to 59.9% at a combination ratio of 1.0 (Fig 19).

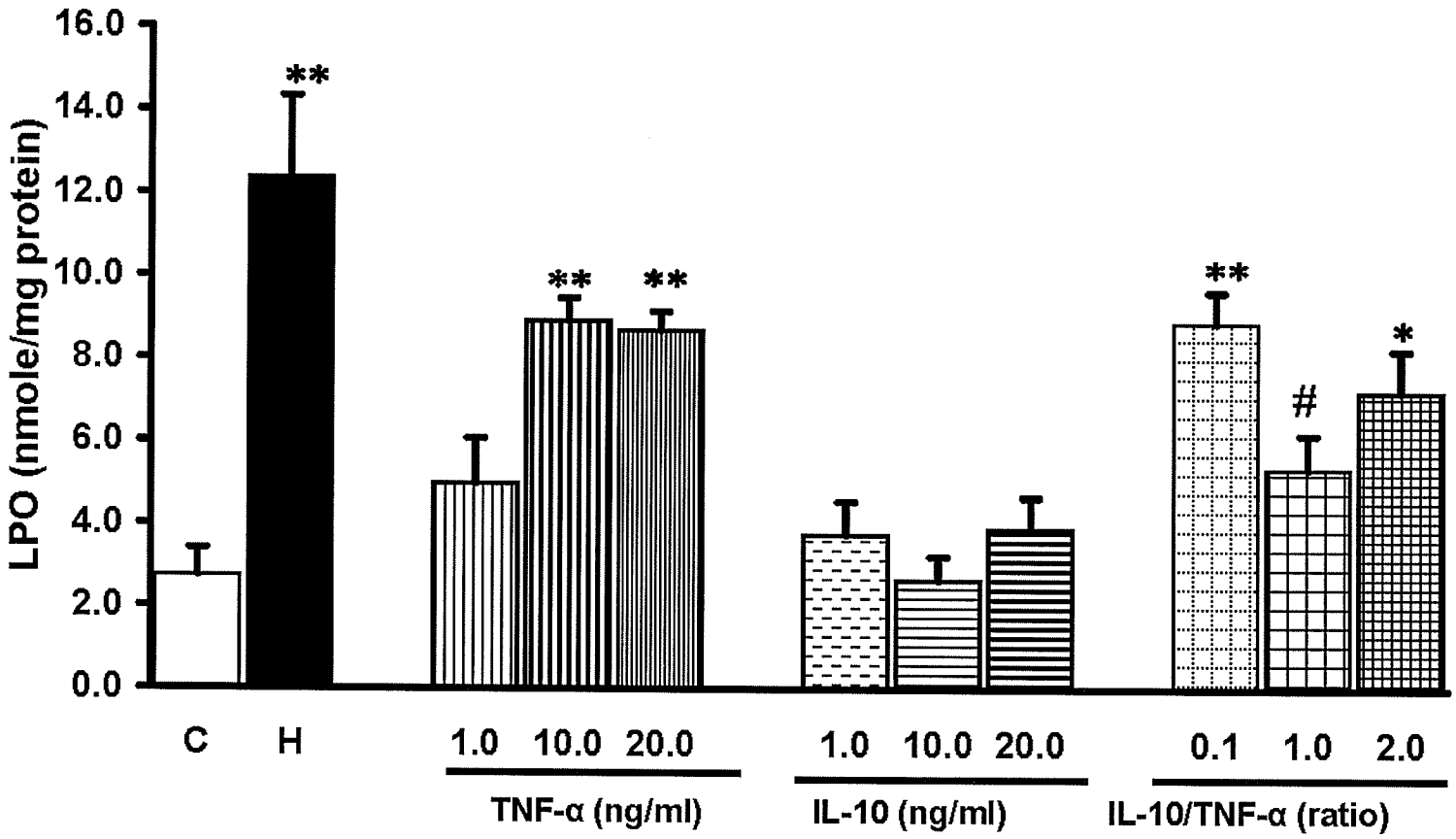


Fig 19: Effects of H_2O_2, TNF-α, IL-10 and IL-10/TNF-α combination treatments on generation of lipid hydroperoxides. For combination treatment, the TNF-α concentration was kept at 10ng/ml, whereas IL-10 was used in three different concentrations (1, 10 and 20 ng/ml). Data are Means ± SEM, N= 5. Significantly different (*$p< 0.05$; **$p<0.005$) vs. Cont; (# $p< 0.05$) vs. 10ng/ml TNF-α. Control (C); H_2O_2 100 μM (H).

Redox ratio was studied in normal and TNF-α treated cardiac myocytes. The baseline value for the redox ratio in normal untreated cardiac myocytes was 25.4. TNF-α treatment reduced the redox ratio both at 10 and 20ng/ml concentration and showed no effect at 1ng/ml concentration. The redox ratio was significantly decreased by 38% after H_2O_2 treatment (Fig 20).

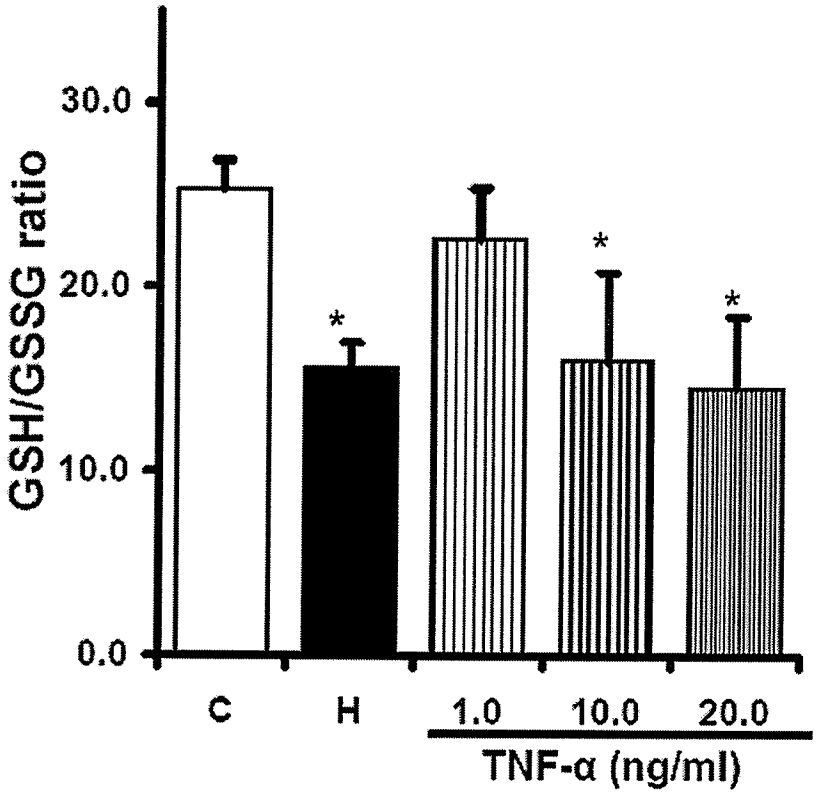


Fig 20: Effects of H_2O_2 and TNF-α treatments on Redox Ratio (GSH/GSSG, ratio of reduced to oxidized gluathione). TNF-α was used in three different concentrations (1, 10 and 20 ng/ml). Data are Mean ± SEM from 5 experiments. Significantly different (*$p < 0.05$) vs Cont. Control (C); H_2O_2 100 μM (H).

2.4 Cell leakage

There was no change in the release of Creatine Kinase (CK) in culture medium after 1ng/ml of TNF-α treatment. Release of CK was significantly increased (189%) at 10ng/ml and 20ng/ml (130%) of TNF-α. IL-10 treatment had no effect on release of CK when compared with control. CK release was significantly increased (285%) after H_2O_2 treatment of the cardiac myocytes (Fig 21A).

There was no significant difference in CK as compared to control with any of the combination treatment. At 10:10 ng/ml CK levels were significantly decreased to 47% of as compared to 10ng/ml of TNF-α levels. The differences in CK levels were insignificant at a ratio of 0.1 and 2.0 when compared to 10ng/ml of TNF-α alone (Fig 21A).

Though there was a 124% increase in the LDH release at 10ng/ml of TNF-α, this change was not significant. Similarly, there was no significant difference in LDH release as compared to control with IL-10 or any combination treatment. LDH release, as measured in the culture medium, was increased by 100 after H_2O_2 treatment of the cardiac myocytes but this increase was insignificant as well (Fig 21B).

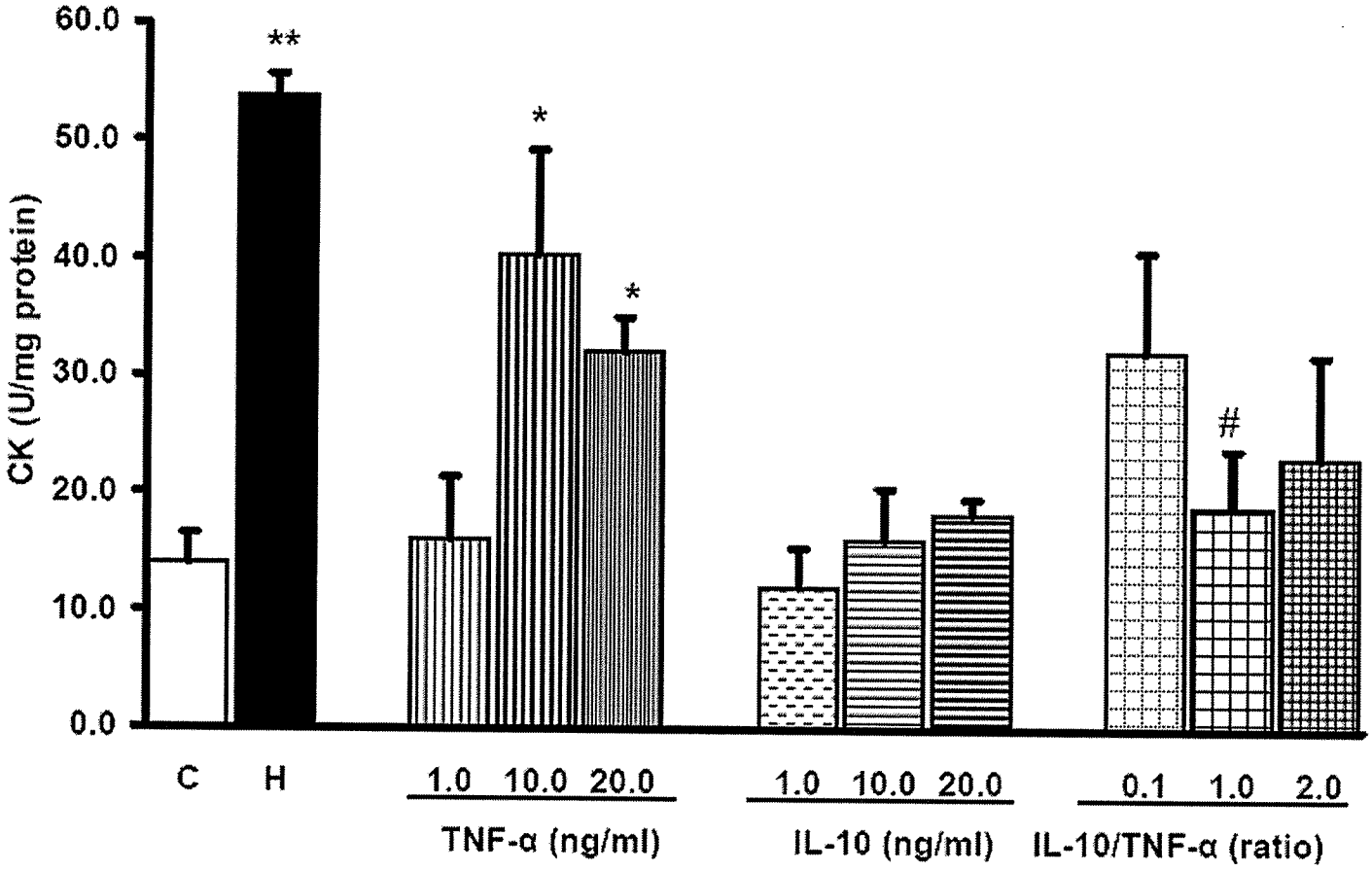


Fig. 21A: Effects of H_2O_2, TNF-α, IL-10 and IL-10/TNF-α combination treatments on Creatine Kinase (CK) release by adult rat cardiac myocytes. For combination treatment, the TNF-α concentration was kept at 10ng/ml, whereas IL-10 was used in three different concentrations (1, 10 and 20 ng/ml). Data are Means ± SEM, N= 5. Significantly different (*$p< 0.05$; **$p<0.005$) vs. Cont; (# $p< 0.05$) vs. 10ng/ml TNF-α. Combination treatment and symbols are the same as in Fig 13A. Control (C); H_2O_2 100 μM (H).

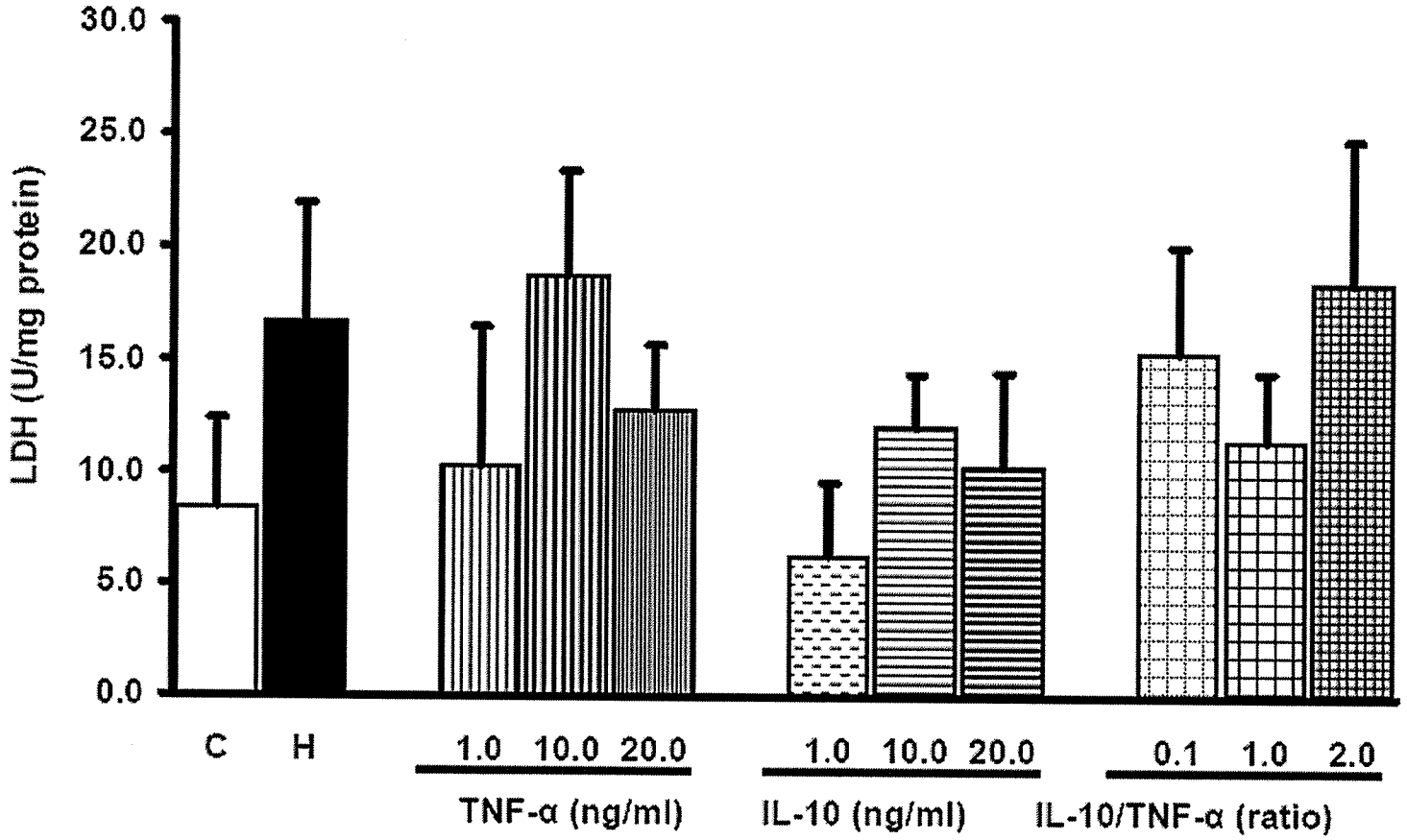


Fig. 21B: Effects of H_2O_2, TNF-α, IL-10 and IL-10/TNF-α combination treatments on Lactate dehydrogenase (LDH) release by adult rat cardiac myocytes. For combination treatment, the TNF-α concentration was kept at 10ng/ml, whereas IL-10 was used in three different concentrations (1, 10 and 20 ng/ml). Data are Means ± SEM, N= 5, significantly different (**$p<0.005$) vs. Cont. -α. Control (C); H_2O_2 100 μM (H).

VIII. DISCUSSION

The study approach was two prong in the first part I analyzed the TNF-α and IL-10 in relation to cardiac function in whole animal. In the second segment, I utilized isolated cardiac myocytes to examine the effect of these two cytokines on oxidative stress and their role in cardiac dysfunction.

1.0 TNF-α, IL-10 and heart failure (*In Vivo* Studies)

Cytokines have been suggested to play an important role in the pathogenesis of cardiovascular diseases. Several clinical studies have examined circulating levels of TNF-α and IL-10 in cardiac patients (Torre-Amione 2005; Giomarelli et al., 2003; Stumpf et al., 2003). In animal studies, changes in TNF-α subsequent to coronary ligation have been examined for only shorter time intervals i.e. 1-4 weeks (Francis et al., 2004; Diwan et al., 2004, Shames et al., 2002), whereas changes in myocardial levels of IL-10 subsequent to myocardial infarction have not been reported.

The present study describes changes in TNF-α and IL-10 at different durations of post myocardial infarction in relation to the cardiac function. Furthermore, levels of membrane bound as well as soluble fractions of TNF-α and IL-10 have been examined separately. Based on the data obtained in this study, it is suggested that a decrease in IL-10 as well as IL-10/TNF-α ratio correlates with a decrease in cardiac function subsequent to coronary ligation.

1.1 Myocardial infarction and heart failure

At 1W PMI, ejection fraction, fractional shortening, ±dP/dt and LVPSP were not different from sham controls. An increase in the LVEDP was the only indication of some cardiac dysfunction at this stage. However, the hemodynamic function declined at 4W PMI and the condition progressed to clear signs of severe congestive heart failure in the 16W PMI animals. These hemodynamic observations were also confirmed by the echocardiographic data. Thus, heart failure, subsequent to myocardial infarction, in this model of coronary ligation was established. Similar findings have also been reported by many others (Dixon et al., 1992, Khaper and Singal, 2001)

1.2 TNF-α and heart failure

Baseline values for the membrane bound fraction of TNF-α (mTNF-α) were 8.17pg/mg protein and for the soluble fraction (sTNF-α) was 4.11 pg/mg protein. These values are in the range for a normal heart, as has also been reported by others (Francis et al., 2004b; Diwan et al., 2004; Shames et al., 2002). At 1W PMI the ratio of mTNF-α to sTNF-α showed an increase to 2.5 from a control value of ~ 2. This increase was mainly due to higher increase in the mTNF-α level in the 1W PMI hearts. Similarly, a ratio of 2.7 has also been reported at 1W PMI in rats (Berthonneche et al., 2005). Over expression of TNF-α in heart failure has been known and is the subject of a recent review (Torre-Amione 2005).

mTNF-α has been suggested to have a more localized effect as compared to sTNF-α which is suggested to have more systemic effects. mTNF-α has also been

implicated in several other disease conditions such as acute hepatitis, rheumatoid arthritis and neurological disorders (Kusters et al., 1997; Abostini et al., 1995; Georogopoulos et al., 1996; Akassoglou et al., 1997). Our data on TNF-α at 1and 4W PMI as well as data from other laboratories (Francis et al., 2004; George et al., 2003; Irwin et al., 1999) are in agreement, showing a rise in this cytokine during early stages of heart failure. Furthermore, our data shows that at later stages of heart failure i.e. at 8 and 16 W PMI TNF-α levels return close to the baseline.

It is known that anti-TNF-α therapy is beneficial only if instituted in early stages of heart failure (Berthonneche et al., 2004). Etanercept, a soluble TNF-α receptor, capable of binding sTNF-α was found to be ineffective in patients with heart failure (Mann et al., 2004). Infliximab, a TNF-α monoclonal antibody was also ineffective in patients with frank heart failure (Chung et al., 2003). It is likely that an early rise in mTNF-α seen in this study has a role in the depressed cardiac function seen in early stages where anti-TNF-α therapy was also seen to be beneficial (Berthonneche et al., 2004). Our findings of lack of any change in TNF-α at later stages of heart failure infact may explain the failure of anti TNF-α therapy in the late stages of failure (Chung et al., 2003; Mann et al., 2004).

Early rise in TNF-α protein may have been due to an increase in mRNA for TNF-α at these stages. Interestingly, mRNA for TNF-α was significantly higher even in the 8W PMI group where TNF-α protein showed a drop towards the control levels. A delayed increase in myocardial mRNA for TNF-α during myocardial infarction has also

been reported by others (Ono et al., 1998). These data suggest that there is a disconnect between transcription and translation of TNF-α at later stages of heart failure and/or there is more degradation of this protein with the severity of the disease.

1.3 IL-10 and heart failure

The baseline level of membrane bound fraction of IL-10 (mIL-10) was about 7 times that of soluble fraction (sIL-10). The levels of mIL-10 were reduced at 1W PMI and this reduction was significant at 4, 8 and 16W PMI. This change correlated with the depressed cardiac function. Other investigators (Ukimura et al., 2003) have reported a direct correlation between the myocardial IL-10 mRNA and heart failure with time. The decrease in IL-10 protein could be due to a drop in the mRNA for IL-10 seen in this study in the failure stages. Interestingly, there was no change in the sIL-10 at any of the time points. Most of the clinical studies show no change in the levels of IL-10 in the plasma (Gullestad et al., 2001) or in the IL-10 production by mononuclear and monocytes in cardiac patients (Waehre et al., 2002; Aukrust et al., 1999). Any lack of change in circulating levels of this cytokine may be due to the lack of change in the soluble fraction of IL-10 in the heart as well as a lack of change in the production of sIL-10 by blood cells. Thus, a change in the mIL-10 in our study highlights that this cytokine protein may have a localized effect. It is important to note that this is the first study where both membrane bound and soluble fractions of TNF-α and IL-10 have been investigated during the progression of heart failure.

1.3.1 Interrelation of cytokines in heart failure

The IL-10/TNF-α ratio was significantly decreased at all post-ligation time points with the lowest level of 0.5 reached in 16W PMI group. Patients with advanced CHF (NHYA, III and IV) also show a low value (0.312) for IL-10/TNF-α ratio (Stumpf et al., 2003). Improved cardiac outcome after dexamethasone, growth hormone or steroid treatment, has been associated with an increase in IL-10 and/or decrease in TNF-α thus shifting the cytokine balance in favor of anti-inflammatory cytokines (El Azab et al., 2002; Adamopoulos et al., 2003; Giomarelli et al., 2003). Growth hormone treatment in idiopathic DCM improved the cytokine IL-10/TNF-α ratio from 1.9 to 3.5 (Adamopoulos et al., 2003). There are reports suggesting the role of imbalance between IL-10 and TNF-α in atherosclerotic lesions along with the occurrence of stable and unstable angina (Mazzone et al., 1999; Waehre et al., 2002; Mallat et al., 1999).

1.4 Function improvement, heart failure and cytokines

In order to further test the validity of the IL-10/TNF-α correlation with the PMI cardiac function, we used Losartan to improve the cardiac function and examine its relationship with the IL-10/TNF-α ratio. Although, activation of the rennin-angiotensin system (RAS) is a compensatory response, over a period of time this compensation becomes detrimental to heart function. Losartan, selectively and specifically inhibits the angiotensin II type 1 (AT_1)-mediated actions (Johnston, 1995) and improves survival and clinical outcomes in humans (Pitt 1997; Eisenberg and Gioia 2006) as well as in animals (Milavetz et al., 1996; Dickstein, 2001). Since maximum rise in TNF-α was seen at 4W PMI, losartan treatment was started immediately after the surgery and was continued for 4 weeks. Improvement of function by Losartan was seen to be associated with a decrease

in TNF-α as well as an increase in IL10. Based on this data, it can be concluded that at 4W PMI there is an inverse relationship between an increase in TNF-α (both membrane bound and soluble fraction) and a decrease in cardiac function. Whereas a decrease in IL-10, as well as the ratio of IL-10/TNF-α, had a direct correlation with a decrease in function. This correlation held true when the function in coronary ligated animals was improved by losartan treatment.

The change in the balance between anti-inflammatory (IL-10) and pro-inflammatory (TNF-α) cytokines in this study suggest the importance of examining the ratio between IL-10 and TNF-α rather then the changes in TNF-α levels alone. Although TNF-α protein did not show any change in later stages of heart failure, there was a great reduction in the IL-10/ TNF-α ratio. This correlated with depressed cardiac function suggesting that even though there was no increase in the inflammatory cytokine, TNF-α, a decrease in anti-inflammatory cytokine, IL-10 may also cause progression of disease by affecting other inflammatory proteins transcriptionaly controlled by NF-κB (Fiorentino et al., 1989). It has been suggested that IL-10 may exert anti-inflammatory properties by inhibiting NF-kB by two ways: 1) Blocking NF-κB translocation by inhibitory κB kinase (IKK) activity thus preventing degradation of inhibitory κB (IκB) (Waehre et al., 2002) and 2) IL-10 may also inhibit DNA binding of NF-κB, already present in the nucleus, with no increase in the nuclear levels of IκB (Schottelius et al., 1999). It is also important to note that for describing the role of complex molecules like cytokines in heart failure, membrane bound versus soluble fractions should also be recorded.

2.0 Cytokine production and oxidative stress (*In vitro* studies)

The available evidence suggests that TNF-α increases oxidative stress whereas IL-10 is known to reduce reactive oxygen species (ROS) (Suzuki et al., 1997; Moore et al., 2001). Thus a decrease in the ratio of IL-10/TNF-α, as seen in this study, can lead to an increase in oxidative stress. It is also known that increased oxidative stress has a detrimental effect on heart structure and function (Kaul et al., 1993; Singh et al., 2000). An imbalance between the production of ROS and the levels of antioxidant enzymes has been well documented during the pathogenesis of heart failure both in animal models and in clinical trials (Ungvari et al., 2005, Singal et al., 2000). Using the isolated cardiac myocytes, we tested the probability that a decrease in IL-10/TNF-α ratio can cause an increase in oxidative stress and cardiac myocyte dysfunction in heart failure subsequent to myocardial infarction.

Different cardiac cells, including myocytes, are capable of producing TNF-α (Comstock et al., 1998; Wright et al., 2002). Although production of IL-10 by immune cells and human skin cells has been documented, there has been no parallel data regarding the production of IL-10 by adult cardiac myocytes in culture. In the present study, by using lipopolysachride stimulation, we documented that adult cardiac myocytes are capable of producing IL-10 as well as TNF-α proteins. Following this conformation, we designed experiments to determine the cardiospecific effects of these two cytokines on oxidative stress.

2.1 Cytokines and oxidative stress

Stimulation of mammalian cells with TNF-α triggers the generation of various ROS. Furthermore, the use of exogenous ROS is known to mimic some of the biological effects of TNF-α (Hennet et al., 1993, Suzuki et al., 1997). Antioxidants such as butylated hydroxyanisole and catalase inhibit TNF-α induced cardiac hypertrophy in cultured cardiac myocytes (Nakamura et al., 1998) suggesting the production of ROS in such system. Thus, exogenous TNF-α can lead to a state of oxidative stress in a number of cell types.

In earlier studies, neonatal cardiac myocytes were used to examine the role of TNF-α induced oxidative stress (Suematsu et al., 2003; Nakamura et al., 1998). However, the consequences of direct exposure of adult cardiac myocytes to TNF-α in terms of changes in antioxidant enzymes and ROS are not known. In the present study, using DCFDA, an increase in ROS was noted in cardiac myocytes exposed to TNF-α. This effect was blocked by IL-10. Production of TNF-α induced ROS was also confirmed by an increase in lipid peroxidation. These data show for the first time that TNF-α not only increases the generation of ROS but also decreases antioxidant enzymes at both the transcription and translation level, thus, creating a state of oxidative stress. This change in oxidative stress was associated with an increase in cell injury as indicated by the observed increase in creatine kinase release in this study.

The fact that oxidative stress can cause myocytes injury was confirmed by the positive control studies using H_2O_2, as an exogenous source of ROS. Previous studies,

using cardiac myocytes, have shown that 50-150 μM of H_2O_2 increased lipid peroxidation, decreased antioxidant enzyme activity and increased cell injury (Kang et al., 2005; Lin et al., 2005). Thus in the present study, we chose 100 μM of H_2O_2 as a positive control to induce oxidative stress and study cell injury in adult cardiac myocytes. It was observed that in adult cardiac myocytes, H_2O_2 caused a significant decrease in protein and mRNA content of all the antioxidant enzymes along with increased intracellular ROS production, lipid peroxidation and cell injury as measured by CK release. A direct damage to proteins, lipids and DNA due to oxidative stress has been reported by others (Kim et al., 2000)

It should be noted that the concentration of TNF-α used in this study is comparable to that used in studies on isolated neonatal cardiac myocytes, monocytes or endothelial cells, reporting an increase in oxidative stress (Suematsu et al., 2003). In adult cardiac myocytes, in my study, TNF-α (≥10 ng/ml) induced an increase in ROS generation, lipid peroxidation and GSSG concentration. This TNF-α induced increase in ROS generation is also consistent with studies in transgenic mice overexpressing TNF-α that have shown an increased production of hydroxy radicals in the myocardium (Machida et al., 2003). In isolated neonatal cardiac myocytes, exogenous TNF-α (10 ng/ml) increased GSSG concentration (Ishii et al., 1992; Marcho et al., 1991). Thus, these data substantiate the concept that, during the pathogenesis of heart disease, increased expression of TNF-α leads to increased generation of ROS in adult cardiac myocytes.

In this study, we noted a significant decrease in the level of MnSOD mRNA and

protein whereas there was no change in Cu/Zn-SOD protein and mRNA. In a previous report, cardiac specific overexpression of TNF-α caused a significant decrease in MnSOD mRNA alone without affecting Cu/Zn-SOD mRNA (Machida et al., 2003). This enzyme selective effect may suggest a higher sensitivity of the mitochondria and/or MnSOD in heart cells. Although, induction of MnSOD mRNA by TNF-α has been reported in some other cell types (Wong and Goeddel 1988) but this increase was not associated with increased protein level (Czaja et al., 1994). The previously reported increased MnSOD may be specific for those cell types and may be a mechanism of resistance to TNF-α in those cells. Cardiac myocytes, on the other hand, which show a decrease in antioxidant enzymes, are likely more sensitive to TNF-α induced injury.

Another finding was that GSHPx protein was decreased and mRNA was increased at 10ng/ml of TNF-α, whereas an opposite trend was seen at higher dose (20ng/ml). However that GSHPx activity was decreased both at 10 and 20 ng/ml of TNF-α. On the other hand, catalase protein was decreased when cells were treated with TNF-α ≥10ng/ml While catalase mRNA was decreased at 20ng/ml of TNF-α concentration. In agreement with our work, TNF-α mediated decrease in catalase activity has been reported in liver (Yasmineh et al., 1991). It is well documented that both catalase and GSHPx are involved in detoxification of H_2O_2. GSHPx is involved in H_2O_2 detoxification at lower concentrations whereas catalase is the only antioxidant for H_2O_2 at higher concentrations. The results from our cardiac myocytes studies indicate that both catalase and GSHPx are involved in the detoxification of ROS generated by lower concentration of TNF-α whereas at higher levels of ROS catalase may have a predominant role.

Animals with cardiac specific overexpression of TNF-α show increased GSHPx mRNA but showed increased ROS generation and cardiac damage (Machida et al., 2003). Thus, the increase in GSHPx mRNA was not protective against ROS generation and lipid peroxidation and consequently the cell injury was still significantly high.

IL-10, an anti-inflammatory cytokine, is known to inhibit the secretion of pro-inflammatory cytokines as well as the release of ROS (Suzuki et al., 1997; Moore et al., 2001). Thus, IL-10 acts as a negative signal against the proinflammatory cytokine induced oxidative burst (Moore et al., 2001, Gougerot-Podicalo et al., 1996). Alveolar macrophages, isolated from IL-10 knockout mouse produce more superoxide anions as compared to those isolated from wild type when stimulated by chitin (Shibata et al., 1998). Furthermore, IL-10 treatment reduced renal ischemia-reperfusion induced lipid peroxidation and improved redox ratio, catalase activity and superoxide dismutase activity (Koken et al., 2004). Cu/Zn-SOD overexpressing transgenic mice show IL-10 associated protection against ozone-induced injury (Fakhrzadeh et al., 2004). In control cardiac myocytes, there was no difference in the levels of intracellular ROS or lipid peroxidation at any concentration of IL-10 used. IL- 10 treatment revealed no changes in protein levels of antioxidant enzymes whereas it increased RNA for these antioxidants at a dose of 20ng/ml. The only change in the levels of antioxidant mRNA at higher IL-10 concentration may be due to some direct effects of IL-10 on MAPK or STAT transcription factors. It is likely that IL-10 has an antioxidant like effect in a biological system where oxidative stress is higher or increased as discussed later.

Addition of IL-10 at a dose of 10 ng/ml inhibited TNF-∝ release from human peripheral blood mononuclear cells (Bolger et al., 2002). Furthermore, IL-10 decreased inflammatory stimulus mediated increase in ROS (Gunnett et al., 2000) and ROS mediated IkB degradation and thus activation of NF-kB (Dokka et al., 2001). IL-10 acts as an internal antagonist to various effects of TNF-α through its effects on NF-κB and other pathways. In our study, exogenous IL-10 mitigated TNF-α induced oxidative stress as well as cell injury thus suggesting that IL-10 could be acting as an internal antagonist to TNF-α induced oxidative stress and the cascade of changes due to this increase.

In the whole animal studies, IL-10/TNF-α ratio was 1 in control hearts. This ratio decreased with the progression of heart failure, suggesting an importance of balance between these two cytokines. Even in isolated myocytes, a ratio of 1 was found to be consistent with normal healthy cardiac myocytes. IL-10 treatment was able to antagonize the deleterious effects of TNF-α in regards to generation of ROS, antioxidant enzymes protein, mRNA, lipid peroxidation and CK release at a ratio of one. It should be noted that at higher concentrations, IL-10 did not provide additive protection. There was a decrease in protection in regards to Cu/Zn-SOD mRNA, MnSOD and catalase proteins and mRNA, Lipid peroxidation and CK release.Not only this, IL-10 in combination with certain other cytokines has been reported to be detrimental. In this regard IL-10 in combination with IL-2 has been shown to increase the frequency of CD8+ cytotoxic cells in mouse (Chen and Zlotnik 1991). When used in combination with IL-18, exogenous IL-10 was shown to enhance IFN gamma secretion by mouse NK cells (Shibata et al., 1998). These observations suggest that higher concentration IL-10 in combination with other

cytokines can activate some mechanisms, which have harmful effects on cardiac myocytes.

3.0 Conclusions

In conclusion, TNF-α may exert both localized and systemic effects primerly during early stages of heart failure whereas decreased IL-10 may have more localized effects during the progression of heart failure. The depressed cardiac function seems to correlates better with a decrease in IL-10 and/or IL-10/TNF-α ratio rather than with TNF-α levels alone. Improved function after losartan treatment correlated with improved IL-10/TNF-α ratio. IL-10 antagonized the oxidative stress component of TNF-α induced injury in isolated cardiac myocytes. Therefore, it is suggested that the reduced IL-10 or IL-10/TNF-α ratio may be a better predictor of the cardiac dysfunction during heart failure subsequent to myocardial infarction.

4.0 Future Studies

Clearly, studies are necessary to further elucidate the subcellular and molecular mechanisms to explain the effects of lower IL-10/TNF-α ratio as well as increased oxidative stress in causing myocardial dysfunction and heart failure. This could inclue the study of NF-κB and MAPK pathways as well as the impact of these changes on apoptosis. It would also be intresting to examine the effects of IL-10 and TNF-α antibody treatment on the pathogenesis of heart failure subsequent to myocardial infarction.

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