BIOCHEMICAL BASIS FOR ALTERATION OF MYOCARDIAL MECHANICS IN OXIDATIVE STRESS AND MARGINAL MAGNESIUM DEFICIENCY

A thesis presented

by

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To

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In partial fulfillment of the requirements
for the degree of

Doctor of Philosophy

Of

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Dedicated to
My parents and teachers

CERTIFICATE

I, L Manju, hereby certify that I had personally carried out the work depicted in the thesis entitled **"BIOCHEMICAL BASIS FOR ALTERATION OF MYOCARDIAL MECHANICS IN OXIDATIVE STRESS AND MARGINAL MAGNESIUM DEFICIENCY"** except where external help was sought and is acknowledged.

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DECLARATION

This is to certify that ***Mrs. L. Manju*** in the ***Division of Cellular and Molecular Cardiology*** of this Institute has fulfilled the requirements of the regulations relating to the nature and prescribed period of research for the Ph.D. degree of the Sree Chitra Tirunal Institute for Medical Sciences and Technology, Thiruvananthapuram - 695 011, Kerala, India. The work relating to her thesis entitled ***"BIOCHEMICAL BASIS FOR ALTERATION OF MYOCARDIAL MECHANICS IN OXIDATIVE STRESS AND MARGINAL MAGNESIUM DEFICIENCY"*** was carried out under my direct supervision.

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Dr. R. RENUKA NAIR
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L. Manju

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SYNOPSIS

INTRODUCTION

The extracellular milieu has a significant influence on the functional response of the myocardium. Studies during past two decades have established the role of magnesium as an ion influencing myocardial performance. Epidemiological studies trace the prevalence of cardiovascular disease and cardiac deaths in soft water areas to magnesium deficiency caused by sub-optimal intake. The role of Mg insufficiency in the precipitation of stress induced changes is often postulated, but the knowledge of underlying mechanisms is limited.

This scientific inquiry has been taken up with a view to understand the mechanical response of the myocardium to oxidative stress when associated with Mg deficiency, based on the hypothesis that " Marginal Mg deficiency augments the myocardial response to stress."

Marginal magnesium deficiency is characterized by decrease in extracellular magnesium with the maintenance of tissue levels. The choice of stress opted for this study is oxidative stress produced by reactive oxygen species (ROS) – superoxide anions and hydrogen peroxide. This study has therefore been designed with the following objectives

1) Record the variation in myocardial mechanics on exposure to ROS and understand the factors mediating inotropic changes.

2) Examine the inotropic response of myocardium to decrease in extracellular Mg and delineate the mechanism of action.

3) Examine the synergistic effect of marginal Mg deficiency and ROS on cardiac inotropy.

METHODOLOGY

1) Recording force of contraction

Adult Sprague-Dawley rats fed with standard diet (200±25 g) was used as the experimental model. Papillary muscles were carefully dissected from the left ventricle. The mural end of the muscle was fixed to a hook and tendinous end attached to a force transducer. Muscles were stimulated electrically at 0.5 Hz at a voltage ~10% above the threshold by pulses of 5 ms duration delivered through two platinum electrodes. After an initial equilibration period of 1 hr in modified Kreb's Ringer Hansleit (KRH) buffer, the muscles were stretched to the length at which the developed force was maximal. Contraction was recorded using a physiograph. The baseline contraction was recorded and the muscles were exposed to different experimental interventions and steady state contraction in response to the treatments were recorded, the response to the treatment was expressed as % variation from the baseline. Two different oxygen radical generators were used for the study - hypoxanthine - xanthine oxidase system (HX+XO) and hydrogen peroxide (H_2O_2).

2) Determination of diastolic calcium levels

In view of the important role of calcium in the maintenance of cardiac contractility intracellular diastolic calcium was determined. Cardiomyocytes

were isolated by retrograde perfusion of isolated heart with Ca free KRH buffer containing EGTA and taurine to relax the cells followed by perfusion with Ca free buffer containing collagenase and trypsin for dissolution of the extracellular matrix to dissociate the cells. The cells were loaded with acetoxy methyl ester of the fluorescent dye Fura-2. The diastolic Ca levels were assayed by spectrofluorimetric method.

3) Role of ion channels and pumps

Role of ion channels and pumps in the mediation of mechanical changes were examined by exposing the papillary muscle to channel blockers first and then introducing the experimental variation. Inotropic variation in presence and absence of the blocker was examined to determine the role of the channel in the mediation of the contractile variation.

4) Biochemical variables influencing mechanical function

The biochemical variables influencing mechanical function was examined in the ventricular portion of Langendorff perfused preparations. The biochemical parameters examined were

a) Thio Barbituric Acid Reactive Substance (TBARS) :- as a measure of lipidperoxidation.

b) Lactate Dehydrogenase (LDH) :- as an indicator of tissue injury and

c) High energy phosphate compounds – Adenosine Tri Phosphate (ATP) and Creatine Phosphate (CP) :- as an index of energy metabolism, along with mitochondrial to cytosolic ratio of ATP.

The Langendorff perfused hearts were allowed to stabilize for 10' with modified KRH. The hearts were then perfused with different experimental interventions for 30'. The venous effluent in each case was collected for LDH assay. Ventricular portion was freeze-clamped in liquid nitrogen and kept at -80°C till analysis.

TBARS was assayed by method of Nichans and Samuelson. ATP was estimated using enzyme assay kit method. CP was assayed by method of Heinz. LDH assay was carried out by method of Worbleskie and La Due and, protein content was assayed by Lowry's method.

Isolation of subcellular fractions was carried out by differential centrifugation. 10% homogenate of the perfused ventricular portion was filtered and the filtrate was centrifuged at 800g to isolate the nuclear pellet. The pellet was saved and the supernatant was centrifuged at 15,000g for 20' to isolate the mitochondrial pellet. The supernatant can be considered as soluble cytosolic fraction. The efficiency of the process and purity of the preparation was checked by assaying the activity of the mitochondrial marker Succinate Dehydrogenase (SDH) in various fractions. It was found that more than 80% of SDH activity was associated with mitochondrial pellet, which confirms the efficiency of the processes. ATP content of subcellular fractions was determined by method of Heinz.

5) Statistical analysis

The data are presented as mean ± SD. Difference in selected means were evaluated by Student's t-test. Group means were compared by ANOVA. Significant difference was said to exist at $p<0.05$.

RESULTS

I) Myocardial mechanics

Inotropic response of the papillary muscle was measured in presence of

i) Two commonly used generators of reactive oxygen species (ROS) – superoxide anion generator HX+XO and H_2O_2

ii) Different concentrations of extracellular magnesium

iii) A combination of ROS and Mg insufficiency.

i) Inotropic response to reactive oxygen species

Both the reactive oxygen species superoxide anions and H_2O_2 produced a negative inotropic response, and the force of contraction was found to be inversely proportional to the concentration of ROS. In presence of 0.5mM HX+0.02U/ml XO the force of contraction was 83.25% and the contractile variation was found to be completely reversible in free radical free medium. Pretreatment with the scavenging enzyme superoxide dismutase did not produce any contractile variation at this concentration. At higher concentration 1mM HX+0.04U/ml XO the contraction was 68% and incomplete recovery was observed in free radical free medium.

Pretreatment with superoxide dismutase provided a partial protective effect. A combination of superoxide dismutase and catalase did not produce any additive effect.

H_2O_2 also produced a significant negative inotropy at a concentration as low as10 μM. At 10 μM concentration the force of contraction was found to be 91.52% of the baseline. Increasing the concentration of H_2O_2 from 10 μM to100 μM was associated with a concentration dependent decrease in the force of contraction. The recovery was found to be complete upto 50 μM, but at 100 μM incomplete recovery was observed in H_2O_2 free medium. Pretreatment with catalase also showed the same pattern.

Addition of free radical scavengers following the induction of contractile variation augmented the negative inotropic response.

ii) Inotropic response to extracellular magnesium

Sequential decrease in the concentration of extracellular Mg from 1.4mM to 0.48mM showed an increase in the force of contraction. Further decrease in the extracellular Mg was associated with a decrease in contractile force. The contractile force at 0.48mM was 58% more than that of the baseline. The magnesium concentration that produced the maximum inotropic response corresponds to serum Mg levels of animals on Mg deficient diet. Hence for further studies 0.48mM Mg was selected for the induction of marginal Mg deficiency.

iii) Synergistic effect of marginal Mg deficiency and ROS on myocardial mechanics

The force of contraction on exposure to ROS was significantly reduced in marginal Mg deficiency when compared to sufficiency. The reduction in force of contraction was found to be higher at higher concentration of ROS. At lower concentration of superoxide anion generator 0.5mM HX+0.02U/ml XO, the recovery was found to be complete in both Mg sufficiency and marginal Mg deficiency. At higher concentration 1mM HX+0.04U/ml XO an incomplete recovery was observed, the recovery being comparatively lower in marginal Mg deficiency. Pretreatment with superoxide dismutase also showed the same pattern. Eventhough H_2O_2 produced a significant negative inotropy at concentration as low as 10 μM, a significant difference between Mg sufficiency and marginal Mg deficiency was observed at 100 μM. In marginal Mg deficiency complete recovery of contraction was obtained only upto 20 μM in H_2O_2 free medium whereas it was 50 μM in Mg sufficiency. From 25 μM onwards incomplete recovery was obtained in marginal magnesium deficiency.

II) Delineation of mechanism responsible for mechanical change

i) Intracellular diastolic calcium

Diastolic Ca levels of cardiomyocytes showed the same pattern as that of contractile response in all the three cases.

ii) Role of ion channels and pumps in the mediation of mechanical change

The impact of individual ion channels and pumps which can possibly induce inotropic variations were assessed using competitive channel inhibitors. The inotropic variation produced by different treatments were compared with the change induced in the absence of antagonist.

The inotropic response to superoxide anions appear to be influenced by SL L type Ca channel, SR Ca pump and Na K ATPase. But the inotropic changes produced by T type Ca channel and SR Ca release channel were not significant. On exposure to H_2O_2 the inotropic response was significantly different in presence of SL L type Ca channel antagonist and SR Ca pump and Ca release channel inhibitors. T type Ca channel and Na K ATPase did not produce any contractile variation in presence of H_2O_2.

The positive inotropic response produced by marginal Mg deficiency was significantly reduced in presence of both L and T type Ca channel blockers indicating that the positive inotropy in marginal Mg deficiency is mediated through the voltage gated Ca channels. Na K ATPase was also found to be activated in marginal Mg deficiency. SR Ca release and uptake were not affected in marginal Mg deficiency.

The negative inotropic response to ROS was found to augmented in marginal Mg deficiency by both superoxide anion and H_2O_2. The augmented negative inotropic response in marginal magnesium deficiency appears to be

mediated by Na K ATPase in the presence of HX+XO and SL T type Ca channel and Ca pump with H_2O_2.

iii) Biochemical variables influencing mechanical function

a) Lipid peroxidation :- The extent of lipid peroxidation was assayed by the measurement of TBARS. The level of TBARS was significantly increased on exposure to ROS. TBARS was increased by 12% in the presence of HX+XO and 18% in presence of H_2O_2. Marginal Mg deficiency did not induce a significant increase in lipid peroxidation. But when marginal Mg deficiency was associated with free radical stress, an augmented response was observed, the increase being 60-66% in marginal Mg deficiency compared to 12-18% in Mg sufficient media.

b) Release of LDH :- LDH assay was carried out in the coronary effluent as a measure of tissue damage. LDH release increased from 19.6 to 35.4 mU/min/g tissue in presence of 0.5mM HX+0.02U XO and to 42.32 mU/min/g tissue on exposure to 100μM H_2O_2. In marginal Mg deficiency 31.92 mU/min/g LDH was released. LDH release also showed an augmented response when marginal Mg deficiency was associated with free radical stress. Marginal Mg deficiency in combination with superoxide anion generator released 74 mU/min/g tissue of LDH and H_2O_2 released 82.38 mU/min/g tissue of LDH.

c) High energy phosphate compounds :- High energy phosphate compounds are the source of energy for myocardial contraction. Hence ATP and CP were

assayed for delineating the possible involvement of these compounds in the induction of contractile variation.

ATP and CP levels were found to be significantly reduced from 26.37 and 39.8 μM/g protein respectively to 19.3 and 22.08 μM/g protein on exposure to 0.5mM HX+0.02U XO and to 16 and 14.03 in the presence of 100 μM H_2O_2. In marginal Mg deficiency ATP level was found to be unaltered with a significant reduction of CP from 39.8 to 21.45 μM/g protein suggesting the possibility of mobilisation of CP to ATP. In oxidative stress associated with Mg deficiency, the high energy phosphates ATP and CP were reduced significantly when compared to sufficiency indicating a synergistic action of oxidative stress and marginal Mg deficiency in the induction of unfavorable tissue response. Mitochondrial to cytosolic ratio of ATP was also assayed in presence of superoxide anion, H_2O_2, marginal Mg deficiency and a combination of marginal Mg deficiency and ROS. The mitochondrial to cytosolic ratio of ATP was significantly higher when compared to control, showing the possibility of impaired transport of ATP from mitochondrial matrix to cytosol. The synthesis of ATP is reduced and/ or utilisation of ATP is enhanced.

CONCLUSION

This study has indicated a dose dependent effect of both superoxide anion generator and H_2O_2 on myocardial inotropy. At lower concentrations the contractile variations produced were completely reversible, whereas at

higher concentrations partial recovery was observed. A basal level of antioxidant reserve is essential for producing defense against ROS as indicated by the protection against ROS produced by pretreatment with scavenging enzymes. The negative inotropic response to oxidative stress is mainly associated with a reduction of high energy phosphate compounds. Significantly higher levels of lipid peroxidation and tissue damage were also observed in free radical stress. In marginal Mg deficiency a positive inotropy was observed. The positive inotropy in marginal Mg deficiency appears to be mediated through voltage gated Ca channels. ATP levels were maintained in marginal Mg deficiency, whereas the transport of ATP from mitochondrial matrix to cytosol was significantly inhibited. Augmented negative inotropic response in marginal Mg deficiency in association with oxidative stress is mediated by enhanced lipid peroxidation and tissue damage associated with reduction of high energy phosphate compounds. Thus the study has experimentally validated the postulation that negative inotropic response of the myocardium to oxidative stress is augmented in marginal Mg deficiency.

ABBREVIATIONS

ADP :	Adenosine di phosphate
ACE:	Angiotensin Converting Enzyme
ATP :	Adenosine tri phosphate
ATPase :	Adenosine tri phophatase
BSA :	Bovine serum albumin
Ca :	Calcium
$[Ca^{2+}]_i$:	Intracellular calcium ion concentration
CHF:	Coronary Heart Failure
CP :	Creatine phosphate
CPCSEA :	Committee for the Purpose of Control and Supervision of Experimental Animals
CVD:	Cardio Vascular Disease
EGTA :	Ethylene glycol-bis (β-amino ethyl ether) N,N,N',N'-tetra acetic acid
FR :	Free radical
GADP :	Glyceraldehyde-3-phosphate dehydrogenase
G-6-PDH :	Glucose-6-phosphate dehydrogenase
HEPES :	N-2-Hydroxy ethyl piperazine sulphonic acid
HF :	Heart failure
HK :	Hexokinase
H_2O_2 :	Hydrogen peroxide
HX :	Hypoxanthine
KRB :	Krebs Ringer buffer
KRH :	Modified Kreb's Ringer Hanseleit buffer
LDH :	Lactate dehydrogenase

MDA :	Malone dialdehyde
Mg :	Magnesium
$[Mg^{2+}]_i$:	Intracellular magnesium ion concentration
$[Mg^{2+}]_o$:	Extracellular magnesium ion concentration
MI:	Myocardial Infarction
NADH :	Nicotinamide adenine dinucleotide reduced form
NADP :	Nicotinamide adenine dinucleotide phosphate
Na pump :	Sodium pump (Na K ATPase)
Na-Ca exchanger :	Sodium calcium exchanger
Na K ATPase :	Sodium potassium adenosine tri phosphatase (Sodium pump)
$NiCl_2$:	Nickel chloride
PGA :	Phospho glyceric acid
PGK :	Phospho glycerate kinase
PMS :	Phenazine methosulfate
PMN:	polymorphonuclear leucocytes
ROS :	Reactive oxygen species
SD :	Standard deviation
SL :	Sarcolemma
SOD :	Superoxide dismutase
SO :	Superoxide anion
SR :	Sarcoplasmic reticulum
TBA :	Thio barbituric acid
TBARS :	Thio barbituric acid reactive substance
TCA :	Tri chloro acetic acid
TEA :	Tri ethanol amine
XO :	Xanthine oxidase

I. INTRODUCTION

I. INTRODUCTION

Most of the changes in cardiac function are mediated through inotropic mechanism. Assessment of ventricular performance in terms of cardiac muscle mechanics is useful in understanding how the behavior of the heart is altered by various physiological stresses. Among the damaging events, which may influence cardiac contraction considerable attention is being devoted to the role of free radicals. Oxygen free radicals have a significant influence on adrenergic receptors and heart sarcolemmal Ca^{2+} pump activity (Dhalla *et al*, 1996b, Kaneko, 1991a, 1991b). Myocardial cells contain enzymatic scavengers of ROS such as SOD, glutathione peroxidase,catalase and coenzyme Q_{10} as well as nonenzymatic antioxidants like α-tocopherol and ascorbate (Burton *et al*, 1985, Kaul *et al*, 1993, Valen and Vaage, 1993). Deleterious effects of active oxygen species may become dominant when the balance between radical formation and removal is disturbed to produce oxidative stress. Substantial evidence suggests an important role of ROS in reperfusion injury (Kaul *et al*, 1993, Kukreja *et al*, 1992). A number of mechanisms have been identified as sources of oxidants in post – ischemic reperfusion, cell injury in inflammation, irradiation injury *etc.* Growing experimental evidence suggests the generation of ROS and its participation in cellular activation and intracellular signal transduction under subtoxic conditions. Free radical mediated damages is known to play a role in the cardio - toxicity of chemotherapeutic agents like doxorubicin.

The interest in the study of Mg deficiency in animals began early in this century, but our understanding of the complex mechanisms leading to injury of heart and other organs remains incomplete. Epidemiological studies trace the prevalence of cardiovascular disease and cardiac deaths in soft water areas to Mg deficiency caused by sub - optimal intake. Mg deficiency has been identified as a risk factor for cardiovascular diseases such as cardiac arrhythmias and coronary heart disease as well as hypertension and diabetes mellitus. It has been inferred that Mg is an important protective agent in acute myocardial infarction, which may work at least in three ways – delaying ischemic injury, reversing coronary flow restriction and aiding the recovery of cardiac metabolism; thus resulting in improved post ischemic recovery of cardiac function.

Investigation on the effects of Mg deficiency on cardiac function gains importance because of the presence of soft water in Kerala. Central Ground Water Board, Thiruvananthapuram observed that the Mg content of potable water of Kerala is less than 5 mg/ L in most of the regions (personal communication). In a survey conducted in the region, serum and red cell Mg was found to be low, significantly in children from low socio - economic group (Nair et al, 1995). Cardiovascular consequences of Mg deficiency is therefore expected to be of importance in this region.

Mg is critical to the proper functioning of many physiologic reactions, including those that are critical to the cardiovascular system. Mg deficiency

has not received the attention it deserves probably due to the absence of clinical symptoms. Mg deficiency concomitant with stress may be of clinical significance, leading to arrhythmic, haemodynamic and ischaemic changes in the heart. Chronic Mg deficiency is accompanied by increased FR generation. Seelig has pointed out that stress conditions if associated with Mg deficiency paradoxically increases the risk of cardiovascular damages including arrhythmia (Seelig, 1994)

Eventhough earlier studies have indicated the separate effects of oxygen derived FRs and Mg deficiency on myocardial damage, the synergistic effect of oxidative stress and Mg deficiency on the myocardium have not been investigated.

In the present study, the mechanism underlying the alteration in the contractile performance in rat papillary muscle transiently exposed to exogenously generated oxygen FRs both in Mg sufficiency and marginal Mg deficiency have been evaluated. The additive action of FR generation in Mg deficiency may be one of the reasons for enhanced sensitivity to stress in Mg deficiency. Clinical and experimental data on cardiac consequences of marginal Mg deficiency being limited, a number of factors need experimental validation. The role of Mg deficiency in the precipitation of stress induced changes have been postulated, but experimental evidence is lacking. This scientific inquiry has been taken up with a view of obtaining a better understanding of the contractile function and associated metabolic changes of

the heart to oxidative stress when associated with Mg deficiency, based on the hypothesis that **"Marginal magnesium deficiency augments the myocardial response to oxidative stress."**

Marginal Mg deficiency is characterised by decrease in extracellular Mg with the maintenance of tissue levels. The choice of stress opted for this study is oxidative stress produced by ROS (SO anion generating system-hypoxanthine + xanthine oxidase and H_2O_2). Although H_2O_2 is not a free radical, it is an unstable intermediate in the univalent pathway reducing oxygen to water, in which ROS are formed. This study has therefore been designed with the following objectives:

1) Record the variation in myocardial mechanics on exposure to ROS and understand the factors mediating inotropic changes.

2) Examine the inotropic response of myocardium to decrease in extracellular Mg and delineate the mechanism of action.

3) Examine the synergistic effect of marginal Mg deficiency and ROS on cardiac inotropy.

In vitro experimental models have been used for the study. Myocardial mechanics were measured in rat papillary muscle as the inotropic response to variation in the extracellular milieu. The mechanism mediating the inotropic changes was delineated using antagonists to channels and pumps likely to be affected by the variables examined.

The major advantage is that, in this multicellular preparation of relatively simple geometry, there is undisturbed extracellular matrix, and cellular connections and energy balance are maintained. Here endothelial cells and cardiomyocytes coexist and interact to sustain optimal performance, hence neurohormonal control and cardiac endothelial control of myocardial performance are well preserved.

In view of the important role of Ca in the maintenance of cardiac contractility, intracellular diastolic Ca was also determined. Diastolic Ca was assessed with the help of calcium sensitive fluorochromes, the fluorescence intensity being measured by the fluorescence - ratio method, using a dual wave length excitation spectroflurometer.

Biochemical changes associated with the inotropic changes were also investigated in FR stress alone, marginal Mg deficiency and a combination of both. Biochemical parameters were measured in ventricular portion of Langendorff perfused whole heart. Thio barbituric acid reactive substance (TBARS) was used as a measure of lipid peroxidation and lactate dehydrogenase (LDH) release as an indicator of tissue injury.

High-energy phosphate compounds adenosine triphosphate (ATP) and creatine phosphate (CP) were quantitated as an index of energy metabolism along with mitochondrial to cytosolic ratio of ATP.

As with any other experimental studies, experimental design of this study is also subject to certain limitations. In isolated papillary muscles

segmental nonuniformity is likely to be present. In contrast to *in vivo* conditions, the muscle preparations are superfused, rather than perfused with a physiological bathing solution. Use of thin muscle strips is expected to minimize this disadvantage.

The use of ion channel modulators for the identification of the mechanism responsible for inotropic changes, suffers a draw back. Most of the channel specific modulators cross - react with other ion channels. Antagonists with minimum cross - reaction have been chosen for this study. Another fact is that when one channel is blocked the other channels show a compensatory response. So the mechanical variation observed in response to an antagonist cannot be regarded as the consequence of blocking of the channels, but a large fraction would be the contribution of channel of interest with minor contribution from related channels. This is also true for pharmacological interventions used in the treatment of cardiovascular diseases that exert their effect by modulating the function of ion channels.

This study is therefore designed with the objective of assessing the inotropic response to variation in extracellular milieu and understand the mechanism of action based on the assumption that the mechanical changes may be mediated either by modulation of ion channels or availability of energy for contraction; or a combination of both of these factors.

II. REVIEW OF RELATED LITERATURE

II. REVIEW OF RELATED LITERATURE

II.1. FREE RADICALS: AN OVERVIEW

Dioxygen is present in all living organisms. Its concentration in different organs and tissues varies to a great extent, the highest being in lungs, skin, and heart and the lowest in intestine and the bile. Free radical stress is used as a generic term describing the involvement of reactive oxygen / nitrogen species in various human diseases. It is involved in numerous pathological processes such as aging, respiratory or cardiovascular disease, cancer, neurological pathologies such as dementia, or Parkinson's disease.

A "free radical" is defined as any atom, group of atoms or molecules with one unpaired electron occupying an outer orbital. Molecular oxygen, O_2, is a triplet in its ground state since it has two unpaired electrons in its outer orbital having parallel or unpaired spins. The parallel electron spin arrangement of O_2 prevents the direct addition of a pair of electrons unless an electron spin inversion occurs (Bellus *et al*, 1978). The one electron reduction of O_2, called the univalent pathway, predominates, and the complete reduction of oxygen involves the addition of four electrons and four protons to each oxygen molecule. The resulting intermediates are the superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($OH^\bullet$). Since these intermediates are very reactive, the modulation of

this reactivity has been a dominating evolutionary pressure since O_2 first appeared in the atmosphere.

II.1.1. Oxidative systems mediating tissue injury

A number of distinct oxidative tissue injury systems have been proposed to play a role in normal metabolic and disease states. Although these systems are clearly interdependent, they will be considered under specific categories

II.1.1.1. Superoxide anion radicals (SO): The chemistry of O_2^- has been intensively studied (Fee *et al*, 1977). Superoxide anion radical has the potential to react as a reducing agent, donating its extra electron, or as an oxidising agent in which it is reduced to H_2O_2. The reactivity of O_2^- with biological compounds in aqueous solution at physiological pH is limited by its spontaneous dismutation rate constant ($K \sim 2x10^{5}$/M/S). The presence of SOD increases this rate intracellularly (Fridovich, 1978). In the microenvironment in which SOD content is low, reactions that have rate constant competitive with spontaneous dismutation would occur. It has been suggested that O_2^- may be reactive in hydrophobic cellular microregions as a base, and at low pH, H_2O, it's protonated form, may play an oxidant role in membranes reacting with fatty acids and other hydrophobic compounds. The ability of O_2^- to cross plasma membrane via anion gaps is a mechanism by which extracellularly generated O_2^- may penetrate cell membranes reaching intracellular targets (Lynch *et al*, 1978). The ability of O_2^- to diffuse

relatively long distances, combined with its ability to reduce transition metals strategically located on or near important macromolecules such as DNA, may result in a selectivity of tissue macromolecular injury that has been called "site specific injury" (Chevion, 1989).

Sources of SO anions :- The source of SO anions can be (a) intracellular and (b)extracellular

(a) *Intracellular sources of ROS* :- Intracellular sources of ROS include mitochondria, enzymatic source and autoxidation reactions.

(i) Mitochondria :- Mitochondria isolated from a number of tissues, including heart, brain, lungs and liver are major sources of O_2^- and H_2O_2 (Boveris, 1977, Nohl *et al*, 1978, Floyd *et al*, 1984, Patole *et al*, 1986, Cino *et al* 1989). By spontaneous and/or enzymatic dismutation, O_2^- is an essential precursor of H_2O_2 in mitochondria (Nohl *et al*, 1978, Chance *et al*, 1979). Flohe (1982) has shown that malate and glutamate support O_2^- generation by intact cerebral mitochondria *in vitro*. Generation of O_2^- by submitochondrial particles have also been demonstrated. Hypoxia is associated with increased succinate-supported release of H_2O_2 by isolated cerebral and lung mitochondria (Cino *et al* 1989). Isolated mitochondria from a wide variety of tissues release extramitochondrial H_2O_2 as the dismutation product of at least a portion of their intramitochondrial O_2^- generation.

(ii) Enzymatic sources :- A number of intracellular enzymes are involved in oxidation reactions in which O_2 is reduced to O_2^-. Xanthine oxidase is the

most extensively studied (Parks *et al*, 1986), but aldehyde oxidase, dehydroorotic dehydrogenase, flavin dehydrogenase, and peroxidases (Kohler *et al*, 1989) are also examples. In normal tissues xanthine oxidase exists predominantly as a dehydrogenase enzyme, using NAD^+ as the electron acceptor (reaction 1). The dehydrogenase enzyme can be reversibly converted into oxidised form by the oxidation of sulfhydryl groups, or irreversibly converted into the oxidase form by proteolysis (Parks *et al*, 1986). Xanthine oxidase in the presence of O_2 oxidizes hypoxanthine and xanthine to xanthine and uric acid respectively, using O_2 as the electron acceptor (reactions 2and 3).

$$\text{Hypoxanthine} + H_2O + NAD^+ \xrightarrow{\text{XO}} \text{Xanthine} + NADH + H^+ \quad (1)$$

$$\text{Hypoxanthine} + O_2 \xrightarrow{\text{XO}} \text{Xanthine} + O_2^- + H_2O_2 \quad (2)$$

$$\text{Xanthine} + O_2 \xrightarrow{\text{XO}} \text{Uric acid} + O_2^- + H_2O_2 \quad (3)$$

At physiological pH and atmospheric O_2 concentration, about 20% of the total electron flux results in O_2^- production. Increasing the oxygen or lowering xanthine concentrations results in increased O_2^- and decreased H_2O_2 generation. The conversion of xanthine dehydrogenase to xanthine oxidase with the subsequent release of O_2^- has been suggested to play a critical role in ischemic-reperfusion injury (Granger *et al*, 1981, McCord, 1985).

(iii) Autoxidation :- The autoxidation of a number of intracellular compounds, including catecholamines, flavins, and ferredoxin result in O_2-release (Misra and Fridovich, 1971, Boveris, 1977)

***(b) Extracellular sources* :-** Extracellular sources of ROS include activated inflammatory cells and myeloperoxidase - dependent tissue injury.

(i) Activated inflammatory cell:- The discovery by Metchnikoff (Metchinikoff, 1905) of the role of the release of cytases (lysosomal products) by phagocytes in inflammation has heralded continuing research into the role of phagocytes in tissue injury. Inflammation may be defined as the organism's response to injury, and therefore, plays a role in all disease process. Specialized inflammatory cells such as polymorphonuclear leucocytes (PMN), macrophages and monocytes, possess formidable bactericidal arsenals. PMN release enzymes and other components from lysosomes, and specific granules release a number of oxidised arachidonic acid products (eicosinoids), and generate a flux of O_2- and resultant family of reactive species. Along with these components, macrophages, monocytes and other immunocompetent cells may release immunomodulating agents collectively known as lymphokines (Masuda *et al*, 1988, Del Maestro, 1991), that may also play an important role in inflammation.

The activation of inflammatory cells by the appropriate stimulus results in a collection of events that allow the release of O_2- and derived products, "the respiratory burst". Oxygen is reduced by an NADPH oxidase (Babior, 1978a, Babior, 1978b) located on the plasmalemmal membrane of inflammatory cells (Dewald *et al,* 1979). Since NADPH oxidase is surface bound, a proportion of the O_2^- flux is released into the extracellular space.

The ability of PMNs to reduce O_2 to O_2^- appears essential to the killing of some type of bacteria since individuals with chronic granulomatous disease, an inherited defect in O_2^-generation is associated with repeated bacterial infection (Babior, 1978a, Babior, 1978b). The majority of the O_2 consumed during the respiratory burst results in O_2^- generation which by spontaneous dismutation and/or SOD catalysis accounts for the H_2O_2 generation

(ii) Myeloperoxidase dependent tissue injury:- Myeloperoxidase catalyses the oxidation of a number of halides by H_2O_2 and such products as hypochlorite and O_2^- have been suggested as the bactericidal element of the system (Foote *et al*, 1980, Kanofsky *et al*, 1984). H_2O_2 generated as a result of NADPH oxidase function is used to oxidise a variety of halides like chloride, bromide and iodide into their corresponding hypohalous acids. Hypochlorous acid is a strong oxidant and capable of oxidizing a whole series of compounds that may be present in the extracellular space or lipid and protein components of plasmalemma. Various aminoacids can be oxidatively decarboxylated by myeloperoxidase in the presence of Cl^-. Controversy continues to revolve around the issue whether or not significant amounts of O_2^- are released during myeloperoxidase catalyzed reactions *in vivo* (Bellus, 1978, Foote *et al*, 1980, Kanofsky *et al*, 1984).

II.1.1.2. Hydrogen peroxide

Two types of reactions result in H_2O_2 generation: the divalent reduction of O_2 by enzymes such as urate, D-amino acid, and xanthine

oxidase and (Allen *et al*, 1989) the spontaneous or enzyme catalyzed dismutation of O_2 (Al-Timimi *et al*, 1977). The majority of the divalent enzymes that result in H_2O_2 generation are localized to specialized organelles called peroxisomes (Chance *et al*, 1979). Mitochondria may be one of the major intracellular source of H_2O_2 generation, although any intracellular sources of O_2^- can result in H_2O_2 production (Flohe, 1982). Hydrogen peroxide has been reported as a major mediator of oxidative stress and a potent mutagen. H_2O_2 can damage genomic DNA indirectly by inducing enzymatic DNA digestion in a pattern consistent with higher order chromatin degradation, ie; excision of chromatin loops and their oligomers from the chromosomes (Konat *et al*, 2001, Mouzannar *et al*, 2001). Konat (2003) has also shown that H_2O_2 can trigger cytoplasmic signalling pathways that activate matrix attachment region associated endonuclease resulting in higher order chromatin degradation, by which the genome is efficiently dismantled. If the concentration of H_2O_2 is sublethal partial higher order chromatin degradation may also occur. Such molecular events can lead to altered protein synthesis.

II.1.1.3. Hydroxyl radical

The $OH^{\cdot}$ is an extremely reactive and unstable oxidizing species, reacting with a wide variety of biological compounds in both hydrophobic and hydrophilic cellular microenvironments. Haber - Weiss (Haber and Weiss, 1934) described a set of reactions while studying Fenton chemistry.

One of these the so called Hydroxyl radical Haber – Weiss reaction, was initially suggested as the mechanism by which the $OH^{\bullet}$ a much stronger oxidant than O_2 or H_2O_2 alone, could be generated by their interaction (Fridovich, 1978).

$$O_2^{-} + H_2O_2 \longrightarrow O_2 + OH^{-} + OH^{\bullet}$$

The Haber – Weiss rection is slow (Koppenol *et al*, 1978). However, the presence of a metal chelate involving iron or copper that can undergo reduction by O_2 was suggested as the reaction series that occurred biologically (Halliwell, 1978b, 1978c, McCord and Day, 1978). The iron catalyzed Haber –Weiss reaction can be represented as follows

$$Me^{n+} \text{ chelate} + O_2 \longrightarrow Me^{(n-1)} \text{ chelate} + O_2^{\bullet}$$

$$Me^{(n-1)+} \text{ chelate} + H_2O_2 \longrightarrow Me^{n+} \text{ chelate} + OH^{\bullet} + OH$$

This oxidant appears to be responsible for at least a portion of the injury caused by $O_2^{-}$and H_2O_2 interaction (Halliwell, 1978b). The generation of the oxidizing species appears to be crucially dependent on the concentration and reactivity of the metal chelates present, and Cu (II) and Fe (IV) have been suggested to be the oxidants involved (Del Maestro, 1991). Hydroxyl radicals participate in hydrogen abstraction and electron transfer reactions also. Unlike $O_2^{-}$and H_2O_2, no enzyme system has been identified which modulate OH reactivity directly.

II.1.1.4. Reactive nitrogen species (RNS)

Potent oxidants can be generated from reactions of NO with other reactive oxygen species. For example NO avidly reacts with superoxide anion at diffusion-limited rates to form the potent oxidant $ONOO^-$ (Beckman, 1996) The presence of $ONOO^-$ and its biological marker 3-nitrotyrosine has been associated with several oxidant related pathological conditions, including the atherosclerotic lesion, endothelial cell dysfunction, ischemia-reperfusion injury, myocardial infarction and heart failure (Buttery *et al*, 1996, Kooy *et al*, 1997, Bauersachs *et al*, 1999, Wattanapitayakul *et al*, 2000a). Clearly the actions of NO *in vivo* may be governed not only by production capabilities, but also by the setting and chemical environment in which it is formed. Reactions of $ONOO^-$ with pathologically relevant molecules have been implicated in oxidative-related cardiovascular disorders. It has also been reported that the $ONOO^-$ biomarker 3-nitrotyrosine is toxic to endothelial cells (Mihm *et al*, 2000).

II.1.2. Free radical modulation

Modulation of free radical reactivity is essential to the survival of aerobic organisms. This modulation involves a complex interactive process between radical generation and a number of enzymatic and nonenzymatic systems localized to hydrophilic and hydrophobic cellular microenvironments that control these reactive species (Dormandy, 1978, Fridovich, 1978, Halliwell, 1978a, Del Maestro, 1980,). In higher organisms,

the controlled release of oxygen derived free radicals by specialized inflammatory cells has been harnessed to perform a bactericidal function (Babior, 1978a, Babior, 1978b,). These same reactive species have also been implicated in the pathophysiology of a variety of disease processes, including inflammation (McCord, 1974, Del Maestro 1980, McCord *et al*, 1980), ischemia-reperfusion injury (Del Maestro, 1980, Dempoulos *et al*, 1980, Granger *et al*, 1981, Hearse *et al*, 1986, McCord, 1985, McCord, 1988), neoplasia (Oberley *et al*, 1981, Gower 1988) and aging (Harman *et al*, 1956, Floyd *et al*, 1984, Miquel *et al* 1984).

II.1.2.1. Scavenging enzymes

A number of enzymatic systems have evolved that can circumvent the electron spin restriction of O_2 reduction. The cytochrome oxidase complex localized to the inner mitochondrial membrane tetravalently reduces the majority of O_2 used by aerobic cells (Antonini *et al*, 1970, Del Maestro, 1980, Cino and Del Maestro, 1989). Mitochondria still appears to be the major intracellular source of both O_2^- and H_2O_2. (Boveris *et al*, 1972, Boveris, 1977, Chance *et al*, 1979, Cino *et al*, 1989). This continued O_2 flux has resulted in the evolution of a variety of superoxide dismutases that function by catalytically scavenging O_2^-. In animal cells, two metalloprotein enzymes, a copper and zinc containing superoxide dismutase (CuZnSOD) and a mangnese containing superoxide dismutase (MnSOD) reduce O_2^- to H_2O_2 (McCord *et al*, 1969, Beauchamp *et al*, 1973, Fridovich, 1975,

Fridovich, 1978, Fridovich, 1983). Marklund (1982, 1984a, 1984b) has described and characterized a mammalian SOD that contains Cu and Zn, but whose major anatomical location appears to be the extracellular space (EC-SOD).

Hydrogen peroxide, the divalent product of O_2 reduction, is decomposed to H_2O by catalase (CAT) and a variety of peroxidases. Glutathione peroxidase (GSH-Px) has been the most intensely studied enzyme of this group (Flohe 1982). These enzymes may be envisaged as a scavenging system in which each enzyme plays an integral role in free radical modulation. A variety of scavenging enzyme patterns have been found in normal (Marklund, 1980, Bannister and Bannister, 1987, Del Maestro, 1987, Del Maestro *et al*, 1989) and tumor tissues (Oberley *et al*, 1981, Marklund, 1982, Del Maestro, 1983), but the reason for this heterogeneity remains unclear. The subcellular concentration and location of these enzymes should be linked to the site of generation of the appropriate substrate and other components of the enzyme scavenging system. In man, the genes for CuZnSOD, MnSOD, CAT, and GSH-Px are located on chromosomes 21,6,11 and 3, respectively (Bannister and Bannister, 1987). Conceptually, a coordinated expression of gene activities would be expected. The cellular and subcellular activities of scavenging enzymes in a tissue are not static, being modulated by a number of factors. These factors include age, differentiation, alterations in intracellular generation of O_2^- and H_2O_2, local

availability of constituent metals and the local concentration of a number of immunomodulating agents called lymphokines.

Thus in summary, although specific enzymatic scavenging systems have evolved to deal with the intracellular products of O_2 reduction, these systems should not be considered static, but capable of responding dynamically to a number of distinct intracellular levels of free radical generation.

II.1.2.2. Hydrophobic mechanisms

The major hydrophobic region associated with cells are their lipid membranes which contain polyunsaturated fatty acids (PUFA). Lipid peroxidation reactions can be initiated by free radical species, resulting in chain propagating lipid radical reactions which can release lipid hydro peroxides (Gardner *et al*, 1983). A variety of hydrophobic scavengers such as tocopherols ($\alpha,\beta,\gamma,\delta$), collectively known as vitamin E and β-carotenes (Burton *et al*, 1984), intercalated into cellular membranes may prevent chain propagating reactions in lipid microenvironments. Vitamin E reacts with lipid hydroperoxides to form relatively stable vitamin E phenoxy radical. Because of its low reactivity it does not continue chain propagation reactions.

Lipid hydroperoxides are substrates for GSH-Px, but this soluble enzyme may not be able to reduce lipid hydroperoxides localized to hydrophobic microenvironments (Grossman *et al*, 1984). The coupling of the action of specific phospholipases, which can remove lipid hydroperoxides

from membrane coupled with subsequent hydroperoxide reduction by GSH-Px may occur. A number of other GSH-dependent systems, glutathione-S-transferases and a GSH- dependent protein derived from liver have been suggested to play roles in hydroperoxide reduction. Both tocopherols and β-carotene may quench singlet oxygen, modulating the ability of this molecule to interact with other cellular constituents (Del Maestro, 1991)

II.1.2.3. Hydrophilic mechanisms

Mechanisms controlling free radical reactivity in ionic or water cellular microenviroments, along with the extracellular space, have been outlined (Del Maestro, 1980). The oxidation of cellular, extracellular and membrane bound proteins can occur by a number of strong oxidants generated by free radical reactions (Del Maestro, 1980). Molecules such as ascorbic acid, cysteine, and reduced glutathione have all been considered to play a role in the prevention of this oxidation and the regeneration of normal protein structure. Ceruloplasmin and transferrin also have scavenging capacity *in vivo*. Ceruloplasmin appears to have the capacity to reduce O2 directly to H2O without release of reactive intermediates (Al-Timimi *et al*, 1977, Biemond *et al*, 1988).

The reactivity of the specific free radical generated, combined with the specific biochemical microenvironment in which it is generated, determines the biomolecular and subsequent cellular injury sustained by the organism. Each intracellular organelle has the hierarchy of scavenging mechanisms

localized to its individual hydrophobic and hydrophilic microregions, along with other mechanisms that may modulate free radical reactivity in that organelle's microenvironment. Intracellular scavenging systems are complex, appear interdependent on other systems, and may respond dynamically to a number of environmental alterations. Extracellular fluids such as plasma, synovial fluid, and cerebrospinal fluid possess low levels of SOD, CAT and GSH-Px and, therefore, may be more vulnerable to free radical injury (Marklund, 1980). The role played by EC-SOD in modulating O_2^- reactivity extracellularly gains importance in this contest. (Marklund, 1982, Marklund, 1984a, Marklund, 1984b,)

II.1.3. Mechanism of free radical mediated tissue injury

Increased free radical generation will occur intracellularly, extracellularly or both intracellularly and extracellularly which has been explained in detail. The major difficulty in deciphering intracellular free radical mechanisms in a complex tissue such as heart or brain is the heterogeneity of the tissue. Three defined free radical hypotheses have been proposed to explain the tissue injury seen in ischemia-reperfusion. They are the mitochondrial hypothesis, xanthine dehydrogenase-xanthine oxidase hypothesis and inflammatory hypothesis.

Mitochondrial hypothesis: During ischemia, the mitochondrial electron transport components dissociate, resulting in the accumulation of ubisemiquinone that autoxidizes on reperfusion, generating increased O_2^- and

H_2O_2 that overwhelms intrinsic scavenging mechanisms and contribute to intracellular injury (Dempoulos *et al*, 1980).

The xanthine dehydrogenase - xanthine oxidase hypothesis: During ischemia, xanthine dehydrogenase undergoes conversion to xanthine oxidase induced by a Ca^{2+} protease (induced by ischemia). Xanthine oxidase acts on hypoxanthine available from purine breakdown, and this results in increased O_2^- and H_2O_2 generation (Granger *et al*, 1981, McCord, 1985).

The inflammatory hypothesis: During ischemia, alterations occur in tissue, which results in the accumulation and activation of inflammatory cells that amplify tissue damage by their release of reactive products and physically plugging microvessels (McCord, 1974, 1988).

III.1.4. Ageing

Ageing is a progressive accumulation of changes with time that, in human occurs over decades. Harman (Harman, 1956) postulated that free radicals play a key role in the aging cascade. This concept has been modified to suggest that progressive free radical induced mitochondrial injury in postmitotic cells is central to the aging process (Miquel *et al*, 1984). The capacity of isolated rat heart mitochondria to release both O_2^- and H_2O_2 increases with age (Nohl, 1986). Isolated heart mitochondria subjected to hyperbaric oxygenation demostrated hydroperoxide accumulation and decreased cardiolipin and other fatty acid components of their membrane (Nohl *et al*, 1981). The scavenging enzyme content of cerebral tissue with

age has been assessed (Del Maestro *et al*, 1987). No change in rat or mouse brain total SOD activity was found between 8 and 32 months of age. The free radical theory of aging has provided valuable insights into the aging process and continued testing of this hypothesis may lead to further understanding of this universal phenomenon.

II.1.5. Oxidative pathways in cardiovascular disease

Cardiovascular disease (CVD) remains the principal cause of death in both developed and developing countries, accounting for roughly 20% of the world wide deaths per year. Since CVD has been viewed historically as a hemodynamic disorder, traditional approaches for its management originally relied heavily on drug therapies that reduce fluid retention and/or systemic blood pressure or enhance cardiac contractility (Wattanapitayakul *et al*, 2001). But hemodynamic status apparently is not a sole determinant in disease progression. For example, inhibition of the renin - angiotensin system, via angiotensin – converting enzyme (ACE) inhibition has provided symptomatic improvement as well as enhanced survival in long term trials (Sharpe, 1999, Yusuf *et al*, 2000). Evidence of regression and delayed progression of coronary artery disease was observed in large-scale, randomized secondary prevention trials of lipid lowering therapy during 1975-1996 (Brown and Zhao, 1999).

An emerging area in cardiovascular research is the apparent significance of biological oxidation mechanisms. Many recent studies have suggested that oxygen-derived free radicals may be important participants in a wide array of cardiac conditions, and several clinical trials have appreciated the use of antioxidants as a therapeutic measure.

Oxidative events in cardiovascular disease

The initial suggestion of oxidative mechanisms during CVD were described in the acute setting of ischemia/reperfusion injury and myocardial infarction (MI). These conditions are associated with a sudden reduction of coronary perfusion and oxygen availability, leading to altered myocardial metabolism, ROS production and cell death. Interestingly, ROS production and associated cellular damage is higher in cardiac tissue during reperfusion relative to ischemic conditions. Ischemia and reperfusion has provided insight into the mechanisms of ROS - induced alteration of cardiac function and disease progression. In addition to cellular and/or tissue evidence of oxidative damage, elevated levels of oxidative stress markers are detected in several pathological conditions of cardiovascular disorders, including hypertension, ventricular hypertrophy, atherosclerosis and congestive heart failure (CHF) (Carlos *et al,* 1998, Keith *et al*, 1998, Miller *et al*, 1998, Suzuki *et al*, 1998, Gokee *et al*, 1999, Harjai, 1999). Despite the diverse etiology of cardiovascular conditions, the enhanced production of ROS and

altered oxygen utilization is apparently a common phenomenon and a participant in disease progression.

The commonly occurring ROS: O_2^-, H_2O_2 and $HO^\bullet$ are found to play a major role in the etiology of cardiovascular disorders. Recent investigations have suggested that reactive nitrogen species peroxynitrite ($ONOO^-$) also plays an important role in cardiovascular dysfunction. While direct evidence of ROS induced cardiac injury during hypoxia or ischemia/reperfusion in humans is lacking (due to inadequate methodology), many studies have shown increases in biomarkers of oxidant production and/or decreases in antioxidant capacity during myocardial ischemia. (Miwa *et al*, 1999, Buffon *et al*, 2000). Furthermore, the administration of antioxidants reduces cardiac cell injury and dysfunction in acute MI (Singh *et al*, 1996), coronary angioplasty (Rajkumar *et al*, 1999) and open heart surgery that mimics myocardial ischemia/reperfusion (Fabiani *et al*, 1993).

As mentioned earlier uncoupling of mitochondrial electron transport chain is a classical mechanism of intracellular oxidant production. The lack of oxygen supply by hypoxia or ischemia disrupts mitochondrial electron transport chain, resulting in an accumulation of toxic metabolites, acidosis, ATP depletion, intracellular Ca^{2+} overload, mitochondrial membrane depolarization, matrix swelling and cell death (Lemasters *et al*, 1997). The generation of large amounts of ROS can overwhelm the intracellular antoxidant defence network, causing activation of neutrophils, lipid

peroxidation, protein modification and DNA breaks (Ambrosio & Tritto, 1999).

Infiltration of activated immune cells into cardiac muscle is a potential mechanism of cardiac oxidant production. The ROS burst during post ischemic reperfusion is accompanied by cell death and tissue injury, which can trigger an acute inflammatory response. The response of immune cell chemoattractants and surface cell adhesion molecules leads to the infiltration of the tissue by immune cells (particularly neutrophils). These activated neutrophils can cause more damage to the tissue by the secretion of several mediators, including ROS, proteolytic enzymes and pro-inflammatory cytokines. It has been shown that inhibition of neutrophil migration reduces reperfusion cardiac injury in ischemic human hearts (Fabiani *et al*, 1993). Further more clinical studies in cardiopulmonary bypass patients have demonstrated that neutrophil activation, leucocyte-platelet aggregation, and cytokine release from cardiac tissue are the primary contributors of an acute inflammatory reaction during post-ischemic reperfusion (Zahler *et al*, 1999). These findings suggest that neutrophils are key participants in at least the early events in acute hypoxia/ischemia. It is important to note that immune cell infiltration during chronic settings of cardiac disease is less well established.

Non-mitochondrial sources of ROS such as oxidative enzymes are equally or perhaps more important in some cardiovascular conditions. The

role of xanthine oxidase has been explained in detail. NADH/NADPH oxidase and nitric oxide synthase are two enzyme systems having important role in cardiovascular pathology.

The enzyme NADPH oxidase is found in phagocytic cells which plays an important role in non specific host defense during infection by generating a large quantity of superoxide (mM level). It has been demonstrated that vascular NADH/NADPH oxidase significantly contributes to superoxide production in all components of vasculature, ie; the endothelium, the medial smooth muscle and the adventitia (Bayraktutan *et al*, 1998; Di Wang *et al*, 1999). Angiotensin II (AngII) may activate superoxide production in vascular tissue via this enzyme. This enhanced production of superoxide has been implicated in the pathogenesis of AngII - induced vascular hypertrophy and endothelial cell dysfunction (Griendling *et al*, 1994; Rajagopalan *et al*, 1996; Ushio-Fukai *et al*, 1998; Wattanapitayakul *et al*, 2000a)

It has recently been recognized that several cytokines eg: interleukin [IL]-1β, IL-6, interferon-γ and tumor necrotic factor [TNF]-α) and growth factors (eg:insulin like growth factor-1 and transforming growth factor- β1) are pro-oxidants, and elevated levels are commonly found in the plasma of patients with heart disease. Increased nitric oxide (NO) production via induction of nitric oxide synthase (NOS) has been suggested as a major mechanism by which cytokines mediate cardiac contractile dysfunction and development of heart disease. Cytokine induced oxidants are regulated by

various stimuli, for example, acute hypoxia followed by reperfusion and in myocardial stunning. These cytokines enhance the expression of a variety of cell adhesion molecules (eg; intercellular adhesion molecule-1, vascular cell adhesion molecule, and monocyte chemoattractant protein-1) in the myocardium, leading to transient leukocyte sequestration and its transmigration to the areas of cardiac injury. Studies also suggest that the heart *per se* is capable of synthesizing biologically active TNF- α, which may be responsible for the progression of heart diseases (Wattanapitayakul *et al*, 2001). Additionally, other potent oxidants can be generated from reactions of NO with other ROS. For example NO avidly reacts with superoxide anion at diffusion-limited rates to form the potent oxidant $ONOO^-$ (Beckman, 1996). The presence of $ONOO^-$ and its biological marker 3-nitrotyrosine has been associated with several oxidant related pathological conditions, including atherosclerotic lesions, endothelial cell dysfunction, ischemia/reperfusion injury, MI and heart failure (Buttery *et al*, 1996, Kooy *et al*, 1997, Bauersachs *et al*, 1999, Wattanapitayakul *et al*, 2000a). The actions of NO *in vivo* is governed not only by production capabilities, but also by the setting and chemical environment in which it is formed. Reaction of $ONOO^-$ with pathologically relevant molecules has been implicated in oxidative related cardiovascular disorders, as given below. Evidences also indicate that the $ONOO^-$ biomarker 3-notrotyrosine is toxic to endothelial cells (Mihm *et al*, 2000).

II.1.6. Consequences of cardiac oxidative events

II.1.6.1. Cardiovascular dysfunction

In both experimental and pathological conditions, impaired vascular function and decreased cardiac function are mediated by ONOO- and other ROS (Miller *et al*, 1998, Ferdinandy *et al*, 1999). ROS have direct impact on myocardial function through inhibition of sarcoplasmic reticulum (SR) Ca^{2+}pump in the cardiac contraction - relaxation cycle (Matsubara and Dhalla, 1996). Benefits from inhibition of superoxide production have also been shown in cytokine-induced cardiac dysfunction (Cheng *et al*, 1999).

Studies by several groups have shown that reduction in bioavailability of NO and endothelial cell dysfunction may be initial events in atherosclerosis and cardiovascular disorders (Ferrari *et al*, 1998 Wattanapitayakul *et al*, 2000a). Additionally endothelial cell dysfunction is one of the earliest events in the pathogenesis of myocardial reperfusion injury, and NO plays an important role in cardioprotection during reperfusion by directly enhancing coronary blood flow and by preventing adhesion of immune cells (Kupatt *et al*, 1996). Early reduction in coronary NO release occurs simultaneously with the oxygen-derived free radical burst observed in ischemia/reperfused heart (Zahler *et al*, 1999), suggesting that superoxide induced reduction in NO may play a role in disease initiation and/or progression. Depending on the tissue availability, NO can act as a "good guy" or a "bad guy". ie; the net concentration of NO at tissue level may

predict its protective effects or toxic effects. Investigations suggest the genetic polymorphism of NOS3 isoform (also known as endothelial NOS) occur in humans and these molecular variations may play a role in NO control and CVD risk (Wattanapitayakul et al, 2000b).

II.1.6.2. Cell death

Two types of cell death – necrosis and apoptosis –are implicated in the oxidative-related cell loss in cardiovascular tissue. Generally, necrotic cell death is associated with inflammatory cell infiltration and subsequent collagen deposition and scar formation. Prolonged ischemic conditions may cause irreversible myocardial cell injury and cell death via necrosis. Transmigration of immune cells from the vasculature into the myocardium results in the release of toxic mediators that induce myocardial cell dysfunction and necrotic cell death (Grisham *et al*, 1998). Unlike apoptosis, necrosis generally does not appear in more chronic oxidative conditions, but occur primarily in prolonged ischemic conditions (Taimor *et al*, 1999).

Apoptotic cell death also known as programmed cell death in general produces ultrastructural and biochemical changes such as cytoplasmic and nuclear condensation, formation of membrane bound apoptotic body and DNA fragmentation. Apoptotic cell death accounts for a great proportion of cell death associated with MI and/or myocardial ischemia/reperfusion. Cell loss through apoptosis contributes to the impairment of cardiac performance, and also plays an important role in the myocardial and vascular remodelling

process. Induction of apoptosis is implicated in atherogenesis and cardiac dysfunction. The oxidative products of ROS and other secondary messenger molecules generated by ROS such as oxidised low density lipoprotein (oxLDL) can also produce DNA fragmentation and apoptosis in macrophages (Kinscherf *et al*, 1998).

ROS induced cardiac apoptosis is mediated through several signalling systems including intracellular Ca^{2+} and further activation of caspases, cytokines, lipid oxidation and proto-oncogene activation (Von Harsdorf *et al*, 1999). Thus ROS-induced changes in cellular signalling and gene expression, leading to apoptosis, involve multiple pathways which are complex and intertwined processes, requiring several selective participants in each pathologic condition or cell type.

II.1.6.3. Altered endogenous antioxidant defenses

Oxidative stress is a reflection of excess intracellular concentration of oxidants. Low levels of glutathione (GSH) are associated with a number of disease conditions known to generate ROS, such as observed in atherosclerosis, heart failure, diabetes, neurodegenerative disorders and acquired immuno deficiency syndrome (AIDS). Increased oxidative stress in CHF patients are associated with increased GSH peroxidase and decreased plasma antioxidant vitamins-vitamin C and E (Keith *et al*, 1998). Another important endogenous antioxidant defense against superoxide is the enzyme SOD. Three isoforms of SOD have been cloned and identified: mitochondrial

manganese containing SOD (Mn-SOD), cytosolic copper/zinc SOD (Cu/Zn-SOD) and extracellular SOD (EC-SOD). Cu/Zn -SOD plays an important role in protecting NO from destruction by superoxide in the endothelium (Harrison, 1997). Reduction in the antioxidant enzyme catalase have also been observed in animal models of cardiac disease (Singal *et al*, 1993; Lin *et al*, 1997). Interestingly, the potent oxidant $ONOO^-$ can inactivate the Mn-SOD via nitration of specific tyrosine residue at its active site further promoting ROS availability (Yamakura *et al*, 1998; MacMillan-Crow and Thompson, 1999).

II.1.6.4. Reactive oxygen species and ion channels

Biochemical evidence support the possible involvement of ion exchange mechanisms in cardiac SL, suggesting that FR injury may have profound effects on the electrical function of the myocardium. Oxygen free radical stress has been reported to induce arrhythmias in isolated perfused hearts (Nakaya *et al*, 1987, Hayashi *et al*, 1989) and alterations in electrical activity in various intact, multicellular and single myocyte preparations of myocardium. Several changes in activity have been described including a fall in resting membrane potential, a decline in action potential amplitude, a decrease in maximal rate of depolarization, a transient increase in plateau amplitude and a marked decrease in action potential duration and delayed after depolarization. The modulation of ion channels have been reported on superfusion of single myocytes with various oxygen radical generating

systems (Barrington *et al*, 1988, Bhatnagar *et al*, 1990, Barrington, 1990, Matsuura and Shattock, 1991a, Coetzee and Opie, 1992, Nakaya *et al*, 1992). Different mechanisms have been proposed and different sites of action within the myocardial cell have been identified as the target of attack to explain the deleterious effects of oxygen free radicals. Most of the workers have concentrated on cardiac sarcolemma. Reactive oxygen species have been reported to affect the membrane phospholipids by initiating peroxidation (Freedman and Crapo, 1982), the latter being associated with a reduction of β adrenoreceptors (Kramer *et al*, 1986). In stunned porcine myocardium though there was substantial evidence of FR injury the β adrenergic system was found to be intact (Fu *et al*, 1992). But Kaneko *et al* using SL membrane preparations from rats observed an increase in the number of β receptors (Kaneko *et al*, 1991a). The same authors reported a decrease in heart SL sulfhydryl groups associated with inhibitory effects on Ca^{2+} activities (Kaneko *et al*, 1991b). Reduction in the number of Ca^{2+} channel activities in the cell membrane upon exposing the myocardium to radical generating systems affecting Ca^{2+} movements in the cardiac cell and a reduction in contractile force cannot be considered to account for the intracellular Ca^{2+} overload due to ROS and thus the effects of ROS on other mechanisms associated with the regulation of $[Ca^{2+}]_i$ seem likely to participate in cellular damage to reactive oxygen species (Kaneko *et al*, 1989). In cardiac SL vesicles Na^+ Ca^{2+} exchange has been found to be very sensitive to membrane

lipid composition (Shi *et al*, 1989). Reduction of SL Na K ATPase activity was observed on exposure to $OH^{\bullet}$ generating systems (Kukreja *et al*, 1990, Taga and Okabe, 1991). Inactivation of Na pump and other SL ion carriers were observed in oxidant induced damage to cardiomyocytes (Xie *et al*, 1990). Huang *et al* (1992) reported an inactivation and degradation of Na K ATPase induced by reactive oxygen radicals. Inactivation of Na pump and other SL carriers were also reported by Xie *et al* (1990) and Shao *et al* (1995). Reversal of Na^+ Ca^{2+} exchanger and activation of Ca^{2+} activated steady state currents (Matsuura and Shattock, 1991b) has also been reported in FR stress. It has been claimed by Jabr and Cole (1995) that ROS may alter ion channel activity by differential mechanisms depending on the compartment, extracellular or intracellular in which they are present. Their experiments suggested that extracellullar oxygen radicals activate a Ca^{2+} sensitive cation current in the absence of changes in $[Ca^{2+}]_i$ probably by modification of extracellular sulfhydryl groups and the mechanisms may be different from the Ca^{2+} dependent activation of nonselective cation current by intracellular oxygen radicals. The same authors have shown that oxygen radicals generated in the extracellular space may not affect SR Ca^{2+} release to the same extent as intracellular oxygen radical stress and that the latter may not alter Na^+ Ca^{2+} exchange to increase Ca^{2+} influx (Jabr and Cole, 1993). However, a ryanodine sensitive rise in $[Ca^{2+}]_i$ was observed in myocytes during extracellular oxygen radical stress (Hayashi *et al*, 1989), consistent

with several studies indicating that SR Ca^{2+} handling is affected by oxygen radical stress by enhanced Ca^{2+} leak from SR (Okabe *et al*, 1989) and depressed SR Ca^{2+} uptake (Kukreja *et al*, 1988). Significant depression of Ca^{2+} uptake was observed in intact SR of digitonin lysed cardiomyocytes exposed to $OH^{\bullet}$ radical generation system. Such a decrease was found to be due to a decrease in SR Ca^{2+} pump mediated Ca^{2+} uptake and not resulting from an increase in the release of Ca^{2+} from SR, as both ATP binding to the SR Ca^{2+} pump and the Ca^{2+} pumping ATPase activity were depressed following $OH^{\bullet}$ treatment (Sulakhe *et al*, 1997). Kaneko and co-workers studied the effect of FRs on Ca/Mg ATPase and ATP independent Ca^{2+} binding activities in rat heart sarcolemma. They observed that xanthine + xanthine oxidase and H_2O_2 had a stimulatory effect on Ca-ATPase activity but a combination of H_2O_2 and Fe^{2+} decreased Ca-ATPase activity (Kaneko *et al*, 1990). This suggests that ROS may influence Ca^{2+} movement in the cell by altering Ca/Mg ATPase and Ca^{2+} binding activities of the membrane and that these effects may be oxygen radical species specific.

II.1.6.5. Reactive oxygen species and energy metabolism

Reactive oxygen species have been implicated in various types of myocardial injury. Primary myocardial ischemic and hypoxic perfusion share many of the same characteristics. Both the forms of injury are associated with a rapid decline in ATP concentrations and increased ATP degradation (Hearse *et al*, 1977, Hess and Manson, 1984). During ischemia ATP is

dephosphorylated to form adenosine via adenosine monophosphate. Adenosine is easily lost from the cell and is further degraded into xanthine and finally to uric acid, which cannot be used as an ATP precursor. Oxygen derived free radicals and their metabolites can cause enzymatic inactivation either directly or indirectly. Hydrogen peroxide can cause an inhibition of ATP synthesis through inactivation of enzymes necessary for glycolysis and oxidative phosphorylation (Spector *et al*, 1988, Goldhaker *et al*, 1989).

II.1.6.6. Altered lipid and protein metabolism

Lipids, both in free and bound forms, are immediate targets of ROS. The overall effects of lipid peroxidation include diminishing membrane fluidity, increasing membrane permeability, destabilizing membrane receptors and inducing immune response to altered phospholipids. Elevated levels of free fatty acids (FFA) are implicated in many pathological conditions as observed in myocardial ischemia, diabetes, hyperlipidemia and cardiac hypertrophy (Spragna and Hickson-Bick, 1999). β-oxidation of FA cannot proceed under oxygen deprived conditions, as observed in hypoxia or ischemia, where FFA and their metabolites become harmful. The metabolic intermediates will accumulate and incorporate into SL, SR and mitochondrial membrane, which will interfere with membrane integrity and function of membrane bound enzymes (Hendrickson *et al*, 1997). In addition to cytosolic FFA, ROS also react with membrane bound PUFA leading to "lipid peroxidation". It has been shown that altered cellular redox state and

increased lipid peroxidation are associated with the transition of cardiac hypertrophy to heart failure (Dhalla *et al*, 1996a). Elevated levels of lipidperoxides were also observed in CHF patients (Diaz-Velez *et al*, 1996, Keith *et al*, 1998).

It is widely accepted that hypertriglyceridemia and abnormal lipoprotein profile are associated with an increase in cardiovascular risk. Endogenous antioxidants in the hydrophilic cellular compartments cannot prevent propagation of the carbon-centered radical and oxidation reaction, which leads to long chain lipid (hydro) peroxidation. These unstable peroxides are degraded rapidly and form secondary products that are toxic to cells. These adducts can interact with LDLs resulting in oxLDLs. These modified LDLs abnormally affect cellular recognition and are chemotactic for circulating monocytes and are toxic to endothelial cells (Diaz *et al*, 1997). The discovery of novel oxLDL receptors-oxLDL receptor-1 and lectin like oxLDL receptor-1- has provided a more mechanistic insight into oxLDL-induced atherogenesis. Expression of these receptors in vascular cells is sensitive to cytokine activation (Kume *et al*, 2000, Minami *et al*, 2000). Circulating oxLDL may enhance the progression of atherosclerosis via regulation of redox sensitive transcription factor through ligand receptor binding (Cominacini *et al*, 2000)

In addition to the direct impact of ROS on cardiac function, they can oxidise amino acid side chains and protein backbone leading to protein-

protein cross linking and protein fragmentation (Berlett and Stadtman, 1997). The protein derivatives used as markers of ROS-mediated protein modifications are: protein carbonyl derivatives and protein nitrotyrosine derivatives. Elevated levels of nitrated proteins are observed in cardiac tissue from animals and patients under various conditions of excess oxidant production, including ischemia/reperfusion, CHF, and myocardial sepsis (Kooy *et al*, 1997, Lopez *et al,* 1997, Luoma *et al*, 1998, Flesch *et al*, 1999).

II.1.6.7. Altered signal transduction

Studies have demonstrated that ROS may indeed act as signal transduction molecules. For example, cellular H_2O_2 was transiently increased upon activation of platelet derived growth factor (Sundaresan *et al*, 1995). Several signalling pathways are affected by the platelet derived growth factor-induced increase in H_2O_2 concentration, because of the effect of H_2O_2 on tyrosine phosphorylation, mitogen activated protein kinase (MAPK) activation, DNA synthesis and chemotaxis. Additionally H_2O_2 activates hypertrophic and apoptotic signalling pathways in cardiac myocytes (Chen *et al*, 2000). ROS activate a wide variety of cellular signalling molecules and pathways, including Ca^{2+}, protein tyrosine kinases, serine/threonine kinases, and phospholipases (Kamata & Hirata, 1999).

II.1.6.8. Altered gene expression

It has become increasingly evident that ROS are more than simply cellular toxicants and that they may be important modulators of cellular gene

expression patterns. For example redox cycling of cysteinyl residues is one of the important mechanisms of ROS-regulated activity of transcription factors and signaling molecules (Dalton *et al*, 1999). ROS induced alterations in gene expression are mediated by activation of transcription activators, such as nuclear factor – κB (NF- κB) and activation protein-1 (AP-1). Changes in early response genes such as erg-1, hsp 70, c-fos, c-junc and c-myc are detected within 30 min after a hypertrophic stimulation (Sen and Packer, 1996). Furthermore lipid peroxidation products can activate NF- κB. Thus ROS induced gene expression are a consequence of changes in cellular signalling pathway through modification of enzyme activities and alterations of molecular structures of biomolecules – proteins, lipids, glycoproteins and nucleotides. NF- κB is important in inflammatory responses since it regulates a number of cytokine genes and their receptors, including TNF- α, IL-1, IL-2, and major histocompatibility complex class I. NF- κB binding sites were also found in a variety of cell adhesion molecules such as colony stimulating factor-1, monocyte chemoattractant protein-1, and vascular cell adhesion molecule-1 (Suzuki *et al*, 1997). ROS act as mediators of transcriptional regulators in signal transduction processes, leading to cell proliferation and transformation (Sun and Oberley, 1996). The field of oxidant related genes and signalling is rapidly expanding, and great deal of new insight into cellular responses to physiological stress has emerged.

II.2. MAGNESIUM : HOMEOSTASIS, METABOLISM AND CARDIOVASCULAR CONSEQUENCES OF DEFICIENCY

Magnesium ion (Mg^{2+}) is abundantly present in mammalian and non mammalian cells. It has been known for a long time that magnesium plays an important role in maintaining the structural and functional integrity of cardiovascular system (Seelig, 1981). Mg deficiency has been shown to produce cardiac and vascular lesions and, over the years, many theories have been proposed to explain the underlying pathogenic mechanisms (Bloom, 1988)

Magnesium is the fourth most common cation in the body and second most common intracellular cation after potassium. The central role of magnesium within the chlorophyll molecule and as a cofactor for enzymes in the 12-transphosphorylation reactions in photosynthesis makes it probably the most important inorganic element in the production of food and fossil fuel. In addition, it has a fundamental role as a cofactor in more than 320 enzymatic reactions involving energy metabolism and nucleic acid synthesis (Fawcett *et al*, 1999). Until recently, the function of magnesium in biological processes was largely ignored to the point where it was described as the 'forgotten' cation. In recent years, there has been an explosion of interest in the physiological and therapeutic properties of this essential element. It is involved in several processes, including hormone receptor binding and gating of calcium channels, trans membrane ion flux, regulation of adenyl cyclase,

muscle contraction and neuronal activity, control of vascular tone, cardiac excitability and neurotransmitter release (Chetan *et al*, 2002).

It has been estimated that refining and processing of food causes a substantial loss of magnesium. For example, the refining and processing of wheat to flour, rice to polished rice and corn to starch depletes magnesium by 82,83 and 97% respectively. Thus modern food technology partially explains why a significant segment of the population has intake of magnesium below recommended dietary amounts and may be predisposed to chronic, latent magnesium deficiency. Drinking water, on the otherhand, remains an important source of magnesium within the ecosystem as a whole. Acid rain causes exchange between magnesium and aluminium in the soil. This, coupled with intensive farming of the soil, has led to a reduction in magnesium within the food chain (Chetan *et al*, 2002).

II.2.1. Biochemistry of magnesium

The importance of Mg in human physiology was realised more than 50 years ago. Magnesium is an activator *in vitro* of a host of enzyme systems that are critical to cellular metabolism. Prominent are enzymes that hydrolyze and transfer phosphate groups, among them are the phosphatases and those concerned with reactions involving adenosine triphosphate. Since ATP is required for glucose utilisation, fat, protein, nucleic acid and coenzyme synthesis, muscle contraction, methyl group transfer, sulfate, acetate and formate activation, by inference the activating effect of magnesium extends

to all these functions. Additionally magnesium is required as a cofactor for oxidative phosphorylation *in vitro* (Potter, 1946, Lehninger and Kennedy, 1948). Magnesium contributes importantly to macromolecular structure. The highly ordered organisation of DNA, RNA and ribosomes is stabilized by the presence of this metal. Maximum stabilization of DNA to thermal disruption occurs when a 1:1 stoichiometry is reached between equivalents of magnesium ion and DNA phosphate residue (Dove and Davidson, 1962).

Magnesium is further involved in protein synthesis by contributing to the binding of mRNA to the 70S ribosome. (Brenner *et al*, 1961, Gros, 1961). Magnesium is also required for *in vitro* synthesis and degradation of DNA (Lehman, 1960, Kornberg, 1961). The metal has also been included in all amino acid activating systems (Hoagland *et al*, 1958, Matthaei and Neirenberg, 1961). Magnesium and calcium have complex interdependent influences on the excitability of the components of the neuromuscular apparatus. Magnesium or calcium depletion leads to increased neuronal excitability and enhanced neuromuscular transmission. In muscle, however, the effects of calcium and magnesium are antagonistic. In the intact animal the overall effect of either low calcium or low magnesium is increased neuromuscular excitability. (Wacker and Vallee, 1958). In pharmacologic doses magnesium has a curariform action on the neuromuscular junction presumably by interfering with the release of acetyl choline from motor nerve terminals (Goodman and Gilman, 1965, Katz and Miledi, 1967). Direct

application of magnesium to central nervous tissue blocks central synaptic transmission and may cause general anesthesia (Somjen *et al*, 1966).

II.2.2. Body content, absorption and regulation of magnesium

Adult human body contains approximately 21 to 28 gm, or approximately 2000 mEq (Widdowson *et al*, 1951) of magnesium. Bone contains about half of the total body magnesium, the remainder being almost equally distributed between the muscle and nonmuscular soft tissues (Aikawa, 1963). Of the non osseous tissues, liver and striated muscle have the highest concentrations, 15-20 mEq /Kg (Aikawa, 1960). The magnesium content of erythrocytes varies from 4.4 to 6.0 mEq/litre (Seller *et al*, 1966, Valberg *et al*, 1965).

Owing to the multiplicity of imprecise methods for the determination of magnesium, normal serum values have ranged from as low as 1.4 to as high as 2.5 mEq/litre . Atomic absorption methods show that mean values of normal serum varies between 1.6 and 2.1 mEq/litre (Alcock, 1960, Wacker *et al*, 1964 and 1965, Briscoe and Ragan, 1967). Careful assessment of the normal serum content by two different analytical methods and certified standards has yielded a value close to 2.0 mEq/litre (Wacker *et al*, 1964).

From recent studies it has been observed that magnesium absorption occurs uniformly throughout the small intestine. Reports have shown an inverse curvilinear relationship between intake of magnesium and fractional

absorption which ranges from 65% absorption at low intake to 11% absorption at high intake. Clinically, this suggests that treating magnesium deficiency with oral supplementation may require an extended period (Warren *et al*, 1968; Chetan *et al*, 2002).

Plasma magnesium is carefully regulated within the narrow range of 1.7 to 2.4 mg/dl (0.7 to 1.0 mmol/l). In contrast to the tight hormonal control of concentration of calcium in blood, kidney is the primary regulator of magnesium balance. Normal intake of magnesium is approximately 300 mg per day, and about one third of the intake is absorbed by gastrointestinal tract. Renal magnesium handling is essentially a filtration reabsorption process even though magnesium secretion has been suggested. Eighty percent of serum magnesium is ultrafiltrable out of which 70 to 80% is ionized. Over a 24 h period, 3500 mg of magnesium is filtered: in humans only 3 to 4% of this amount is excreted in urine. About 20 to 30% of the filtered magnesium is reabsorbed along the proximal tubule. The thick ascending limb of Henle's loop is known to be the major site of magnesium reabsorption in the renal tubule (50 to 60%) and the principal locus of renal control of magnesium excretion. The most striking change in renal magnesium handling occurs in response to alteration in plasma magnesium concentration. Recent evidences suggest that the cells within the distal tubule, possibly the thick ascending limb of Henle, are capable of adapting to magnesium and calcium availability through receptors that sense the

concentration of these cations. Thus when the magnesium status is suboptimal, these receptors sense the need for magnesium retention and cause more reabsorption (Chetan *et al*, 2002).

Renal magnesium wasting may be either primary, due to renal defect or secondary, representing the response of the kidney in a normal manner to a variety of systemic and local factors that increase magnesium losses. Several drugs particularly diuretics, thiazides, cisplatin, gentamycine, cyclosporin etc. can cause magnesium loss into urine by inhibiting magnesium reabsorption in the kidney (Chetan *et al*, 2002).

II.2.3. Hormonal modulation of magnesium

Despite early proposals for the existence of a specific hormonal control of magnesium homeostasis (Romani and Scarpa, 1990), no single endocrine factor that controls circulating or urinary magnesium has been identified. Among many extensive and excellent reviews dealing with magnesium homeostasis, one describes magnesium as the body's 'orphan ion' because of an apparent lack of a specific endocrine control similar to that existing for calcium, sodium and potassium. A number of hormones, including parathyroid hormone and calcitonin, vitamin D, insulin, glucagon, antidiuretic hormone, aldosteron and sex steroids have been reported to influence magnesium balance notwithstanding the possibility that these may not be primary regulators of magnesium homeostasis. Recent observations suggest that these hormones act through a common second messenger,

adenosine 3',5'-cyclic monophosphate to enhance magnesium transport and modulate magnesium excretion at that nephron site (Chetan *et al,* 2002).

II.2.4. Magnesium deficiency

Marginal magnesium deficiency is common and multifactorial. Numerous research reports and clinical commentaries regarding magnesium deficiency have appeared in recent years. Magnesium deficit can be categorised into two types: magnesium deficiency and magnesium depletion. Dietary amounts of magnesium are marginal and little alteration in magnesium intake may increase the prevalence of magnesium deficiency (Fawcett *et al*, 1999). Magnesium depletion may be due to dysregulation of factors controlling magnesium status: intestinal hypo – absorption of magnesium, reduced uptake and mobilization of bone magnesium, urinary leakage, hyperadrenoglucocorticism by decreased adaptability to stress, insulin resistance and adrenergic hyporeceptivity. Magnesium deficiency in aging largely results from various pathologies and treatment to elderly persons. Osmotic diuresis caused by glucosuria (as in diabetes mellitus), mannitol and urea results in urinary magnesium wasting. It has been suggested that aging, stress, and various disease states may increase magnesium requirement (Shills, 1988).

Magnesium deficiency has been demonstrated in 7 to 11% of hospitalized patients and is found to coexist in upto 40% of patients with other electrolyte abnormalities, particularly hypokalemia and to a lesser

extent hyponatremia or hypocalcemia. (Whang *et al*, 1984)The co-existence of secondary electrolyte abnormalities plays a key role in the clinical features of magnesium depletion. Although hypomagnesemia reliably indicates magnesium deficiency , a normal plasma magnesium concentration does not exclude magnesium depletion. Experimental observations to date support the view that magnesium and potassium metabolism are closely linked. Concentrations of magnesium and potassium have been inversely correlated. The mechanism behind this interrelationship may be the magnesium dependency of the activity of Na K ATPase, a physical influence *per se* by low magnesium concentration on the cellular membrane leading to leakage of potassium and/or interaction between magnesium and the secretion of aldosterone. Chetan *et al* (2002) has noted the functional role of Mg in the pathogesesis of diabetes. The incidence of subclinical deficiency is common in diabetes mellitus and cardiovascular disorders.

II. 2.5. Magnesium homeostasis in myocardium

The concentration of total magnesium in cardiac ventricular myocytes ranges between 11 and 17mM (Polimeni and Page,1973, Murphy *et al*,1989, Romani *et al*,1993) depending on the technique used and animal species investigated. Majority of cellular magnesium is present as a complex with ATP bound to cytosolic proteins(Page and Mc Callister,1973) or metabolites(Garfinkel *et al*,1986) or internalized within intracellular structures (Polimeni and Page,1973, Garfinkel *et al*, 1986), so

that only a small fraction of total free magnesium is available within the cytosol. The values of cytosolic free magnesium reported to be present in cardiac ventricular myocytes under physiological conditions vary considerably, because of the indirect techniques used (Paradise *et al*, 1978, Hess *et al*, 1982, Blatter and Mc Guigan, 1986, Fry, 1986) and/or the difficulty in proper quantitation (Polimeni and Page, 1973, Gupta *et al*, 1984, Fry, 1986, Murphy *et al*, 1989, Romani *et al*, 1993). Following table summarises the data from various laboratories which measured the absolute value for cytosolic magnesium by different techniques.

Table.1. Total and free magnesium in mammalian cardiac cell

Total Mg^{2+}	**Free Mg^{2+}**	**Method**	**Reference**
17mM	1.0mM	^{28}Mg	Polimeni & Page,1973
	1.5-3.6 mM	Mg Flux	Paradise *et al*, 1978
	0.8 mM	^{31}P NMR	Gupta *et al*, 1984
	2.5 mM	^{31}P NMR	Wu *et al*, 1981
	3-3.5 mM	Mg^{2+} electrode	Hess *et al*, 1982
	0.4 mM	Mg^{2+} electrode	Blatter & McGuigan, 1986
	2.4 mM	Mg^{2+} electrode	Fry, 1986
17 mM	0.44 mM	^{13}C-NMR citrate /isocitrate ratios	Masuda *et al*, 1990
11-12 mM	0.5-0.7mM	Atomic absorbance	Romani *et al*, 1993
0.5 mM		Mag-Fura	Murphy *et al*, 1989

Although the basal value of magnesium broadly ranges between 0.4 and 3.5mM, it is unanimously accepted that the cytosolic free magnesium concentration does not change at all, or change only minimally, even under drastic hormonal stimulation (Murphy *et al*, 1989). The observation that amount of magnesium transported across the membrane is equal to or larger than the whole content of free magnesium within the cytosol and that negligible changes in the cytosolic free magnesium content are detectable, lead to the conclusion that magnesium redistributes among intracellular organelles, and that the observed fluxes of magnesium across the plasma membrane change the total but not the free cellular magnesium (Romani et al, 1995).

Intracellular mechanisms involved in magnesium extrusion and redistribution in cardiac cells involves several hormonal events which can be summarised as follows. The release of norepinephrine or epinephrine activates the β adrenergic receptors of cardiac cells and through adenylate cyclase, enhances the cellular level of cAMP. cAMP binds to mitochondrial adenine nucleotide translocase and induces the extrusion of magnesium and ATP from mitochondria. Magnesium accumulation, instead, appears to be mediated by the activation of muscarinic receptors, possibly through activation of protein kinase C pathway and/or the down regulation of the cAMP pathway. Several aspects of the whole picture need to be further elucidated. In fact, it is uncertain if magnesium extrusion across the plasma

membrane of cardiac cells depends on the increase in cellular cAMP level or, alternatively, on the increase in cytosolic ATP.Mg^{2+} content following their extrusion from mitochondria. In addition, no clear evidence has been provided about the nature of the extrusion mechanism located at the sarcolemmal level, though the operation of an amiloride-sensitive Na^{+}/Mg^{2+}exchanger is inferred (Romani *et al* 1993)

Studies by Weglicki et al have shown that while serum Mg levels are reduced (hypomagnesemia), cardiac tissue as well as aortic Mg levels remain unaltered (Weglicki *et al*, 1996). There is ample evidence to support the postulation that vasoconstriction associated with elevated blood pressure and vascular endothelial injury may be the earliest pathogenic events in Mg deficiency (Shivakumar, 2001). In hypomagnesemia, vascular cells, especially the endothelium, would be the first to 'sense' and respond to a fall in circulating Mg. Several animal and clinical studies point to significant alterations in vascular tone, and elevated blood pressure in response to changes in Mg concentration (Turlapty and Altura, 1980, Altura and Altura, 1985a, Altura and Altura, 1985b). Pioneering studies by Turlapty and Altura have demonstrated that reduced Mg in the coronary vasculature environment exerts profound influences on coronary vascular tone and reactivity, supporting the hypothesis that hypomagnesemia could produce vasoconstriction, resulting in coronary arterial spasm. This in turn could produce ischemic changes in the myocardium resulting in perivascular

lesions. A role for enhanced Ca^{2+} influx in promoting vasoconstriction when extracellular Mg^{2+} is lowered has been suggested. There is experimental evidence that acute Mg deficiency produces a pro-inflammatory state marked by elevated circulating levels of vasoactive pro-oxidants and mitogenic factors. (Weglicki *et al*, 1992, Weglicki *et al*, 1996).

Linking these observations, one may hypothesise that a fall in serum Mg may produce a temporal sequence of events involving vasoconstriction, hemodynamic alterations, and vascular endothelial injury followed by myocyte damage and finally alterations in mechanical response. Such a sequence of events would explain the cardiovascular lesion of Mg deficiency even when myocardial or vascular tissue levels of Mg are well preserved.

It is generally believed that myocardial Mg is well preserved except in extraordinary conditions, as upon administration of certain types of drugs like diuretics. On the contrary, hypomagnesemia, defined as suboptimal serum levels of Mg can be more common in a population, particularly in regions with soft water (Altura and Altura, 1985a and Shills, 1988). In fact, the overall incidence of hypomagnesemia in hospitalized patients is reported to range from 7 to 52% (Altura and Altura, 1985a). The cardiovascular consequences of Mg deficiency may be latent or patent depending on its severity and other compounding factors, including reduction in tissue levels of Mg (Shivakumar, 2001).

The consequences of Mg deficiency have engaged the attention of researchers the world over since the first description of clinical Mg deficit in the humans way back in 1934 (Hirschfelder and Haury, 1934, Seelig, 1981, Durlach, 1988, Rayssiguier *et al*, 2001). Association between Mg deficiency and cardiovascular disease was discussed as early as 1933s (Zwillinger, 1935) and dietary deficiency of Mg has since been demonstrated to produce cardiac and atrial lesions in experimental animals (Heggtveit *et al*, 1964, Werner *et al*, 1964, Heggtveit, 1969 and Bloom, 1988). Incisive investigations on animal models of Mg deficiency and clinical studies over the last three decades have resulted in the emergence of Mg as an important cardiovascular cation. Several animal models of Mg deficiency have been used to examine the cardiovascular sequelae of Mg deficiency. Durlach *et al* have, however noted that due care is often not exercised in the interpretation of data from clinical studies and animal models. Extrapolations, as from the pharmacological effects of Mg to its physiological properties, can lead to incorrect conclusion. (Durlach *et al*, 1992a and 1992b). In many clinical settings, atrial arrhythmias have been related to Mg deficiency and successful treatment of arrhythmias with parenteral magnesium infusion is reported (Arsenian, 1993). However, the aforementioned beneficial effects of magnesium administration on angina and arrhythmias may reflect the pharmacological action of the element and should not be interpreted as the correction of an underlying deficiency. The influence of magnesium on

vascular tone and cardiac arrhythmias described above can be related to its effects on cardiac ion channels (Iseri, 1984, White and Hartzell, 1989).

II.2.6. Vascular lesions in magnesium deficiency

Magnesium deficiency is reported to produce vascular lesions which include intimal thickening, thinning and fragmentation of the elastic membrane and calcification (Rayssiguier *et al*, 1992a). Fibrinoid necrosis of small coronary arteries has been described in magnesium deficient hamsters. It has been proposed that a small increase in calcium influx in vascular smooth muscle cell during magnesium deficiency may lead to increased vascular tone and spasm whereas greater influx of calcium might cause fibrinoid necrosis (Bloom, 1988). Magnesium deficiency enhances vascular lipid infiltration and intensifies cardiovascular lipid deposition and lesions in animals on atherogenic diet whereas magnesium supplementation prevents atherogenesis (Rayssiguier and Gueux, 1986, Rayssiguier *et al*, 1992a and 1992b). Such observations have contributed to the view that magnesium deficiency may be involved in development of ischemic heart disease (Rasmussen *et al*, 1989). The cardiac lesions of magnesium deficiency, which are in the nature of a cardiomyopathy, have been the subject of several incisive investigations over the past several decades. In animals, a diet deficient in magnesium to the extent that it does not cause death but retards growth and development, produces cardiac and atrial lesions (Bloom, 1988, Kartha *et al*, 1998) Typically the morphologic finding is one of focal necrosis

and calcification progressing to fibrosis (Bloom, 1988). Early myocardial lesions develop predominantly in the small coronary and intramyocardial arteries. Bloom reported hyperplasia of smooth muscle cells, and intimal edema and chronic inflammtory changes in the media and advetitia in the small arteries of hamsters on a magnesium deficient diet (Bloom, 1988). When dietary deficiency of magnesium was combined with high saturated fats, more typical atherogenic lesions were observed in the large coronary arteries. Heggtveit et al demonstrated an array of myocardial changes in rats as early as two weeks on a magnesium deficient diet (Heggtveit *et al*, 1964, Heggtveit , 1969). Focal myocardial necrosis, manifesting subendocardially, enlarge, develop areas of calcification and become numerous as deficiency advances. Vascular dilatation and hyperaemia are prominent in the early lesions of magnesium deficiency. As it advances,fragmentation, vacuolization and eventual myocyte loss are noticed along with progressive increase in macrophages, fibroblasts and collagen. Electronmicroscopy reveals prominent early mitochondrial swelling and loss of internal fine structure. The lesions of myocardial mitochondria in magnesium deficiency resembles those of ischemia and catecholamine induced cardiomyopathy. Interestingly, in the rodent model of acute magnesium deficiency, there is a marked drop in serum magnesium levels (hypomagnesemia)whereas myocardial magnesium levels remain unaltered over several weeks, implying that these changes are not related to a reduction

in myocardial magnesium level (Shivakumar and Kumar, 1997, Kumar *et al*, 1997a).

II.2.7. Molecular basis of magnesium dependent electrical and mechanical variations

Extracellular Mg can modulate intra and extracellular concentrations of other ions by affecting transport systems, by regulating phosphorylation or dephosphorylation of transporters or associated regulatory proteins. The existence of Mg^{2+} transport in rat ventricular muscle has been confirmed by Polimeni and Page (1972 and 1973). Efflux of Mg is generally against the electrochemical gradient and studies favor an electroneutral antiport mechanism. Evidence has accumulated for the existence of Na^{+}/Mg^{2+} exchange mechanism in the cardiac muscle and its contribution to regulation of $[Mg^{2+}]_i$ (Fry,1986, Romani *et al*, 1993, Grow *et al*,1995, Tashiro and Konishi, 2000). The presence of a Mg^{2+} pump in cardiac muscle is excluded in light of the recent findings (Spah and Fleckenstein, 1979, Anand-Srivastava *et al*, 1982). Fluctuations of $[Mg^{2+}]_i$ seen in ischemia, hypoxia and other pathological conditions is not mediated by change in $[Mg]_o$ (Murphy *et al*, 1989, Headrick and Willis, 1991, Fatholahi *et al*, 2000). Decrease in cardiac tissue magnesium was not significant in chronic hypomagnesemia (Kartha *et al*, 1993 and 1998, Kumar *et al*, 1996, Singh *et al*, 1997). Large proportional changes to $[Mg^{2+}]_o$ influences $[Mg^{2+}]_i$ only to a small degree. Insufficient dietary intake is not accompanied by a change in cytosolic Mg^{2+}.

Hence cytoplasmic free Mg^{2+} is expected to remain unaffected in Mg deficiency (Nair and Nair, 2002)

Both $[Mg^{2+}]_o$ and $[Mg^{2+}]_i$ alters the functioning of a number of K channels and plays a significant role in modulating the activation and inactivation curves by voltage shifts (Fry and Proctor, 1993). Fischer and Girous (1987) found that the activity of Na K ATPase was less in rats fed a Mg deficient diet although the concentration of the enzyme was unaffected. This would partly explain the changes in electrolyte levels within the cells in Mg deficiency – higher levels of Na^+ and Ca^{2+} and lower levels of Mg^{2+} and K^+ (Durlach, 1988). Chronic insufficient dietary intake of Mg can lead to hypokalemia in addition to hypomagnesemia. A disturbance in potassium homeostasis can adversely affect the electrical stability of the myocardium. Lower $[K^+]_o$ will lead to an unstable membrane potential consequent to a decrease in K^+ conductance, and the cells become more excitable as the resting membrane potential approaches the threshold potential (Nair *et al*, 2002).

Variation in $[Mg^{2+}]_o$ affects the functioning of Na^+ channels and shifts the activation and inactivation curves. There is positive evidence for the presence of Na^+ Mg^{2+} exchange system in cardiac cells (Romani *et al*, 1993, Handy *et al*, 1996, Wei *et al*, 2002). The trans membrane Na^+ and Mg^{2+} gradients are coupled such that an alteration of one gradient will affect the other. Reduction of $[Mg^{2+}]_i$ enhances Na^+ Ca^{2+} exchanger activity (Wei *et al*,

2002). Raised $[Mg^{2+}]_o$ attenuates both the Na^+ and Ca^{2+} currents and shifts the activation curve to more positive potential (Fry and Proctor , 1993, Shetty *et al*, 1988).

Cardiac contractility is influenced by both $[Ca^{2+}]_i$ and Ca^{2+} transients. Studies using different experimental systems have consistently shown that $[Mg^{2+}]_o$ has a blocking effect on voltage gated Ca^{2+} channels (Williams, 1970). Reduction of Ca^{2+} inward current in presence of Mg^{2+} can also be mediated by its competition with Ca^{2+} binding sites of sarcolemma. (da Salva and Williams, 1991). Single channel studies have shown that the presence of $[Mg^{2+}]_o$ converts long -lasting channel openings to briefer events without altering the unitary conductance, and unlike other divalent cations that show this property, the block is highly voltage dependent, with hyperpolarisation increasing the degree of block (Lansman *et al*, 1986). Thus in the resting cell Mg^{2+} would prevent Ca^{2+} influx, but at depolarising potential the blocking influence of Mg^{2+} would be reduced and Ca^{2+} would permeate the channel (Fry and Hall, 1990). Reduction of $[Mg^{2+}]_o$ is therefore expected to promote a rise in basal levels of SR Ca. Patch-clamp studies showed that Mg^{2+} acts on both L and T type Ca^{2+} channels, and Mg^{2+} has been dubbed as "nature's calcium antagonist" (Millane and Camm, 1992). An inverse relation between Mg and Ca was observed in the myocardial tissue of patients with heart disease (Elwood *et al*,1980).

II.2.8. Energy metabolism and magnesium

Magnesium is a cofactor for the activity of many intracellular enzymes involved in glycolysis, lipid hydrolysis, oxidative phosphorylation etc. The production of high energy phosphates at a rate approximating that of ATP utilisation requires continuous oxygen supply and a large number of mitochondria. The large mitochondrial content of the myocyte (about 35% of the cell volume) underscores the demand on this organelle for energy supplied by aerobic metabolism. The adenylate cyclase-cAMP system exerts a regulatory role on a number of proteins which play a significant role in cardiac contractility. Magnesium facilitates the formation of cAMP from ATP and also activates the breakdown of cAMP by the cytoplasmic phosphodiesterase, thus ensuring that the cyclic nucleotides are raised only during receptor activation, for example the phosphorylation of Ca^{2+} channel. Matrix free Mg regulates oxidative phosphorylation and affects the K^+/H^+ antiporter that controls the mitochondrial volume together with K^+ uniport. Magnesium inhibits swelling and uncoupling of mitochondria caused by mitochondrial permeability transition (Bierley *et al*, 1994, Zoratti and Szabo, 1995).

Studies reporting a negative inotropic response to Mg deficiency have associated the functional change to the adverse effect on myocardial energetics consequent to reduction in oxygen and nutrient delivery to the cardiac myocytes. Degenerative changes of mitochondria in myocardial cells

were observed in rats on Mg deficient diet. Mitochondria were enlarged with a concomitant decrease in the density of the matrix, but the other organelles were unaffected. The biochemical and structural changes have been attributed to excessive uptake of Ca^{2+} by mitochondria, consequent to an increase in the content of intracellular calcium (Nishiyama *et al*, 1989, Ikeda *et al*, 1997). Reduction of atrial intracellular Pi and adenine nucleotide was accompanied by elevation of creatine phosphate (Savabi *et al*, 1988). Saks *et al* (1975) have reported that at limited Mg^{2+} concentrations creatine phosphate is preferably synthesised while at high Mg^{2+} concentrations ATP synthesis takes place.

Apart from the variation in energy metabolism, vascular changes that affect myocardial perfusion in Mg deficiency can also affect cardiac contractility. Damage to coronary arteries in Mg deficiency can restrict the supply of nutrients and oxygen to the myocardium. Perfusion of isolated rat hearts with low Mg^{2+} resulted in significant reduction in coronary blood flow, concomitant with decreased oxygen consumption, and reduction in energy turn over due to reduced delivery of oxygen (Altura and Altura, 1990, Altura and Altura, 1993). Decrease in oxymyoglobin and an uncoupling between oxygen consumption and cardiac contractility was also observed (Wu *et al*, 1989). Species related differences in heart's susceptibility to Mg deficiency is known to exist (Arsenian, 1993). Sudden death in ischemic heart disease of Mg deficiency has also been attributed a vascular mechanism

(Turlapty and Altura, 1980, Ku and Ann, 1987). Magnesium deficiency therefore induces a negative energy balance, both by degenerative changes in the mitochondria and inefficient myocardial perfusion with adverse effect on myocardial contractility.

II.3. SYNERGISTIC EFFECT OF OXIDATIVE STRESS AND MAGNESIUM DEFICIENCY ON THE MYOCARDIUM

Several studies have reported enhanced stress sensitivity in Mg deficiency. When Mg deficiency exists, stress increases the risk of cardiovascular damage including arrhythmias (Seelig, 1994). Chronic suboptimal intake of Mg results in reduced tolerance to cold stress and noise stress resulting in the development of calcified cardiac lesions in rats (Laloraya *et al*, 1989, Parihar and Dubey, 1995, Prakash *et al*, 1998). Unfavorable effects of Mg deficiency are also apparent in aging, (Suh *et al*, 2001) various disease states (Lunec, 1991, Baines *et al*, 1997) and in pharmacological interventions (Hetch, 1986). From an experimental study carried out in rats Gunther (1991) suggested that in Mg deficiency intracellular Ca and Fe contents are increased and more catecholamines are released particularly when Mg deficient animals are additionally stressed. The same author observed that heart collagen contents are particularly enhanced suggestive of cardiac hypertrophy when Mg deficient rats were subjected to noise stress (Ising *et al*, 1976, Gunther, 1991). Magnesium deficiency was found to predispose postischemic hearts to enhanced

oxidative injury and functional loss (Kramer *et al*, 1994). Gunther *et al* (1994) have also shown that in vitamin-E depleted rat hearts MDA formation due to oxidative stress was considerably enhanced by Mg deficiency. Gunther and Hollriegl (1989) have shown an increased lipid peroxidation in liver mitochondria from Mg deficient rats. Depressed antioxidant defense in rat heart in experimental Mg deficiency has been reported by Kumar *et al* (1997a) which has been shown as an implication for the pathogenesis of myocardial lesions. Shivakumar and Kumar (1997) reported that Mg deficiency enhances oxidative stress and collagen synthesis *in vivo* in the aorta of rats. Potentiation of free radical production associated with myocardial infarction in Mg deficiency has been proved by Kharb and Sing (2000). *In vitro* studies in endothelial cells have shown an enhanced free radical induced intracellular oxidation and cytotoxicity in Mg deficiency (Dickens *et al*, 1992). Studies by Kumar *et al* (1997b) provides evidence of increased lipid peroxidation and net deposition of collagen in the myocardium in response to dietary deficiency of Mg in rats, were the tissue Mg levels remain unaltered. Freedman *et al* (1991) reported that in Syrian hamsters Mg deficiency increases the susceptibility of the cardiovascular system to oxidative stress. There is a large volume of literature suggesting that Mg deficit contributes to the aging process and to the vulnerability to age-related diseases. One of the biological changes associated with aging is an increase in FR formation with subsequent damage to cellular processes.

Prime targets of the FR are unsaturated lipids in cell membrane, amino acids in proteins and nucleotides in DNA. The accumulation of unrepaired oxidative damage products may be a major factor in cellular aging (Rayssiguier *et al*, 1993). In a rodent model of hypomagnesemia, the alteration of antioxidant enzyme activities in heart indicates a free radical mediated mechanism of cardiac lesion formation (Kuzniar *et al*, 2001). Some of the defense systems against oxidative stress have been found to be compromised during Mg deficiency. In rats the level of glutathione in RBCs was significantly reduced after two to three weeks on Mg deficient diet (Wegliki *et al*, 1996). Similar changes were also observed in Mg deficient hamsters (Freedman *et al*, 1992b). Studies have shown that endogenous antioxidant defense is compromised during Mg deficiency (Stafford *et al*, 1993, Mak *et al*, 1994). Cardiotoxicity to Cd was found to be higher in Mg deficient rats. Supplementation of cadmium to Mg deficient rats however alleviated myocardial necrosis and Ca overload, compared to hearts of rats on Mg sufficient diet (Nishiyama *et al*, 1990).

This review reveals that Mg deficiency augments the adverse effect of oxidative stress. However, reports on the combined effect of oxidative stress and Mg deficiency on cardiac function are lacking.

III. DESIGN OF THE STUDY

III. DESIGN OF THE STUDY

The investigation is aimed at a better understanding of the inotropic response of the myocardium to reactive oxygen species and variation in extracellular magnesium. Experiments were designed to examine the mechanical response of the myocardium to variation in the extracellular milieu and delineate the mechanism of action. Mechanical variations are generally mediated by variation in ion transients or myocardial energetics. Studies were carried out on isolated rat heart preparations of adult Sprague-Dawley rats. Mechanical parameters were recorded using papillary muscles. As calcium is the major ion influencing cardiac mechanics, the influence of ROS and $[Mg]_o$ on channels and pumps likely to modulate Ca^{2+} transients were assessed using channel specific inhibitors. Intracellular level of diastolic Ca^{2+} was determined in isolated myocytes using the fluorescent probe fura-2 AM. High energy phosphates are the source of energy for myocardial contraction. Hence the levels of ATP and creatine phosphate was determined along with an assessment of tissue damage as determined by LDH assay. The level of TBARS was determined as a measure of lipid peroxidation. The biochemical parameters were assayed in the ventricular tissue of Langendorff perfused preparations following different treatment protocols. All parameters were assessed separately on exposure to ROS and alteration in $[Mg]_o$ to understand the independent effect of the two variables. The synergistic effect of oxidative stress and Mg deficiency were assessed subsequently.

Major objective of this study was to assess inotropic and biochemical response of the myocardium to different stress situations. In keeping with the objectives, the experimental protocol was designed as follows:

(i) Inotropic response to ROS and variation in $[Mg]_o$

Inotropic response of papillary muscles were recorded for,

a) Different concentrations of superoxide anions and H_2O_2

b) Different levels of $[Mg]_o$ and

c) A combination of ROS and Mg deficiency.

(ii) Intracellular levels of diastolic Ca^{2+}

Since contractile changes are mediated through cytosolic free Ca, diastolic Ca levels were measured in isolated cardiomyocytes.

(iii) Ion channels and pumps influencing cardiac contractility

Inorder to delineate the mechanism mediating the inotropic changes, response of the papillary muscle to different ion channel and pumps mediating Ca flux was assessed using channel specific inhibitors.

(iv) Biochemical changes

Mechanical changes may also be mediated by biochemical variations. Biochemical parameters assessed in this study were

a) High energy phosphate compounds - ATP and CP as an index of energy metabolism.

b) LDH level as an index of tissue injury

c) TBARS as an indicator of lipid peroxidation

III.1. MATERIALS

III.1.1. Fine chemicals

Acetoxy methyl ester of fura-2, ATP kit, caffeine, catalase, collagenase type-I, ethylene glycol-bis (β-aminoethyl ether) N,N,N',N'-tetra acetic acid (EGTA), hypoxanthine, ouabain, ryanodine, superoxide dismutase, taurine, thiobarbiuric acid, trypsin, verapamil and xanthine oxidase were obtained from Sigma.

III.1.2. Routine chemicals

Sodium chloride, potassium chloride, calcium chloride, magnesium chloride, magnesium sulphate, glucose, sodium bicarbonate, disodium hydrogen phosphate, potassium dihydrogen phosphate, sodium hydroxide, sodium potassium tartrate, hydrochloric acid, trichloroacetic acid, perchloric acid, potassium carbonate, methyl orange, sodium pyruvate, dichlorophenol indophenol, phenazine methosulfate (PMS) and potassium cyanide were obtained from Sisco Research Laboratories, India.

III.1.3. Tissue culture dishes

35 mm cell culture dishes were purchased from Nunc, USA

III.1.4. Equipments used

Physiograph, Stimulator and Force transducer, (Biodevices, India) Platinum electrode (fabricated), Fiber optic illuminator (Nikon, Japan), CO_2 incubator (Forma scientific, UK), High speed refrigerated centrifuge (Hitachi, Japan), Weighing balance (Sartorious, Germany), pH meter

(Labindia,India), Spectrofluorometer (Shimadzu, Japan), Inverted microscope with phase contrast optics (Diaphot TMD , Nikon, Japan), Ice-machine (Hoshozaki, Japan), Laminar flow hood (CLAS, India) and EASY pure UV/UF compact reagent grade water system (Barnstead, USA), Spectrophotometer (Shimadzu, Japan).

Experimental animals:- Sprague-Dawley rats maintained in the animal house of the Institute were used for the study. The animal house is certified by CPCSEA, and the Institutional Animal Ethics Committee approved the study.

III.2.COMPOSITION OF BUFFERS AND REAGENTS USED

III.2.1. Adenosine diphosphate - 21.2 mmol/l

10 mg ADP- disodium salt in 1ml water

III.2.2. ATP assay kit

NADH preweighed vial containing 0.3 mg disodium salt, 3-phosphoglyceric acid buffer-18 mmol/l, glyceraldehyde-3-phosphate/3-phosphoglyceric phosphokinase enzyme mixture, 12% W/V trichloroacetic acid solution.

III.2.3. Calcium free buffer for fura-2 AM loading, pH 7.4

140 mM NaCl, 3.8 mM KCl, 1.0 mM KH_2PO_4, 1.2 mM $MgSO_4$, 10 mM glucose, 10 mM HEPES

III.2.4. Calcium chloride solution 4.0 mM

III.2.5. Creatine Kinase

1900 KU/l – 5 mg of the enzyme preparation in 1 ml water.

III.2.6. Dichlorophenol indophenol (DCIP)

15 mg/ 20 ml of water

III.2.7. Glucose-0.5 mol/l

9.91g glucose in TEA solution made up to 100 ml

III.2.8. Glucose-6-phosphate dehydrogenase

87.5 KU/l – commercially available enzyme preparation was diluted according to requirement.

III.2.9. Hexokinase

70 KU/l – commercially available enzyme preparation was diluted according to requirement.

III.2.10. Kreb's Ringer phosphate buffer (KRB) for cardiomyocytes pH, 7.4

117 mM NaCl, 5.7 mM KCl, 4.4 mM $NaHCO_3$, 1.5 mM NaH_2PO_4, 1.4 mM $MgCl_2$, 21.1 mM HEPES, 11.7 mM glucose

III.2.11.Magnesium chloride - 0.1 mol/l

2.033 g $MgCl_2.6H_2O$ in water and made up to 100 ml

III.2.12. MDA standard

Stock standard-1,1,3,3-tetramethoxy propane-8.3 µl/100 ml distilled water. Working standard-1 ml stock diluted to 100 ml

III.2.13. Methyl orange indicator

50 mg methyl orange in 100 ml water.

III.2.14. Modified Kreb's Ringer Hansaleit (KRH) buffer for papillary muscle, pH 7.3

133 mM NaCl, 3.6mM KCl, 1.2mM $MgCl_2$, 1.5mM $CaCl_2$, 16mM glucose, 3mM HEPES

III.2.15. Nicotinamide-adenine dinucleotide phosphate (NADP)

12.7 mmol/l- 10 mg NADP disodium salt in 1ml water

III.2.16. Nicotinamide adenine dinucleotide reduced form

0.002 M NADH solution

III.2.17. Perchloric acid - 6%W/V

5.2 ml $HClO_4$ diluted to 100 ml with water

III.2.18. Phenazine methosulfate (PMS)

0.2% in water

III.2.19. Phosphate buffer, pH 7.4

0.1 M KH_2PO_4, 0.1 M Na_2HPO_4

III.2.20.Phosphate buffer, pH 7.6

0.05 M KH_2PO_4 and 0.05 M K_2HPO_4

III.2.21. Potassium carbonate - 5 mol/l

72 g K_2CO_3 in water made up to 100 ml

III.2.22. Potassium cyanide solution

190 mg / ml water

III.2.23. Sodium pyruvate

0.01 M sodium pyruvate

III.2.24. Sodium succinate, 0.5 M

III.2.25. Sucrose solution 0.35M

III.2.26. TBA reagent

3 g trichloroacetic acid, 75 mg thiobarbituric acid, 19.58 ml distilled water, 0.42 ml concentrated HCl, dissolved using magnetic stirrer, prepared freshly.

III.2.27.Triethanolamine buffer (TEA), pH 7.5-7.6

50 mmol/l triethanolamine, 1 mol/l NaOH

III.3. EXPERIMENTAL MODELS USED IN THE STUDY

III.3.1. Papillary muscle preparation and measurement of force of contraction

In papillary muscles endothelial cells and cardiomyocytes coexist and interact to sustain optimal performance. In this multicellular preparation of relatively simple geometry, the undisturbed extracellular matrix and cellular connections and energy balance are maintained. Inorder to evaluate myocardial performance the isolated papillary muscle therefore serves as an excellent model.

Adult Sprague-Dawley rats of either sex weighing 200±25 g were used for the study. Animals were euthenized under deep ether anesthesia. A thoracotomy was performed and the hearts were rapidly excised into modified KRH buffer, pH 7.4 at a temperature 37 °C. Papillary muscles were dissected out from the left ventricle (Fig.1) into the oxygenated buffer. The

mural end of the muscle was fixed to a hook and tendinous end attached to a force transducer. Muscles were stimulated electrically at 0.5 Hz at a voltage ~10% above the threshold by pulses of 5 ms duration delivered through two platinum electrodes. After an initial equilibration period of 1 hr in modified KRH buffer the muscles were gradually stretched in a stepwise manner till the maximum force of contraction was achieved. These papillary muscle preparations remained stable over the experimental period. The baseline contraction was recorded using a physiograph (fig.2). Fig.3. shows the experimental set up used for the measurement of force of contraction. A typical tracing and the calculation of the time for contraction (t) and half relaxation ($t_{1/2}$) are shown in fig.4.

III.3.2. Isolation of cardiomyocytes

Cardiomyocytes were isolated by the method standardised in the laboratory (Nair and Nair, 1997). Isolated heart was placed in oxygenated KRB containing 1mM Ca and 25 IU/ml heparin. Then the heart was cannulated via aorta and perfused retrogradely with the same solution at a flow rate of 10 ml/min for 5 minutes, in order to flush the blood and to ensure proper perfusion. Calcium free buffer containing 0.1 mM EGTA and 20 mM taurine was perfused subsequently for 5 minutes, inorder to relax the myocardium by depletion of calcium. This was followed by perfusion with Ca, EGTA and taurine free buffer for 5minutes to wash out EGTA. The same Ca free solution containing 0.06% (W/V) collagenase type I and 1.25%

(W/V) fatty acid free fraction V BSA was perfused by recirculating for 25-30 minutes. By this time heart becomes flaccid losing its toughness because of the dissolution of extracellular matrix. The ventricular portion was minced and incubated in the buffer with 0.05% collagenase type I and 0.8% BSA for 10 minutes with gentle tituration. Cell suspension was strained through 300 μM gauze to remove undissociated pieces of tissue. The suspension was centrifuged at 20 g for 2 minutes at room temperature to selectively isolate the myocardial cells from nonmyocardial cells. Healthy viable myocardial cells thus isolated will have rod shaped morphology with clearly visible cross striations and well defined cell edges. (fig.5).

III.3.3. Langendorff preparation

The hearts were rapidly excised into ice-cold modified KRH buffer. Aorta was immediately cannulated and hearts were perfused in a retrograde manner at constant flow rate with modified KRH buffer. The perfusate was continuously oxygenated at 37°C. The heart was allowed to stabilize for 10 minutes and then perfused according to different protocols for 30 minutes. The ventricular portion was then freeze clamped in liquid nitrogen and kept at -80 °C till analysis. The coronary effluent in each case was collected for the assay of lactate dehydrogenase.

III.4. EXPERIMENTAL DESIGN

III.4.1. Inotropic response of papillary muscle to reactive oxygen species

Reactive oxygen species opted for the study were superoxide anion (SO) generating system and hydrogen peroxide (H_2O_2). Superoxide anion generating system used in the study was hypoxanthine + xanthine oxidase (HX + XO). Pathophysiologicaly relevant concentrations of the superoxide anion generator was used for the study. The levels of SO anion produced by purine (2 mM) and XO (0.02 U/ml) is reported to be comparable to *in vivo* production by stimulated neutrophils (Tate *et al*, 1982). Xanthine oxidase activity in the heart has been determined to be of the order of 0.06 U/g tissue (Battelli *et al*, 1972). Therefore the SO anion levels produced using hypoxanthine and 0.01-0.02 U/ml of XO would be within a physiological range for myocardial production. Different concentrations of the SO anion generating system used were – 0.5mM HX + 0.01 U/ml XO, 0.5mM HX + 0.02 U/ml XO and 1mM HX + 0.04 U/ml XO. Concentrations of H_2O_2 used were 10, 20, 50 and 100 μM. These concentrations were used for the inotropic studies, and based on the observations of the study concentrations of ROS for biochemical studies were selected.

After stabilization of the muscle in modified KRH for 1h the baseline contraction was recorded without any treatment, and taken as the control. The SO anion generating system or H_2O_2 was then added to the system. The contractile variations stabilize within 10 to 20 minutes. The stable

contraction was recorded and the percentage change in the force of contraction was calculated compared to control.

III.4.2. Inotropic response of papillary muscle to variation in extracellular magnesium.

After stabilization of the muscle in modified KRH for 1h the papillary muscles were exposed to different concentrations of $[Mg^{2+}]_o$ ranging from 0.32 mM to 1.4 mM. Inotropic response to variation in $[Mg^{2+}]_o$ was recorded after stabilization and the change was calculated as percentage of baseline (1.2 mM).

III.4.3. Synergistic effect of ROS and marginal Mg deficiency on myocardial inotropy

After stabilization of the muscle for 1h, inotropic response of papillary muscle was measured in marginal Mg deficiency in presence of HX+XO and H_2O_2.

III.4.4. Recovery of contraction in fresh medium

After treating the muscle with different experimental interventions the superfusate was substituted with fresh medium and stable contraction was recorded.

III.4.5. Inotropic response to the presence of antioxidant enzymes

The muscles were pre treated with the scavenger enzymes and then the reactive oxygen species were added. The scavenging enzymes used for SO anions was 100 U SOD and that for H_2O_2 was 120 U catalase. A combination of SOD and CAT was also examined.

III.4.6. Inotropic response to channel modulators

Ion channel antagonists are commonly used to assess the functional contribution of different channels to varying physiological and experimental conditions. In myocardium there are specific pathways for transport of Ca across the sarcolemma and sarcoplasmic reticulum. The impact of individual channels can be assessed using competitive channel blockers. The different channel blockers used for this study are given in table.2. The muscles were exposed to specific channel antagonists and the force of contraction was recorded. The muscle was then exposed to Mg deficiency and/or ROS. The inotropic variation produced by the treatment was compared with the change induced in the absence of the antagonist. Variation in the pattern of response is an indication of the involvement of the channel / pump in the induction of the mechanical response to the treatment.

III.4.7. Measurement of intracellular calcium level

In an attempt to correlate the mechanical change with the variation in calcium, the diastolic Ca levels were measured by dual – excitation fluorescent method, using ion sensitive cell permeant fluorescent probe, acetoxy methyl ester of fura-2. Fig.6. is a photomicrograph of cardiomyocyte after loading fura-2 AM. The ratio of fluorescence intensity elicited at two different wavelengths was determined and taken as the measure of the cytoplasmic level of calcium. The ratiometric method has the advantage that the measurements are not influenced greatly by changes in dye concentration

or cell volume. The intracellular Ca was measured in Mg deficient and sufficient media in presence and absence of ROS. The cells were loaded with 50 μM concentration of the probe and then exposed to the different treatments for 30 minutes in KRH buffer

in a CO_2 incubator. Loaded cells were then washed with KRH buffer without the probe by mild centrifugation at 20 g for 2 minutes to remove extracellular and bound dye. The pellet was then suspended in KRH buffer with normal levels of ions under study and placed into quartz cuvette. The fluorescence was measured in the spectrofluorometer at the emission wavelength of 490 nm and excitation wavelengths 340 nm and 380 nm (Cobbold and Rink, 1987).

III.4.8. Estimation of MDA

Malone dialdehyde was estimated by thio barbituric acid reactive substance assay of Nichans and Samuelson (1968). Tissue homogenate was prepared in 0.1 M Tris-HCl buffer (pH 7.5). 1 ml of the homogenate was combined with 2 ml of TCA-TBA reagent and mixed thoroughly. The solution was heated for 15 minutes in a boiling water bath. After cooling the mixture was centrifuged at 1000 X g for 10 minutes and the absorbance at 535 nm was recorded.

III.4.9. Assay of LDH

Lactate dehydrogenase was assayed in the coronary effluent immediately after collection using the standard kinetic assay of the rate of

decline in NADH absorbance at 340 nm as lactate is generated from pyruvate under non- limiting substrate conditions (Taylor *et al*, 2003).

III.4.10. Assay of adenosine tri phosphate (ATP)

Assay of ATP was carried out using commercially available kit (Sigma Chemical co.). The assay is based on the principle that, the enzyme phosphoglycerate phosphokinase catalyze the conversion of ATP into ADP along with the conversion of 3- phosphoglycerate to 1,3- diphosphoglycerate. The enzyme GAPD present in the reaction mixture catalyzes the conversion of 1,3- diphosphoglycerate to glyceraldehyde-3-phosphate along with the oxidation of NADH to NAD. By determining the decrease in absorbance at 340 nm that results when NADH is oxidised to NAD, a measure of ATP originally present is obtained.

A homogenate of the perfused ventricular portion was prepared in 12% TCA. The homogenate was mixed well and allowed to stand for approximately 5 minutes in an ice bath and centrifuged at 3000 rpm for 5-10 minutes to obtain a clear supernatant. Into 0.3 mg NADH vial the solutions were pipetted in the following order: 1.0 ml PGA buffered solution, 1.5 ml water, 0.5 ml supernatant. The vial was capped and inverted several times to dissolve the NADH. The contents were decanted into a cuvette. The initial absorbance (A_1) vs water was used as reference at 340 nm. Into the cuvette 0.04 ml GAPD/PGK enzyme mixture was pipetted and mixed by inversion. The cuvette was replaced in the instrument and the absorbance recorded

continuously until minimum absorbance reading had reached. This was recorded as final (A_2). The difference in absorbance ΔA ($A_2 - A_1$) was used for calculating ATP content, and ATP was expressed in μmol/g protein.

III.4.11. Assay of ATP in subcellular fractions

Inorder to examine whether there is a difference in the mitochondrial synthesis and/or transport of ATP to cytosol, the ATP content of mitochondrial and cytosolic fractions were assayed. Subcellular fractions such as nuclear, mitochondrial and soluble cytosolic fractions were isolated by differential centrifugation. A 10% homogenate of the perfused ventricular portion was prepared in 0.35 M sucrose solution in ice-cold condition and filtered through a cheese cloth. The residue was discarded. The supernatant was centrifuged at 800 g for 10 minutes. The nuclear pellet was saved and the supernatant was again centrifuged at 15,000 g for 20 minutes. The mitochondrial pellet and soluble cytosolic fractions were stored separately. Aliquots of different fractions were used for estimation of protein. The pellet was dissolved in suitable volume of $HClO_4$ and used for ATP estimation by method of (Heinz *et al,* 1985). An aliquot of the solutions were taken and one drop of methyl orange indicator was added and neutralized with K_2CO_3. The solution was placed for 15 minutes in ice-bath and ATP was determined immediately in the supernatant after equilibration at 25°C.

Assay conditions :- Wavelength 339 nm; light path 10 mm; final volume 0.635 ml; room temperature; measurement against air.

Assay procedure

Sample - 0.1 ml

TEA solution - 0.4 ml

NADP solution - 0.01 ml

$MgCl_2$ solution - 0.05 ml

G-6-PDH - 0.005 ml

Glucose solution - 0.05 ml

The absorbence was recorded and the determination of ATP was initiated by the addition of

HK - 0.005 ml

The change in absorbence is proportional to the amount of ATP in the sample.

III.4.12. Assay of succinate dehydrogenase (EC.1.3.99.1)

The purity of mitochondrial preparation was tested by assay of SDH. SDH activity was assayed by method of Arrigoni and Singer (1962). The assay system consists of 0.9 µl of phosphate buffer pH 7.6, 3 µmoles of KCN, 0.8 µmoles of $CaCl_2$, 0.04 µmoles of DCIP, and 100 µl of the enzyme solution in a total volume of 1 ml. 50 µmoles of succinate and 0.05 ml of 0.2% PMS were added and the decrease in absorbance at 600 nm was recorded. The amount of dye reduced was calculated where 16.2 is the millimolar extinction coefficient. The enzyme activity was expressed as

units/mg protein where one unit enzyme activity represents one nano mole of dye reduced per minute.

III.4.13. Assay of creatine phosphate

Enzymatic method was preferred for the assay of creatine phosphate (Heinz, 1985).

Principle

Creatine phosphate + ADP $\xrightarrow{CK^{++}}$ creatine + ATP

ATP + D- glucose $\xrightarrow{HK}$ D- glucose 6- phosphate + ADP

D- glucose 6- phosphate $\xrightarrow{G\text{-}6\text{-}PDH}$ D- gluconolactone-6-phosphate +NADPH + H^+

Preparation of the sample

A weighed portion of freeze clamped tissue was added to a known volume of ice-cold perchloric acid solution (about 1 g to 9 ml). The tissue was ground rapidly and centrifuged in the cold 3000 g for 10'. An aliquot of the decanted solution was taken and one drop of methyl orange indicator was added and neutralized with K_2CO_3. The solution was placed for 15 minutes in ice-bath and creatine phosphate was determined immediately in the supernatant after equilibration at 25°C.

Assay conditions :- Wavelength 339 nm; light path 10 mm; final volume 0.635 ml; room temparature; measurement against air.

Assay procedure

Sample - 0.1 ml

TEA solution - 0.4 ml

NADP solution - 0.01 ml

$MgCl_2$ solution - 0.05 ml

Glucose solution - 0.05 ml

ADP solution - 0.005

G-6-PDH - 0.005 ml

HK - 0.005 ml

Mix, read till constant A or ΔA linear with time. Read A_1

CK solution - 0.01 ml

Mix, read every two minutes for ~ 15'; or monitor the reaction by extrapolation with time and take final A_2. $\Delta A = A_1 - A_2$. The change in absorbence was directly proportional to the creatine phosphate concentration in the sample.

III.4.14. Assay of protein

Protein content was assayed by Lowry's method (Lowry et al, 1951). The TCA precipitate of the sample was dissolved in a suitable volume of 0.1N NaOH. 50 µl of the sample was made up to 300 µl with distilled water. 1 ml of alkaline copper reagent was added and allowed to stand for 10'. Then 0.1 ml of diluted Folin's – Ciocalteaue – phenol reagent was added, kept for

30' and the absorbence read at 660 nm. The concentration of protein was calculated using BSA as standard.

III.5. STATISTICAL ANALYSIS

The data are presented as mean ± SD values for each set. Each experimental observation was based on a minimum of 4 replicates. ANOVA was applied for comparison between groups wherever necessary. The difference between selected means were evaluated via unpaired Student's t-test. A level of $p < 0.05$ was selected to indicate statistical significance.

Table.2. Ion channel modulators – their specificity and concentrations used

Channel	Modulator	Concentration	References
SL – L - type Ca channel	Verapamil	1 μM	Tada et al, 1982; Yang et al, 1996
SL – T- Type Ca channel	$NiCl_2$	40 μM	Hagiwara et al, 1988; Marban et al, 1995
Na-K – ATPase	Ouabain	0.3 mM	Kennedy et al, 1986
SR-Ca pump	Caffeine	10 mM	O'Neill et al, 1990
SR-Ca release channel	Ryanodine	1 μM	Seguchi et al, 1986; Su and Chang, 1993

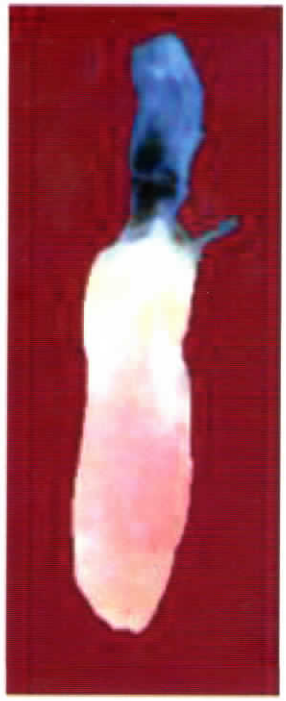


Fig.1. Isolated papillary muscle

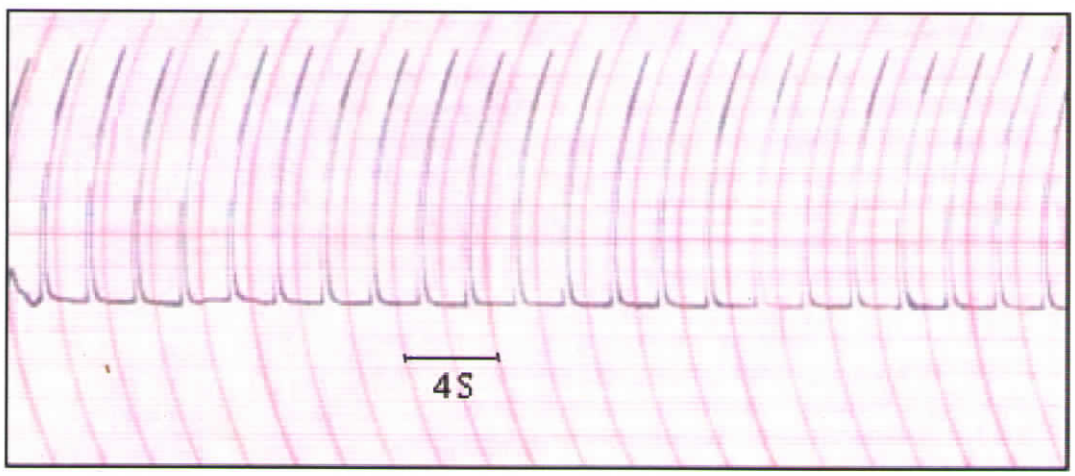


Fig.2. Representative tracing of papillary muscle contraction

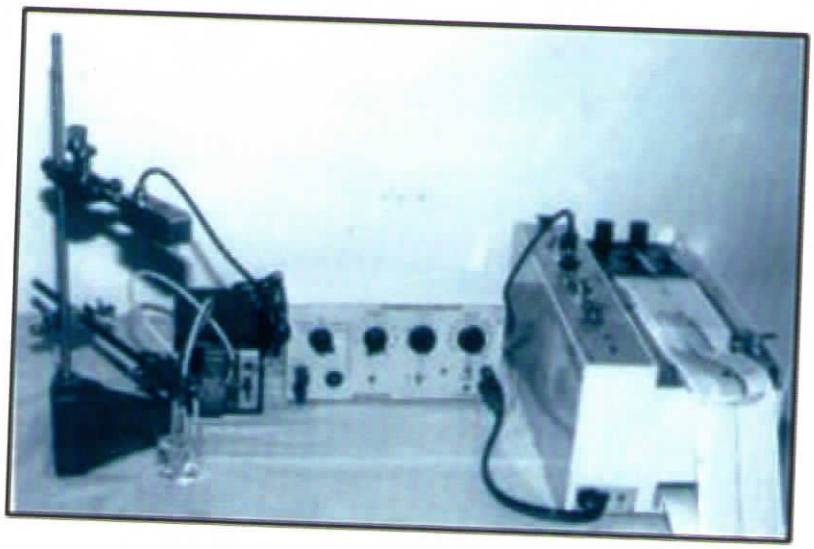


Fig.3. Set up for measurement of papillary muscle contraction

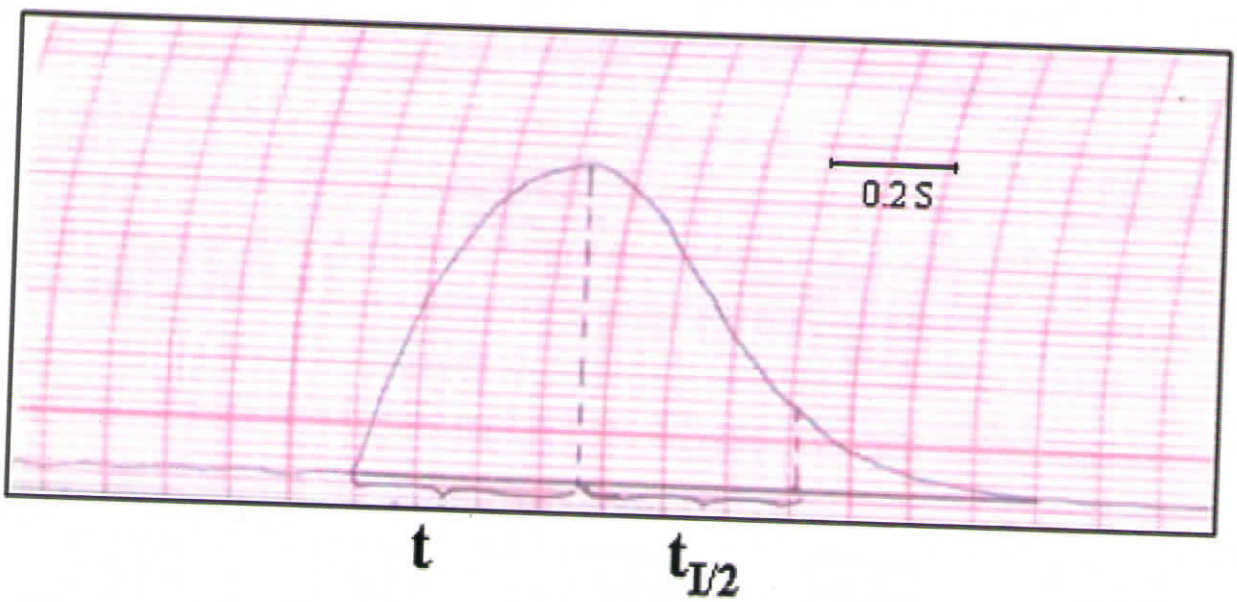


Fig.4. Measurement of time for contraction (t) and half relaxation($t_{1/2}$)

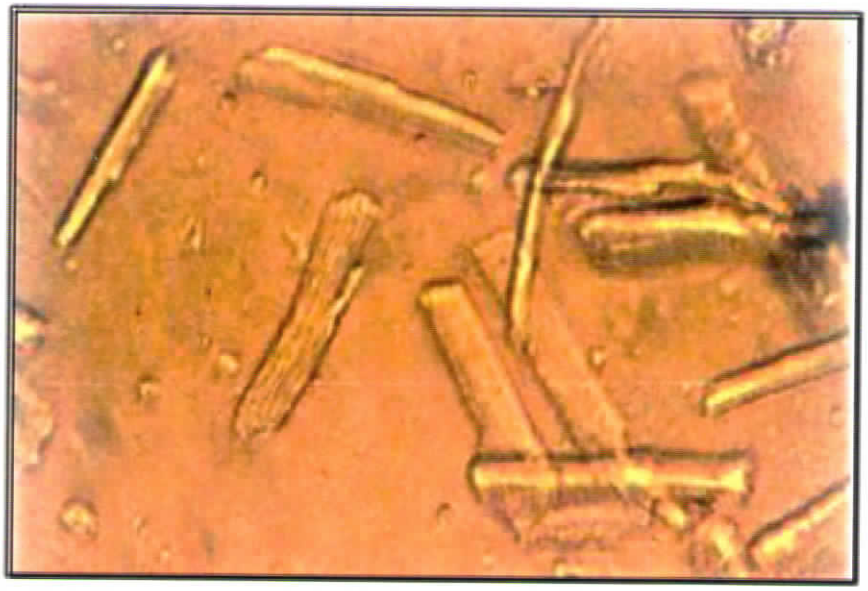


Fig.5. Isolated cardiomyocytes Magnification 170X

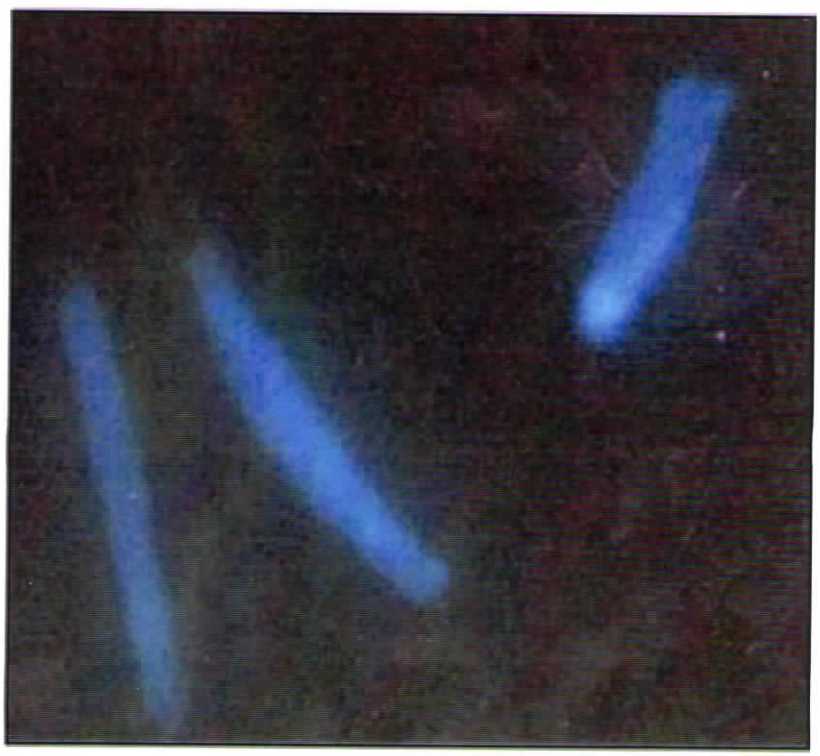


Fig.6. Fura-2 AM loaded cardiomyocytes Magnification 320X

IV. RESULTS

IV. RESULTS

The experimental results are presented under the following headings:

Inotropic response of the myocardium and diastolic levels of Ca on exposure to

a) Reactive oxygen species

b) Marginal magnesium deficiency.

c) A combination of oxidative stress and marginal magnesium deficiency.

Delineation of the mechanism responsible for contractile variation.

a) Role of sarcolemmal and sarcoplasmic reticular ion channels and pumps regulating Ca transients.

b) Biochemical variables with possible influence on contractile function.

IV.1. MYOCARDIAL MECHANICS

Myocardial mechanics were studied in papillary muscles isolated from left ventricle and mounted vertically in a chamber to contract isometrically. Muscles having identical geometry were selected for the study.

IV.1.1. Inotropic response of the myocardium to reactive oxygen species

Inotropic response of the papillary muscle was measured in the presence of two commonly used generators of reactive oxygen species:

superoxide anion generator hypoxanthine + xanthine oxidase (HX+XO) and hydrogen peroxide (H_2O_2).

IV.1.1.1. Inotropic response to HX+XO

Inotropic response of papillary muscle was measured in the presence of three different concentrations of the superoxide anion generating system. Increasing the concentration of SO anion generating system from 0.5mM HX +0.01U/ml XO to 1mM HX+0.04U/ml XO was associated with a concentration dependent decrease in force of contraction. At 0.5mM HX+0.01U/ml XO practically no contractile variation was produced ie; the force of contraction was comparable to control. At 0.5mM HX+0.02U/ml XO the force of contraction was found to be 83% of the baseline, but at a higher concentration, 1mM HX+0.04U/ml XO the contractile force decreased to 68% of the baseline (fig.7) and plate.I. Diastolic Ca^{2+} showed the same pattern as that of contractile response (fig.8).

The contractile variation produced up to 0.5mM HX+0.02U/ml XO was found to be completely reversible on transfer to medium without the superoxide anion generator, but at higher concentration the recovery was incomplete with contraction being 87% of the baseline (fig.9).

Pretreatment with the superoxide anion scavenger SOD protected the muscle from producing any contractile variation upto 0.5 mM HX+0.02 U/ml XO. But at higher concentration the contraction was 86% of the baseline (fig.9). A combination of SOD and catalase did not produce an additive

effect. The addition of SOD after the induction of contractile variation was found to augment the negative inotropic response. The force of contraction reduced to 65 % of the baseline on post treatment with superoxide dismutase.

On treatment with 0.5mM HX+0.02U/ml XO there was no significant difference in time for contraction and half relaxation when compared to control, but at higher concentration, time for contraction was significantly reduced with a prolongation of time for half relaxation (fig.10).

To rule out the independent effects of HX and XO on contractility, the inotropic response to these compounds were examined. It was observed that neither HX nor XO independently affected myocardial contractility.

IV.1.1.2. Inotropic response to hydrogen peroxide

Hydrogen peroxide also induced significant negative inotropy at concentration as low as 10 μM. A concentration dependent increase in negative inotropy was observed on increasing the concentration of H_2O_2 from 10 μM to 100 μM (fig.11) and plate.II. Intracellular Ca^{2+} also showed the same pattern as that of contractile response (fig.12). Post treatment of the muscle with catalase decreased the force of contraction to 68% of the base line at 50 μM H_2O_2.

The recovery of contraction was found to be complete upto 50 μM H_2O_2, but at 100 μM H_2O_2 recovery was 78% in H_2O_2 free medium. Pretreatment with 120 U catalase did not produce any contractile variation

upto 50 μM. At 100 μM H_2O_2, on pretreatment with 120 U catalase, the force of contraction was 95% of the baseline (fig.13).

The time for contraction and half relaxation were also examined in presence of H_2O_2. At concentrations upto 50 μM, both time for contraction and half relaxation were found to be unaltered when compared to control. At higher concentration, 100 μM, both time for contraction and half relaxation were found to be reduced significantly (fig.14).

The contractile variations in response to both the ROS were recorded in both male and female rats, and it was found that the contractile response produced by both male and female were comparable, suggesting that there is no gender difference in the response to reactive oxygen species.

IV.1.2. Inotropic response to variation in extracellular magnesium

The contractile response to variation in extracellular magnesium $[Mg^{2+}]_o$ was studied by exposing the papillary muscle to different levels of Mg^{2+}. A positive inotropy was observed with a decrease in $[Mg^{2+}]_o$ (fig.15) and plate. III. Maximum contraction was recorded when $[Mg^{2+}]_o$ was 0.48 mM. The extent of increase in contractility was 58% more than that of the control (1.2mM). This also corresponds to the serum Mg levels of animals on Mg deficient diet. Hence for further studies 0.48 mM was taken as marginally magnesium deficient, whereas 1.2 mM Mg was considered Mg sufficient. Diastolic Ca level also followed the same pattern as that of contractile response (fig.16). Time for contraction was found to be unaltered

in marginal Mg deficiency whereas the time for half relaxation reduced significantly (fig.17).

IV.1.3. Synergistic effect of ROS and marginal Mg deficiency

IV.1.3.1. Effect of HX+XO in marginal Mg deficiency

The force of contraction of papillary muscle to HX+XO was found to be significantly reduced in marginal Mg deficiency at concentration as low as 0.5mM HX+0.02U/ml XO and the negative inotropy was found to be augmented at higher concentration of the SO anion generating system (fig.18) and plate.IV. Diastolic Ca^{2+} levels also followed the same pattern as that of inotropic response (fig.19).

At lower concentration of SO anion generating system, 0.5 mM HX+0.02 U/ml XO the recovery of contraction in fresh medium was complete both in Mg sufficiency and marginal Mg deficiency. At higher concentration, the recovery was 87% in Mg sufficiency whereas it was only 80% in marginal Mg deficiency. Pretreatment with the scavenger SOD (100 U) also showed a force of contraction close to the baseline in both Mg sufficiency and marginal Mg deficiency at lower concentration. But at higher concentration, 1 mM HX+0.04U/ml XO, in presence of 100 U of SOD, the force of contraction was 86% in Mg sufficiency while it was only 77% in marginal Mg deficiency (fig.20). These results were obtained only when the antioxidant enzyme was added before producing the contractile variation.

Addition of the scavenger after producing the contractile variation augmented the negative inotropism. In presence of 0.5mM HX+0.02U/ml XO, post treatment of the muscle produced a force of contraction 65% of the baseline in Mg sufficiency and 58%of the baseline in marginal Mg deficiency.

Eventhough the time taken for contraction was reduced in both the concentrations, the reduction was significant at the higher concentration, 1 mM HX+0.04 U/ml XO. The time taken for half relaxation was prolonged in both higher and lower concentrations of HX+XO, the increase being statistically significant only at lower concentration (fig.21).

IV.1.3.2. Effect of H_2O_2 in marginal Mg deficiency

In presence of H_2O_2 papillary muscle showed a dose dependent reduction of contractile force. Eventhough a significant negative inotropy was observed from 10 μM onwards, a significant difference between Mg sufficiency and marginal Mg deficiency was observed only at higher concentration, 100 μM (fig.22) and plate.V. Diastolic Ca^{2+} level also showed the same pattern as that of contractile response (fig.23). Hence for further studies 100 μM H_2O_2 was opted for producing oxidative stress.

In Mg sufficiency the contractile variation produced upto 50 μM was completely reversible whereas in marginal Mg deficiency complete recovery was observed only upto 20 μM. At 25 μM recovery was 94% and at

50 μM it was 90%. At100 μM H_2O_2 in Mg sufficiency the recovery in fresh medium was 96% whereas in marginal Mg deficiency it was78%.

In Mg sufficiency on pretreatment with the scavenger, catalase the force of contraction was close to the baseline upto 50 μM. In marginal Mg deficiency pretreatment with the scavenger protected the muscle from the induction of contractile variation upto 20 μM. At 25 μM the force of contraction was 94% and at 50 μM it was 89% and 100 μM, 85% (fig.24).

Addition of the scavenger after the induction of negative inotropy augmented the negative inotropic response. In Mg sufficiency at 100 μM H_2O_2 the force of contraction was 64% when catalase was added after producing the contractile variation by H_2O_2, whereas it was 52% in Mg deficiency.

Time taken for contraction and half relaxation were unaltered at 10 μM H_2O_2, but it was reduced significantly at 20 μM. At higher concentrations, 50 μM and 100 μM H_2O_2, the changes in both the time for contraction and half relaxation were statistically insignificant (fig.25).

IV.2. DELINEATION OF MECHANISM RESPONSIBLE FOR CONTRACTILE VARIATION

IV.2.1. Role of ion channels and pumps in the mediation of mechanical change

The impact of individual ion channels and pumps which can possibly induce inotropic variations were assessed using competitive channel inhibitors. The inotropic variation produced by different treatments were

compared with the change induced in the presence and absence of the antagonist.

IV.2.1.1. Ion channels and pumps mediating the inotropic response to free radical stress

Inotropic response to free radicals appears to be modulated by SL L-type Ca channel as indicated by the reduction in negative inotropic response in presence of SL L type Ca channel blocker verapamil. The negative inotropic response was reduced in the presence of SR Ca pump inhibitor caffeine, but augmented in the presence of Na K ATPase inhibitor ouabain. The T type Ca channel blocker, $NiCl_2$ and SR Ca release channel blocker, ryanodine did not influence the response to HX+XO (fig.26)

As observed for HX+XO, the negative inotropic response in the presence of caffeine was significantly reduced on exposure to H_2O_2. Unlike the changes observed with HX+XO the response to H_2O_2 was significantly enhanced in the presence of SL L type Ca channel blocker, verapamil, and SR Ca release channel blocker, ryanodine. The pattern was comparable in the presence of Na K ATPase blocker ouabain but the variation was not statistically significant for H_2O_2 Sarcolemmal T type Ca channel blocker, $NiCl_2$ also did not affect the contractile variation in presence of H_2O_2 (fig.27). The results therefore indicate similarities as well as differences in the site of action of the two reactive oxygen species.

IV.2.1.2. Ion channels and pumps influencing inotropic response to marginal Mg deficiency

The negative inotropic response to L type blocker verapamil and T type blocker $NiCl_2$ was augmented in marginal Mg deficiency. The positive inotropic effect in the presence of Na K ATPase inhibitor ouabain was also significantly higher. The inotropic response to SR Ca pump inhibitor and ryanodine was not significantly affected by Mg deficiency (fig.28)

IV.2.1.3. Influence of antagonists of ion channels and pumps on inotropic response to ROS synergistically with marginal Mg deficiency

The negative inotropic response to ROS was augmented in Mg deficiency by both HX+XO and H_2O_2. The mechanism mediating the inotropic response as observed using channel antagonists was comparable for some and different for others. A significant interaction between marginal Mg deficiency and HX+XO was observed only in the presence of Na K ATPase inhibitor ouabain with a reduction in the negative inotropic response. The contractile response was comparable in the presence of verapamil when exposed to HX+XO in sufficient or low Mg. The enhanced negative inotropic response to caffeine and attenuated response to $NiCl_2$ and ryanodine were not statistically significant (fig.29).

H_2O_2 when associated with marginal Mg deficiency produced a significant difference in inotropic response in presence of SL T type Ca channel blocker, $NiCl_2$ and SR Ca pump antagonist caffeine. The inotropic change in the presence of inhibitors of all other channels and pumps were not

statistically significant but comparable with that of HX+XO for caffeine and the patterns were different for $NiCl_2$ and ryanodine (fig.30).

IV.2.2. Biochemical variables influencing mechanical function

The biochemical variables influencing mechanical functions were examined in Langendorff perfused heart.

IV.2.2.1. Lipid peroxidation

The extent of lipid peroxidation due to ROS was assayed by measurement of TBARS. The level of TBARS was significantly increased on exposure to ROS in Mg sufficient medium. Both HX+XO and H_2O_2 produced a significant enhancement in the level of TBARS. In HX+XO treated myocardium TBARS was increased by 12% and in H_2O_2 treated myocardium by 18%. The increase induced by both the ROS were comparable (fig.31). The extent of lipid peroxidation produced in marginal Mg deficiency though slightly more than that in Mg sufficient medium, the difference was statistically not significant suggesting that marginal Mg deficiency does not induce significant lipid peroxidation (fig.32).

ROS associated with marginal Mg deficiency produced a significantly higher level of TBARS when compared to sufficiency. In Mg sufficiency the extent of lipid peroxidation was increased by ~ 12% in presence of SO anion whereas the increase was ~ 73.65% in marginal Mg deficiency when treated with same concentration of HX+XO. H_2O_2 also showed the same trend. In

Mg sufficiency H_2O_2 produced a TBARS level of 18% above the control while in marginal Mg deficiency it was 73.08% (fig.33).

IV.2.2.2. Release of LDH

Lactate dehydrogenase release into coronary effluent was assayed as a marker of tissue injury. In HX+XO treated myocardium LDH was increased by 45% and in H_2O_2 treated myocardium by 54% when compared to untreated control. (fig.34).

In marginal Mg deficiency LDH was increased by nearly 37% when compared to Mg sufficiency (fig.35).

A combination of SO and marginal Mg deficiency produced an enhancement of 73.63%, whereas in sufficiency it was 45%. H_2O_2 when associated with marginal Mg deficiency produced an enhancement of LDH by 76.18%, while that in Mg sufficiency was 54% (fig.36).

IV.2.2.3. High energy phosphate compounds

High energy phosphate compounds are the source of energy for myocardial contraction. Hence the levels of ATP and CP were assayed for delineating the possible involvement of these compounds in the induction of contractile variation.

IV.2.2.3.1. Myocardial ATP

Adenosine tri phosphate was assayed in the ventricular portion of Langendorff perfused whole heart. Myocardial ATP content was reduced by 27% on exposure to HX+XO and 39% in the presence of H_2O_2 (fig.37).

In marginal Mg deficiency the level of ATP was comparable to that of control (fig.38).

Superoxide anions when associated with marginal Mg deficiency produced a significant reduction in the level of ATP when compared to sufficiency. Myocardial ATP content was reduced by 27% in sufficiency, whereas it was reduced by 48% in marginal Mg deficiency on treatment with HX+XO. Hydrogen peroxide also produced a similar pattern of reduction in the level of ATP. In Mg sufficiency 39% reduction in ATP was produced by H_2O_2 while in deficiency it was 63% (fig.39).

In an attempt to examine whether the synthesis and/or transport of ATP was affected, the mitochondrial to cytoplasmic ratio of ATP was assayed. The first step in this protocol was isolation of subcellular fractions. The purity of isolated fractions were checked by determining the activity of the mitochondrial marker succinate dehydrogenase in isolated fractions. About 87% activity of the marker enzyme was associated with the mitochondrial pellet, thereby confirming the purity of the preparation (fig.40).

The mitochondrial level of ATP was 22.48 nmole/mg protein in control and 18.34 and 15 nmoles/mg protein respectively on perfusion with HX+XO and H_2O_2. The mitochondrial ATP level in marginal Mg deficiency was 20.9 nmoles/mg protein. In marginal Mg deficiency mitochondrial ATP was 12.68 and 10.01 nmoles/mg protein on treatment with HX+XO and H_2O_2

respectively. The results indicate that mitochondrial ATP content is significantly reduced in presence of ROS with additive effect in Mg deficiency.

In HX+XO as well as H_2O_2 treated myocardium, the mitochondrial to cytosolic ratio of ATP was significantly higher when compared to control, which shows the possibility of impaired transport of ATP from the mitochondrial matrix to cytosol in free radical stress (fig.41).

In marginal Mg deficiency also the mitochondrial to cytosolic ratio of ATP was significantly higher than the control (fig.42).

When exposure to ROS (both SO and H_2O_2) was associated with marginal Mg deficiency, the mitochondrial to cytosolic ratio of ATP was found to be higher when compared to sufficiency. Both the synthesis and transport of ATP may be affected, thereby reducing significantly the available ATP for myocardial contraction (fig.43).

IV.2.2.3.2. Myocardial creatine phosphate

The high energy phosphate compound creatine phosphate was also assayed in Langendorff perfused myocardium. CP is the storage form of ATP which is mobilized for the generation of ATP.

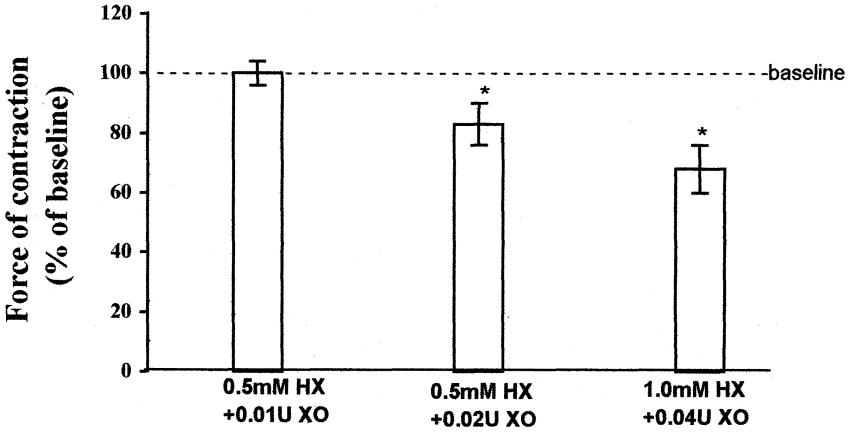
The reduction of CP was relatively higher on exposure to H_2O_2 compared to superoxide anion generator. In HX+XO treated myocardium CP was reduced by 45% and in H_2O_2 treated myocardium by 65% when compared to control (fig.44).

In marginal Mg deficiency eventhough the ATP content of the myocardium was comparable to control, CP was significantly reduced suggesting the possibility of mobilization of CP for the maintenance of ATP level (fig.45).

Combination of marginal Mg deficiency and FR stress produced a further reduction in the level of CP. The reduction in CP level in Mg sufficiency associated with SO anion was nearly 45% whereas in deficiency it was nearly 78%. H_2O_2 produced a reduction in CP level of nearly 65% in Mg sufficiency, whereas the reduction comes to be about 80% in marginal Mg deficiency (fig.46).

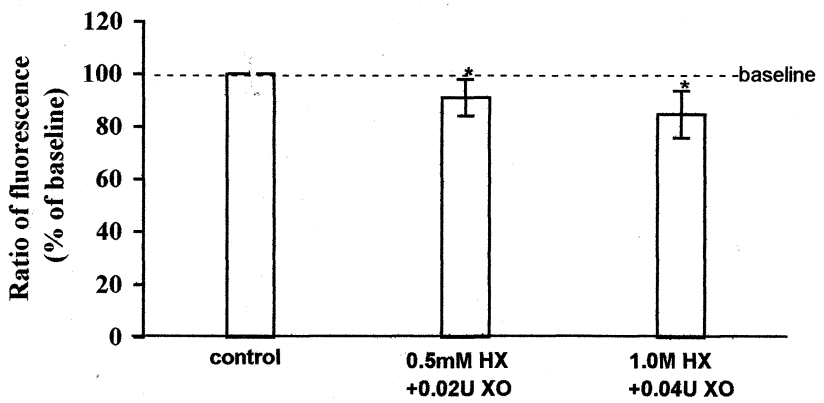
The observations of this study indicate that the negative inotropic response to ROS is augmented in marginal Mg deficiency. In addition to the influence of membrane channels and pumps modulating contraction, the energy available for contraction is significantly compromised when oxidative stress is associated with suboptimal levels of extracellular magnesium.

Fig.7. Effect of HX+XO on the inotropic response of papillary



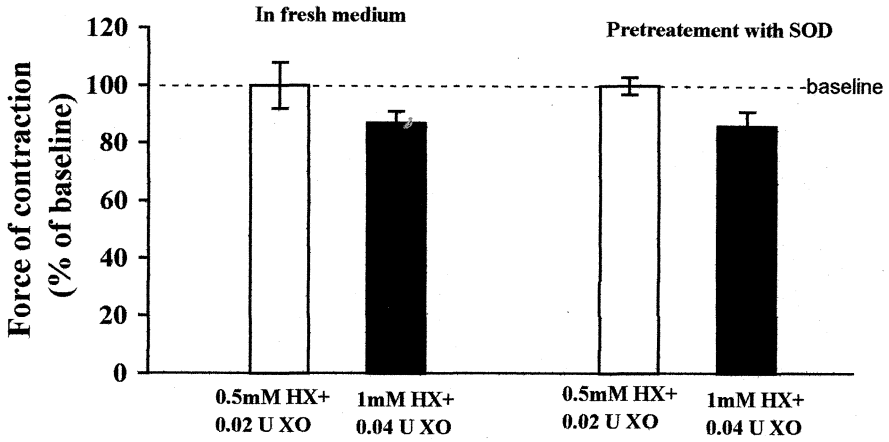
Values are mean ± SD; n = 15-23 preparations
***p<0.05 Vs baseline**

Fig.8. Effect of HX+XO on diastolic calcium level



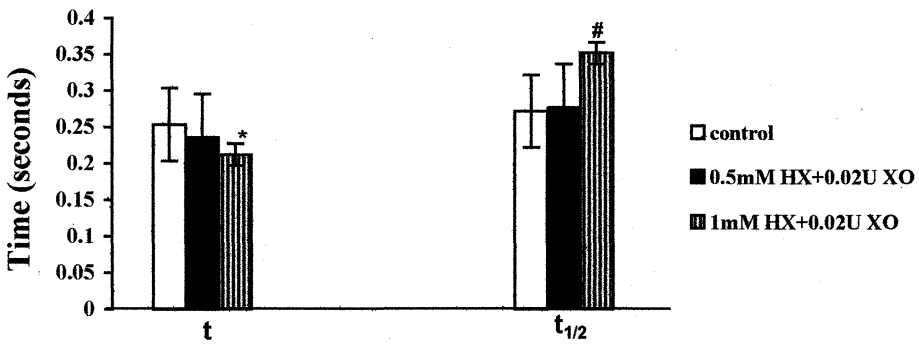
Values are mean ± SD; n = 4-6 preparations
***p<0.05 Vs control**

Fig.9. Recovery of contraction of HX+XO treated muscle



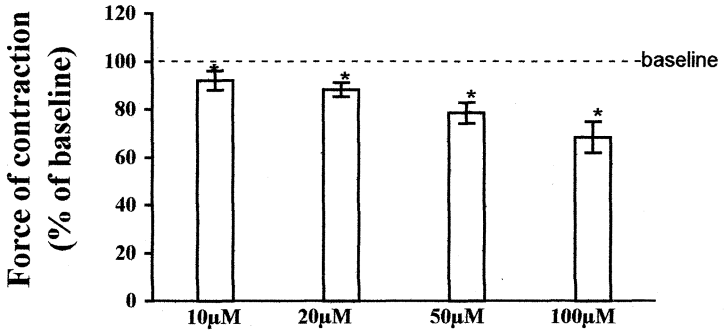
Values are mean ± SD; n = 4-8 preparations

Fig.10. Effect of HX+XO on time for contraction (t) and half relaxation ($t_{1/2}$)



Values are mean ± SD; n = 20-25 preparations
*p<0.05 Vs control, #p<0.005 Vs control

Fig.11. Effect of H_2O_2 on force of contraction

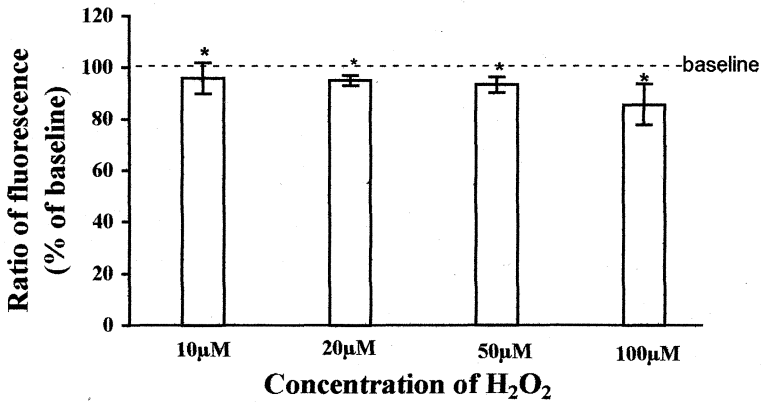


Concentration of H_2O_2

Values are mean ± SD; n = 8-12 preparations

*p<0.05 Vs baseline

Fig.12 Effect of H_2O_2 on diastolic Ca level

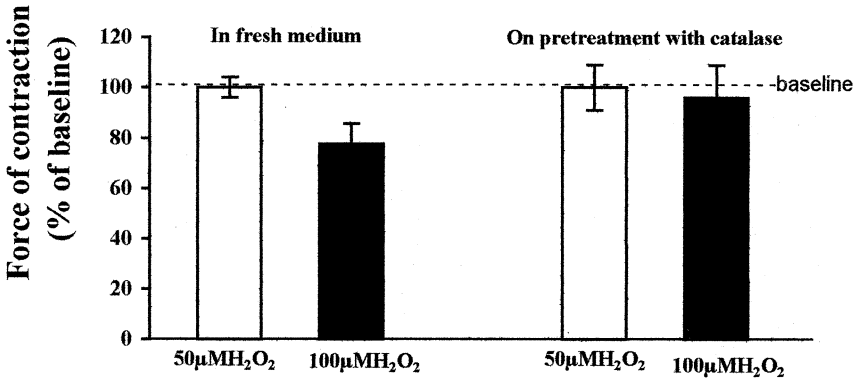


Concentration of H_2O_2

Values are mean ± SD; n = 6-8 preparations

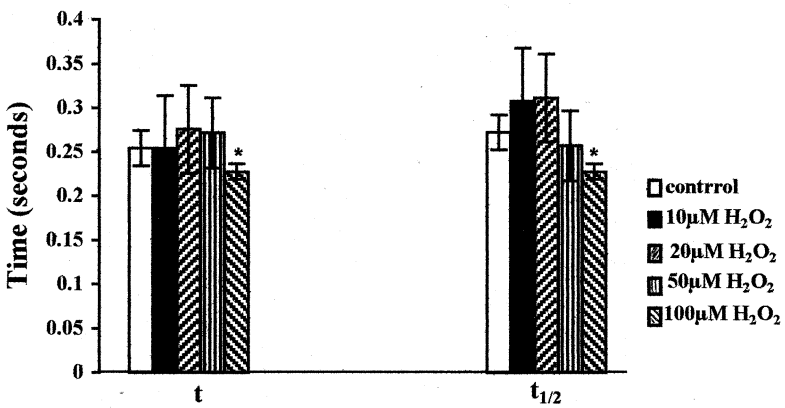
*p<0.05 Vs baseline

Fig.13. Recovery of contraction of H_2O_2 treated muscle



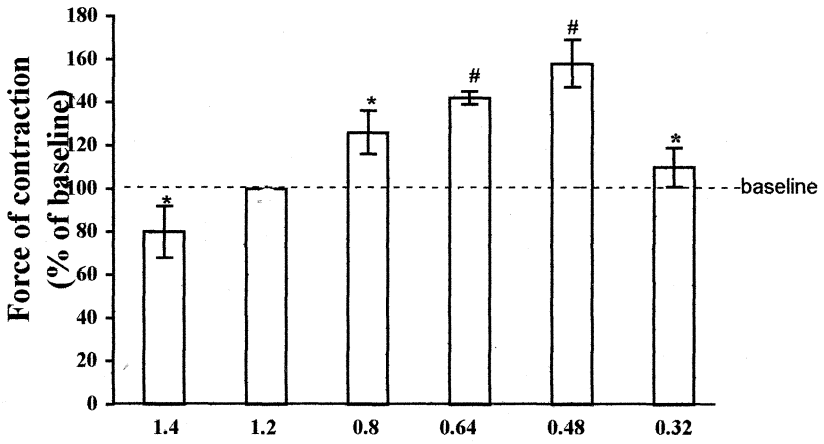
Values are mean ± SD; n=6-9 preparations

Fig.14. Effect of H_2O_2 on time for contraction and half relaxation



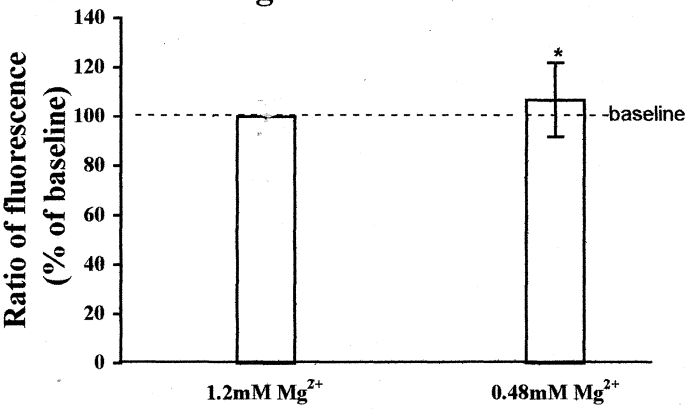
Values are mean ± SD;
n=10-12 preparations
*p<0.005 Vs control

Fig.15. Inotropic response of papillary muscle to variation in extracellular Mg



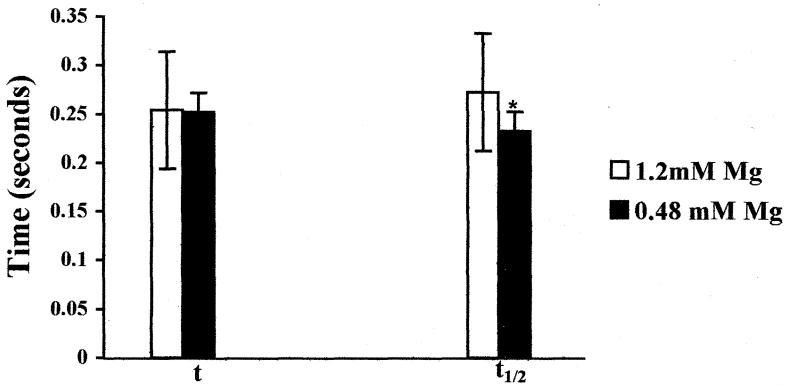
Values are mean ± SD; n = 10-13 preparations
*p<0.05 Vs baseline, #p<0.001 Vs baseline

Fig.16. Effect of extracellular Mg on diastolic Ca level



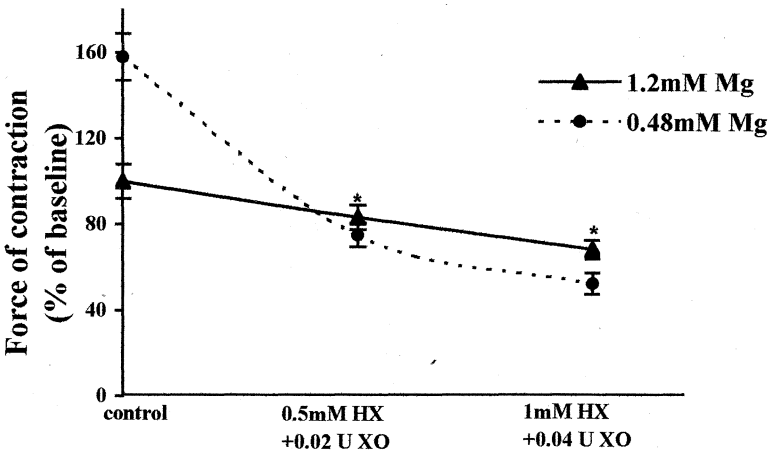
Values are mean ± SD; n = 10-12 preparations,
*p< 0.05 Vs baseline

Fig.17. Effect of extracellular Mg on time for contraction and half relaxation



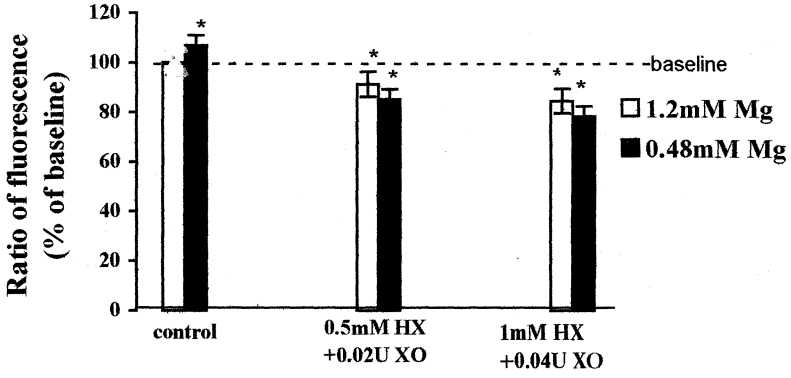
Values are mean ± SD; n=10-13 preparations. *p<0.05 for 0.48mM Vs 1.2 mM

Fig.18. Effect of extracellular Mg on inotropic response to HX+XO



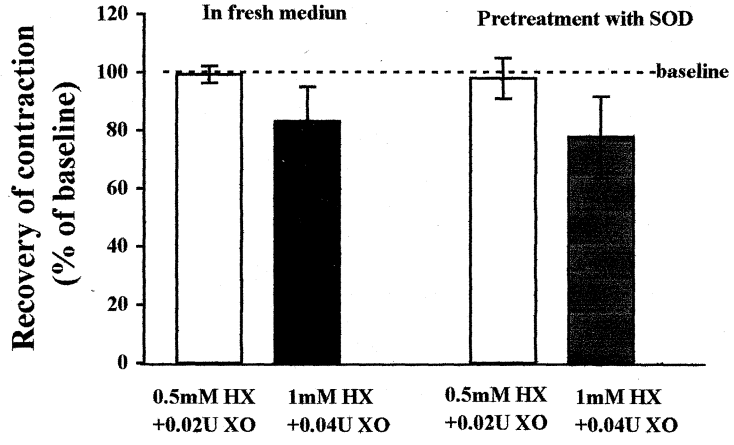
Values are mean ± SD; n = 15-18 preparations
***p<0.05 0.48mM Vs 1.2 mM**

Fig.19. Effect of extracellular Mg on diastolic Ca level in response to HX+XO



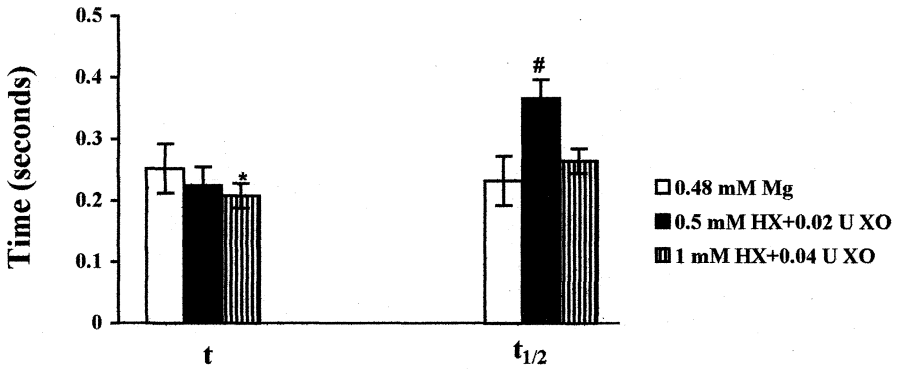
Values are mean ± SD; n = 8-10 preparations *p<0.05 Vs baseline

Fig.20. Recovery of contraction of HX+XO treated muscle in Mg deficiency



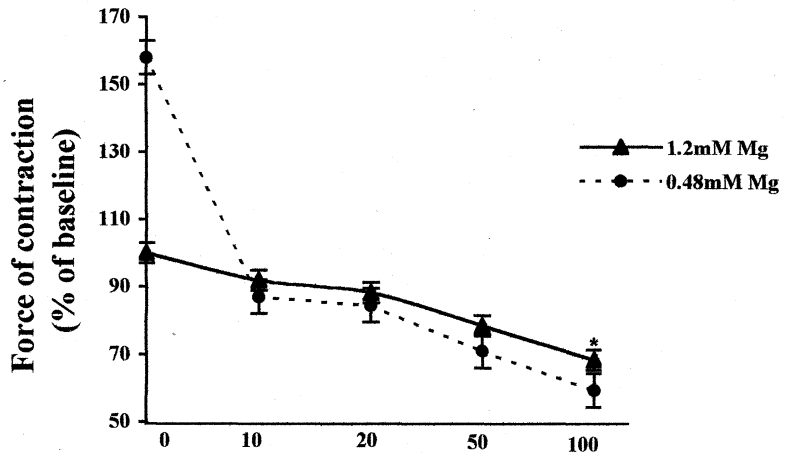
Values are mean ± SD; n = 8-10 preparations

Fig.21. Effect of extracellular Mg on time for contraction and half relaxation



Values are mean ± SD; n = 15-18 preparations
***p<0.05 Vs control, #p<0.005 Vs control**

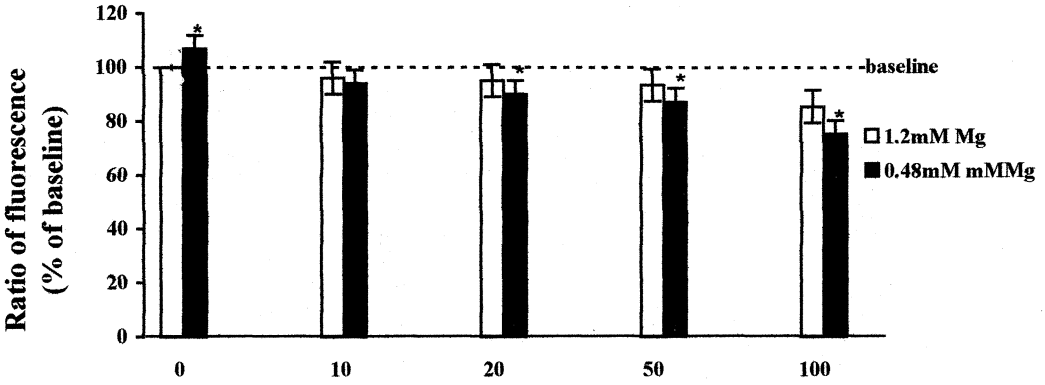
Fig.22. Effect of extracellular Mg on the inotropic response to H_2O_2



Concentration of H_2O_2 (μM)

Values are mean ± SD; n = 10-15 preparations
***p< 0.05, 0.48mM Mg Vs 1.2m M Mg**

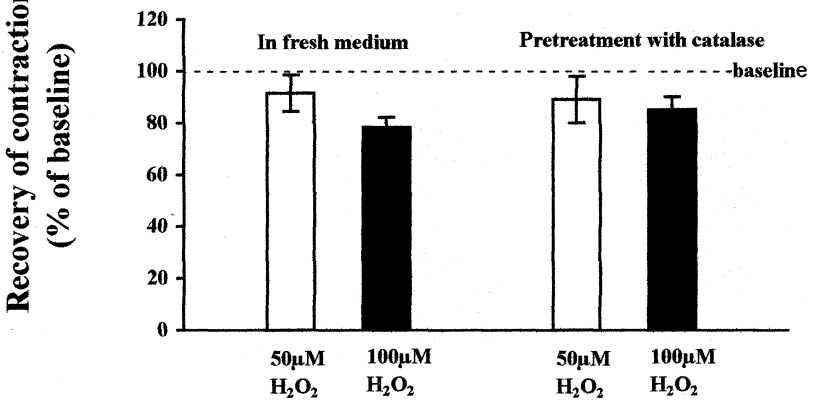
Fig.23. Effect of extracellular Mg on diastolic Ca level in response to H_2O_2



Values are mean ± SD; n = 6-9 preparations

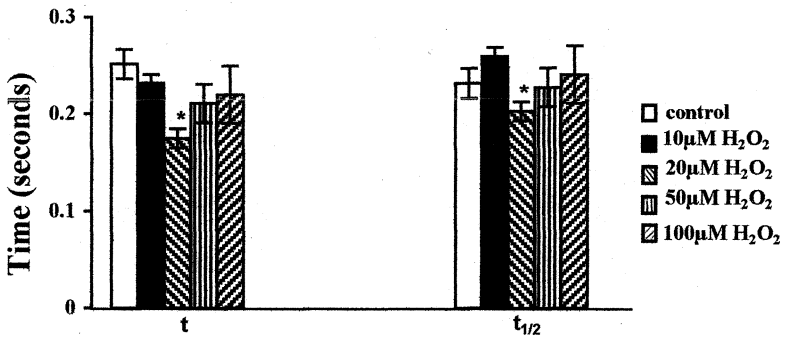
*p< 0.05 Vs baseline

Fig. 24 Recovery of contraction of H_2O_2 treated muscle in Mg deficiency



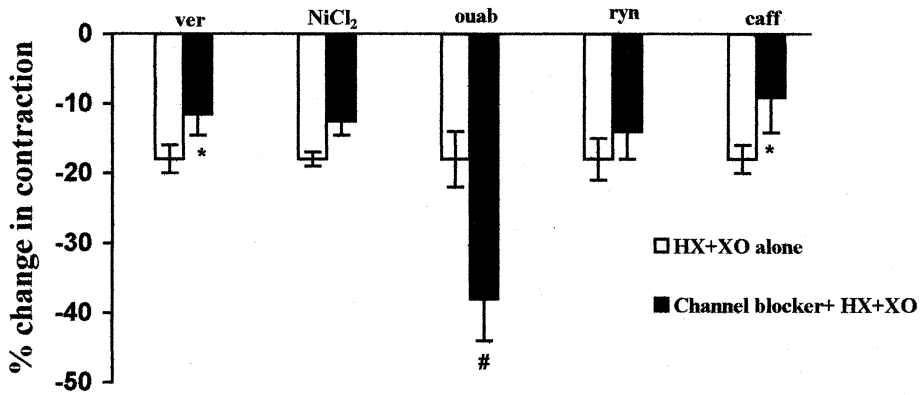
Values are mean ± SD; n = 6-9 preparations

Fig.25 Effect of extracellular Mg on time for contraction and half relaxation in response to H_2O_2



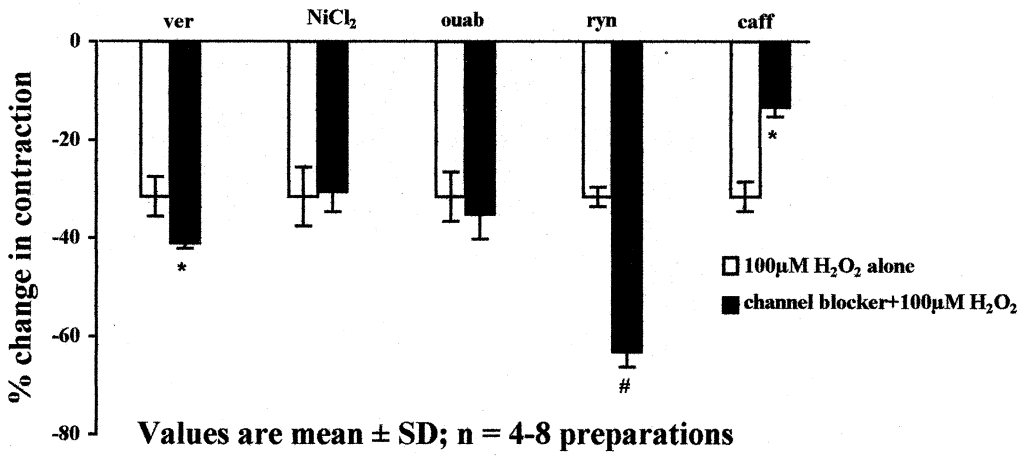
Values are mean ± SD; n = 23-25 preparations
***p< 0.005 Vs control**

Fig.26. Effect of ion channel blockers on response to HX+XO



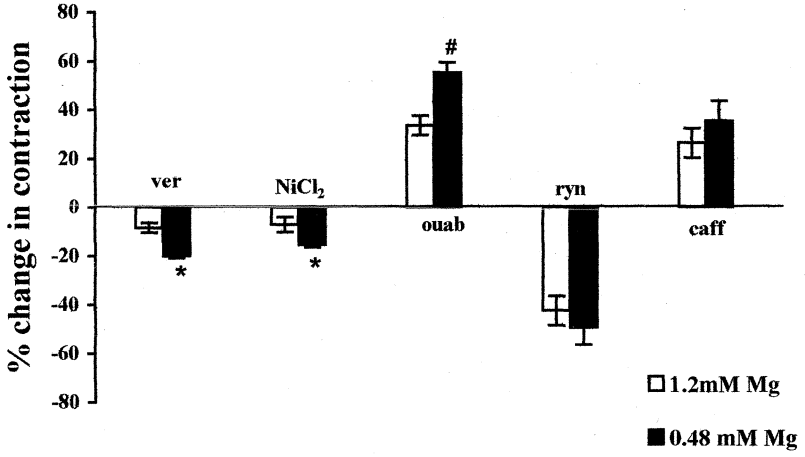
Values are mean ± SD; n = 4-8 preparations
*p<0.05 Vs HX+XO, #p< 0.001 Vs HX+XO

Fig.27. Effect of channel blockers on response to H_2O_2



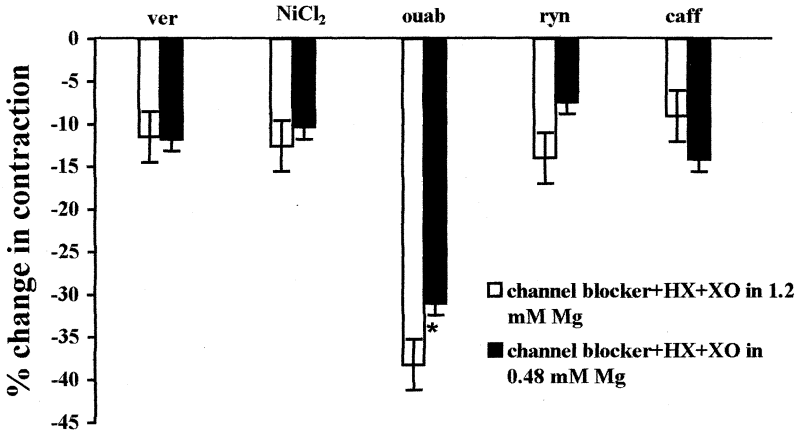
Values are mean ± SD; n = 4-8 preparations
*p<0.05 Vs HX+XO, #p< 0.001 Vs HX+XO

Fig.28. Differential response to channel blockers in Mg deficiency



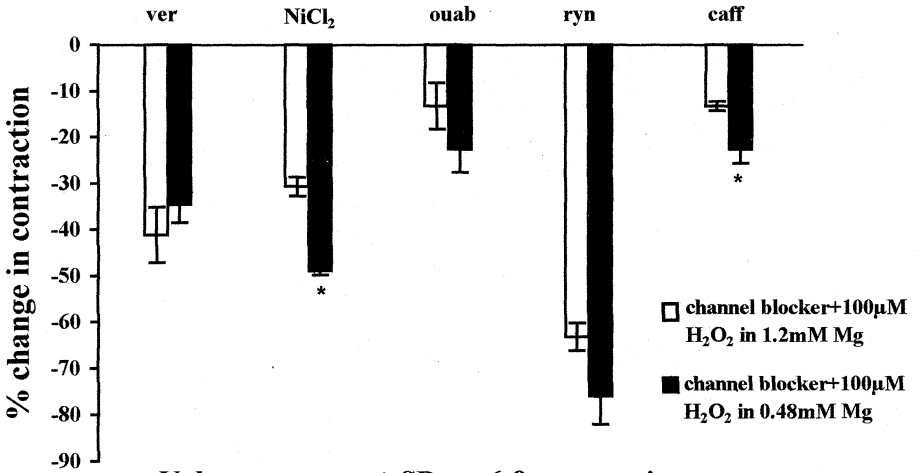
Values are mean ± SD; n = 4-8 preparations
***p<0.001 Mg deficiency Vs Mg sufficiency**
 # p<0.001 Mg deficiency Vs Mg sufficiency

Fig.29. Effect of channel blockers on response to HX+XO in Mg deficiency



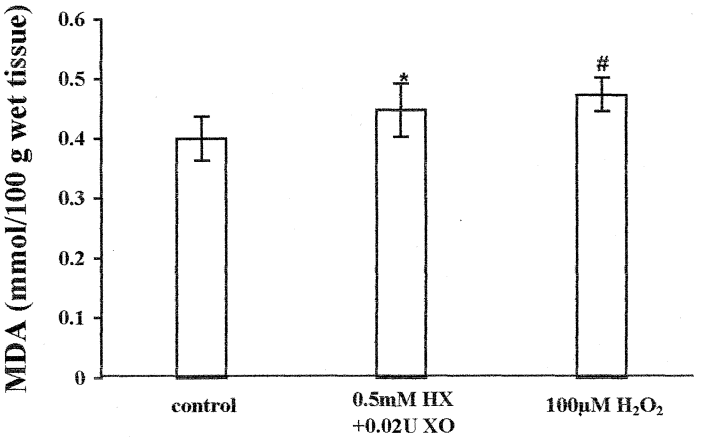
Values are mean ± SD; n=5-8 preparations
*p<0.001 Mg deficiency Vs Mg sufficiency

Fig.30. Effect of channel blockers on response to H_2O_2 in Mg deficiency



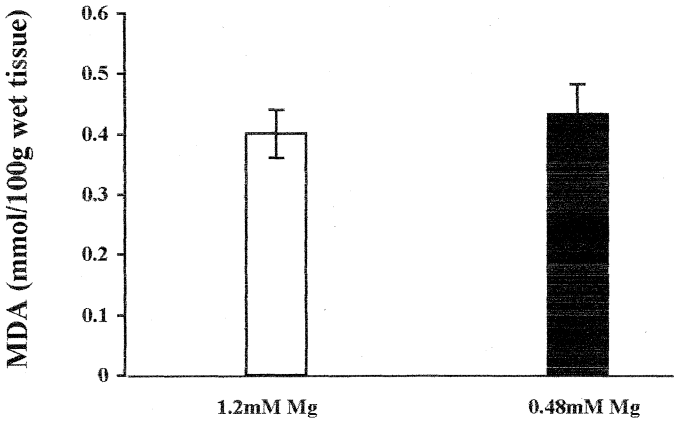
Values are mean ± SD; n=6-9 preparations
*p<0.005 Mg deficiency Vs Mg sufficiency

Fig.31. Effect of ROS on TBARS level



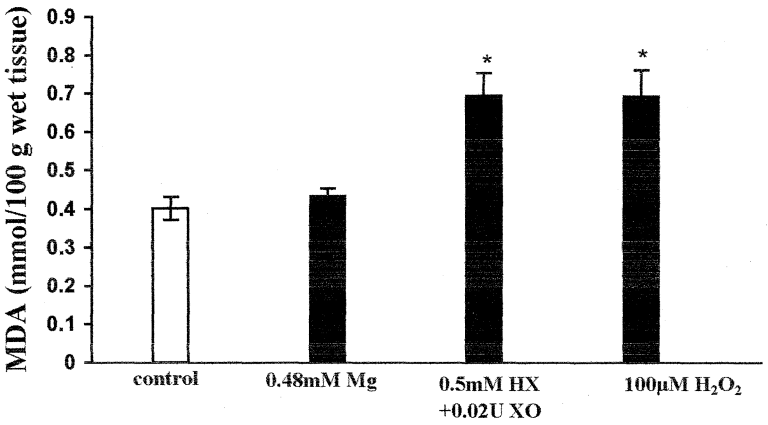
Values are mean ± SD; n = 5-7 experiments
*p<0.05 Vs control, #p<0.01 Vs control

Fig.32. Effect of extracellular Mg on TBARS level



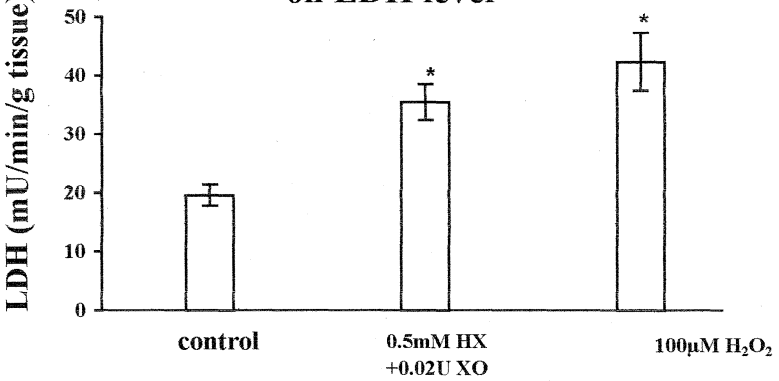
Values are mean ± SD; n = 4-7 experiments

Fig.33. Combined effect of Mg deficiency and ROS on TBARS



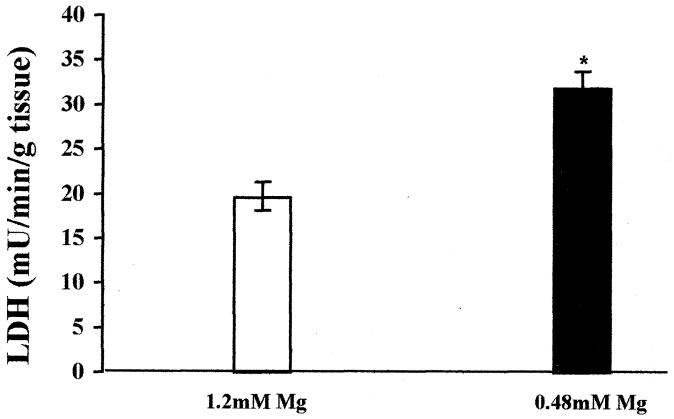
Values are mean ± SD; n = 4-7 experiments
*p<0.005 Vs control

Fig.34. Effect of ROS on LDH level



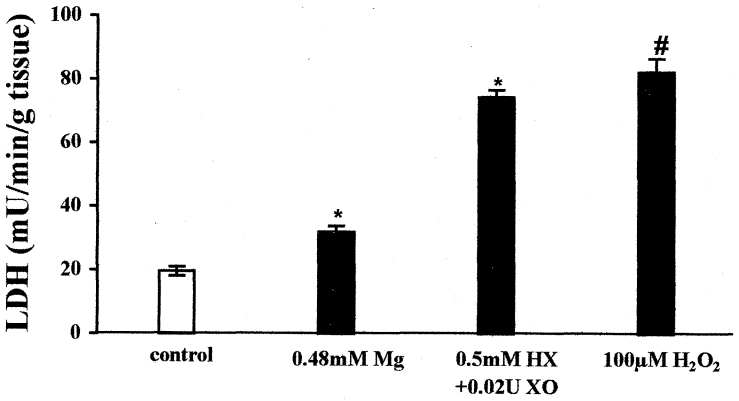
Values are mean ± SD; n = 4-7 experiments
*p<0.05 Vs control

Fig.35. Effect of extracellular Mg on LDH level



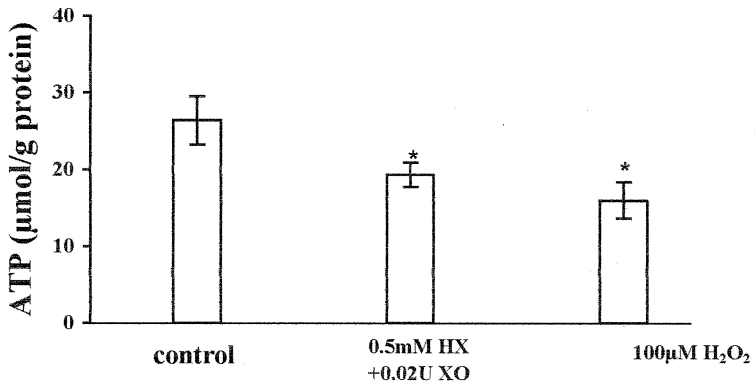
Values are mean ± SD; n = 4-7 experiments
***p<0.005 Vs control**

Fig.36. Combined effect of Mg deficiency and ROS on LDH



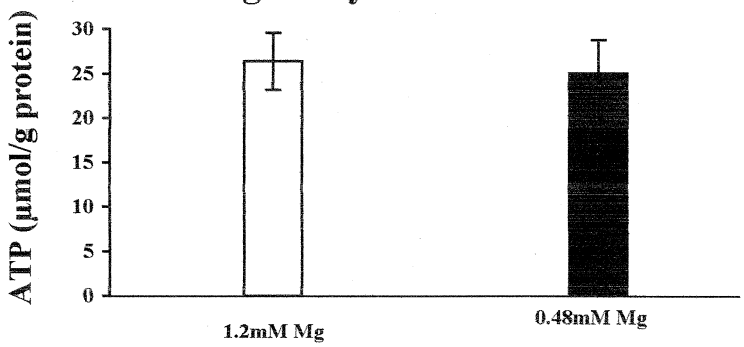
Values are mean ± SD; n = 4-7 experiments
***p<0.005 Vs control**
#p<0.001Vs control

Fig.37. Effect of ROS on myocardial ATP level



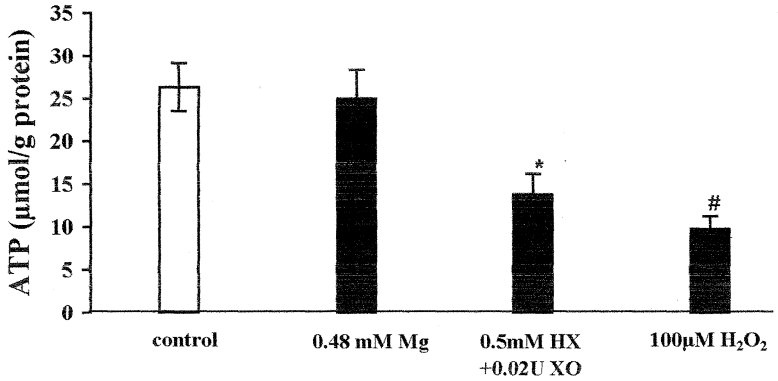
Values are mean ± SD; n = 4-7 experiments
*p<0.05 Vs control

Fig.38. Effect of extracellular Mg on myocardial ATP



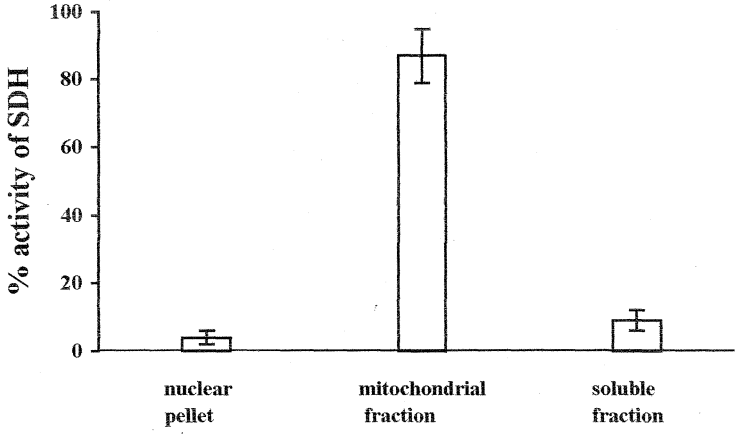
Values are mean ± SD; n = 4-7 experiments

Fig.39. Combined effect of ROS and Mg deficiency on myocardial ATP



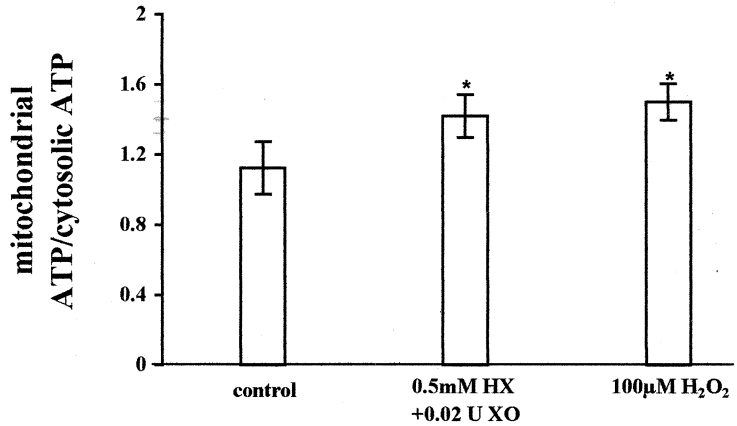
Values are mean ± SD; n = 8 preparations
*p<0.005Vs control,#p,0.001 Vs control

Fig.40. Specific activity of mitochondrial marker-SDH in various subcellular fractions



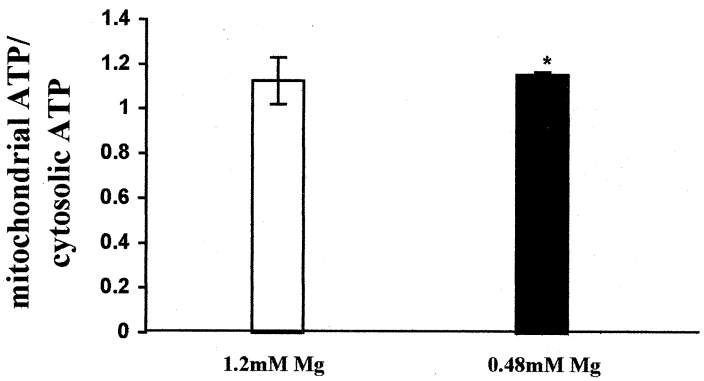
Values are mean ± SD; n = 8 preparations

Fig.41. Effect of ROS on mitochondrial to cytosolic ratio of ATP



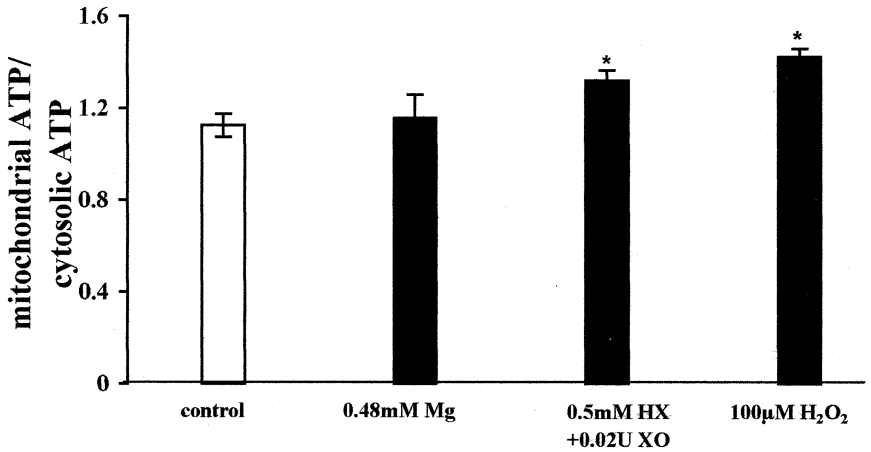
Values are mean ± SD; n = 4-6 preparations
***p<0.05 Vs control**

Fig.42. Effect of extracellular Mg on mitochondrial to cytosolic ratio of ATP



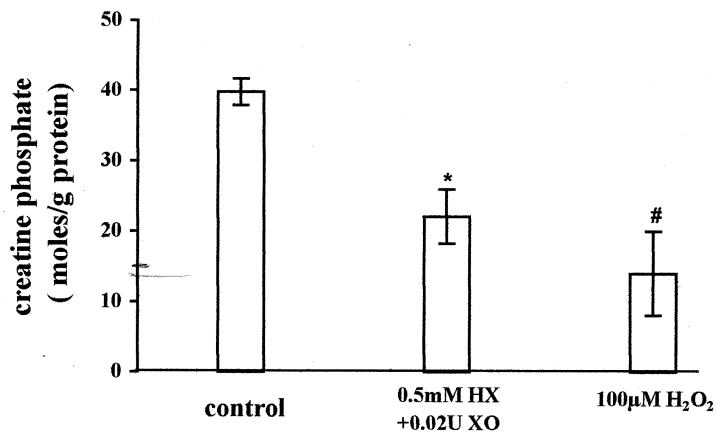
Values are mean ± SD; n = 5-8 preparations; *p<0.05 Vs control

Fig.43. Combined effect of Mg deficiency and ROS on mitochondrial to cytosolic ratio of ATP



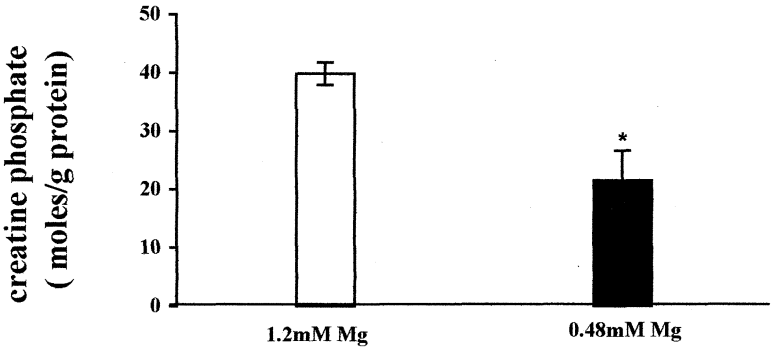
Values are mean ± SD; n = 4-6 preparations
*p<0.05 mitochondrial ATP/cytosolic ATP of ROS treated Vs control

Fig.44. Effect of ROS on myocardial creatine phosphate



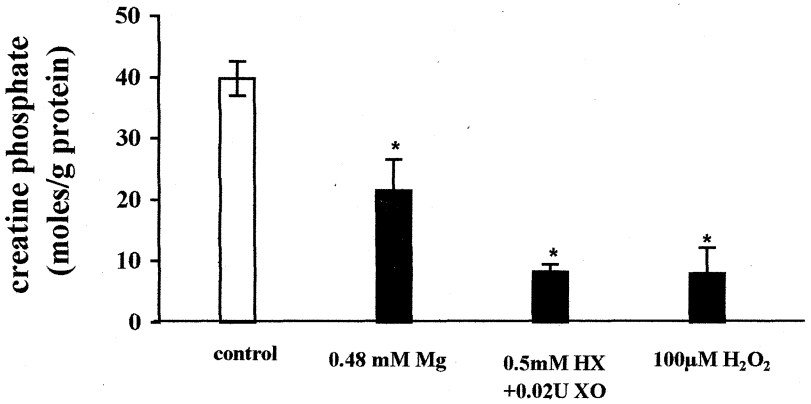
Values are mean ± SD; n = 6-9 experiments
*p<0.005 Vs control, #p<0.001 Vs control

Fig.45. Effect of extracellular Mg on myocardial creatine phosphate



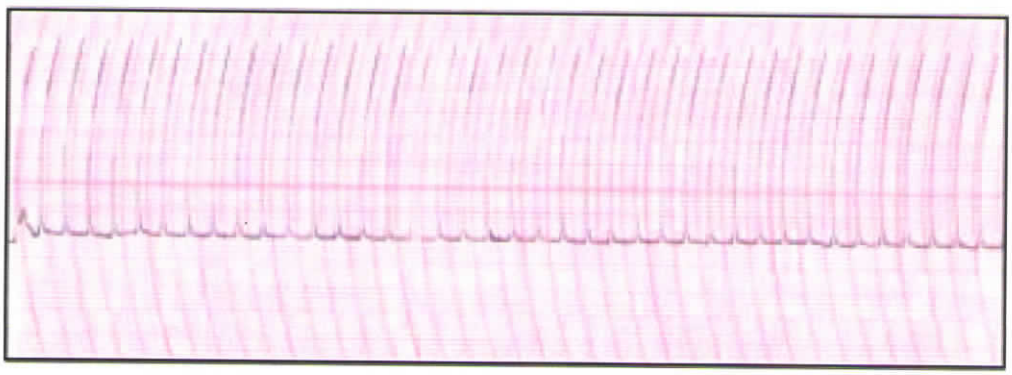
Values are mean ± SD; n = 6-7 experiments
***p<0.05 Vs control**

Fig.46. Combined effect of Mg deficiency and ROS on myocardial creatine phosphate

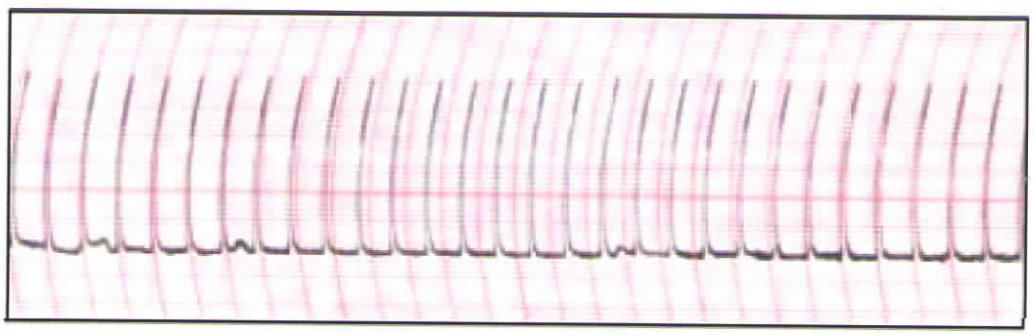


Values are mean ± SD
n = 6-9 experiments, *p<0.005 Vs control

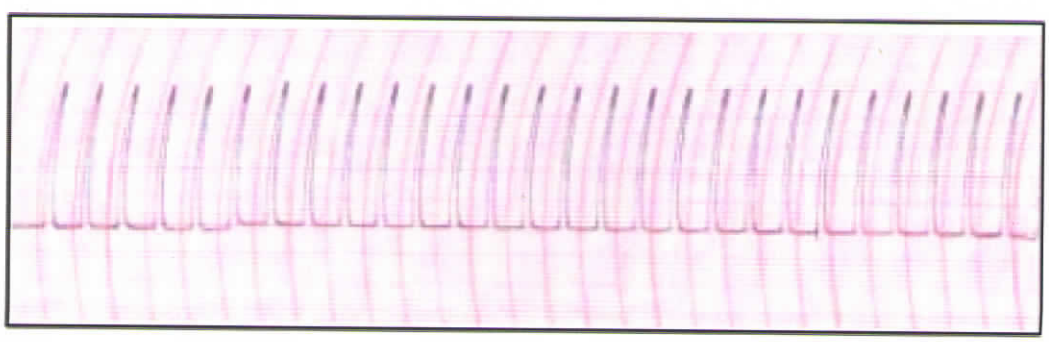
Plate.I. Tracing of the effect of HX+ XO on force of contraction



Control

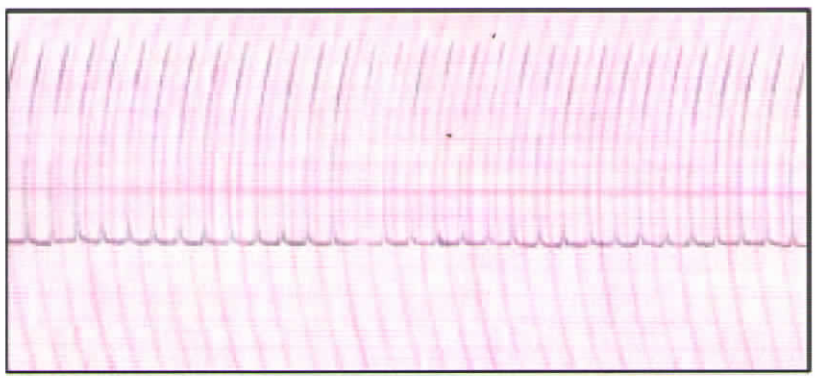


0.5mM HX+ 0.02U XO



1mM HX+0.04U XO

Plate.II. Tracing of the effect of H_2O_2 on force of contraction



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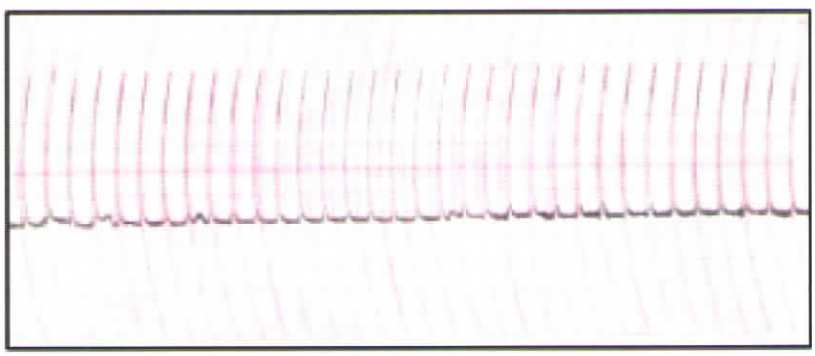
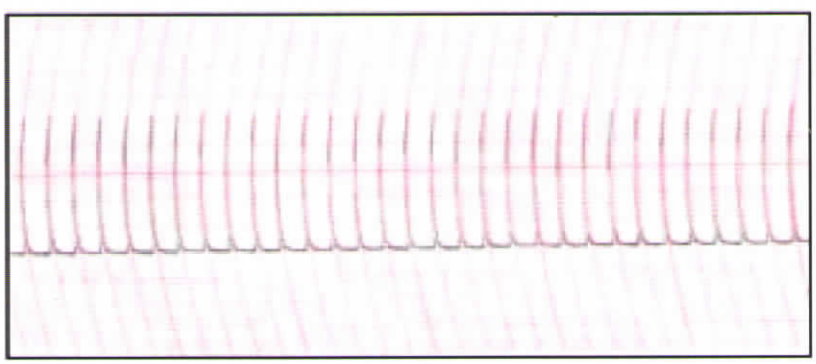
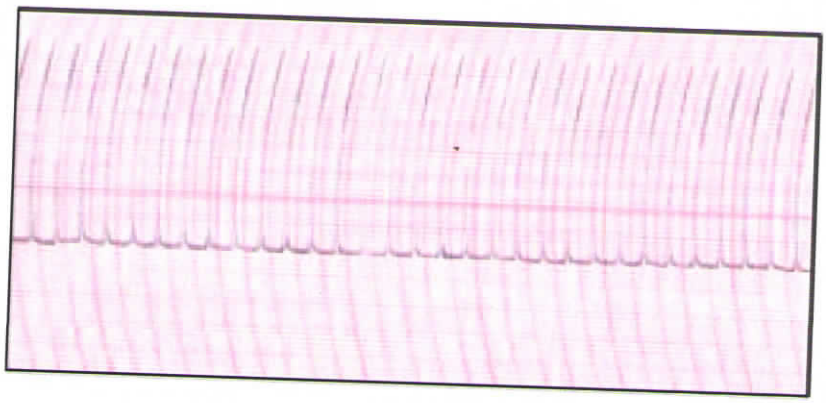


50 µM H_2O_2

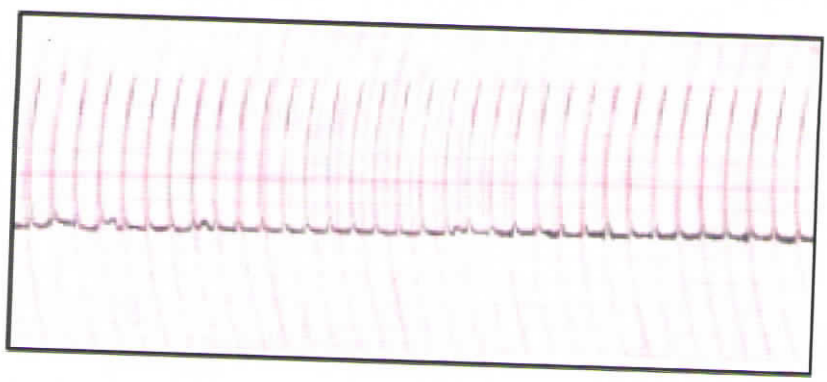


100 µM H_2O_2

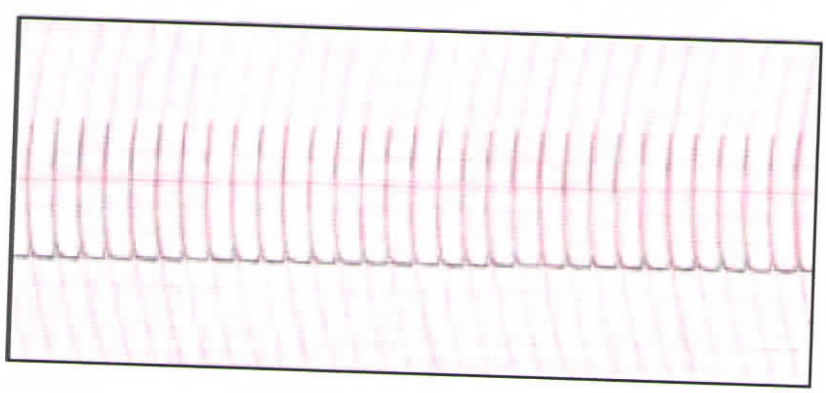
Plate.II. Tracing of the effect of H_2O_2 on force of contraction



Control

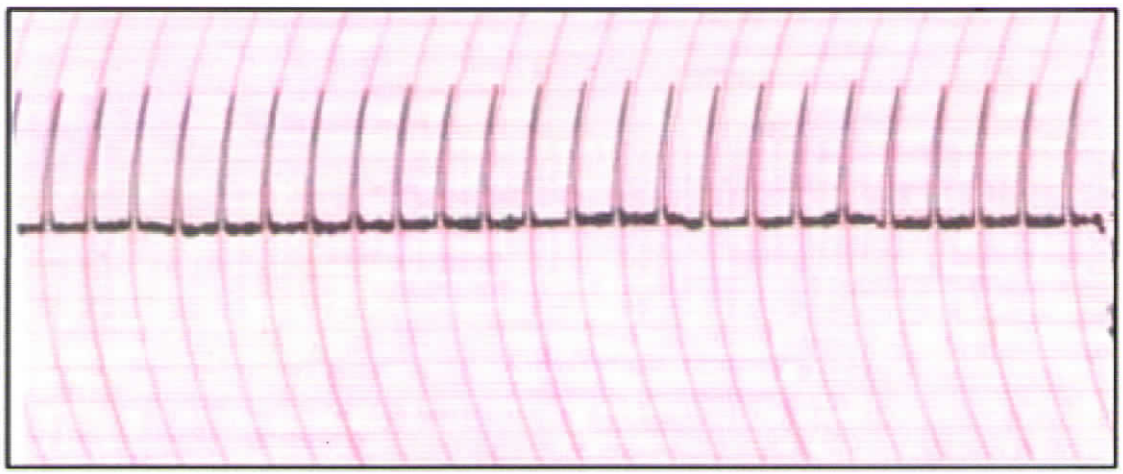


50 µM H_2O_2

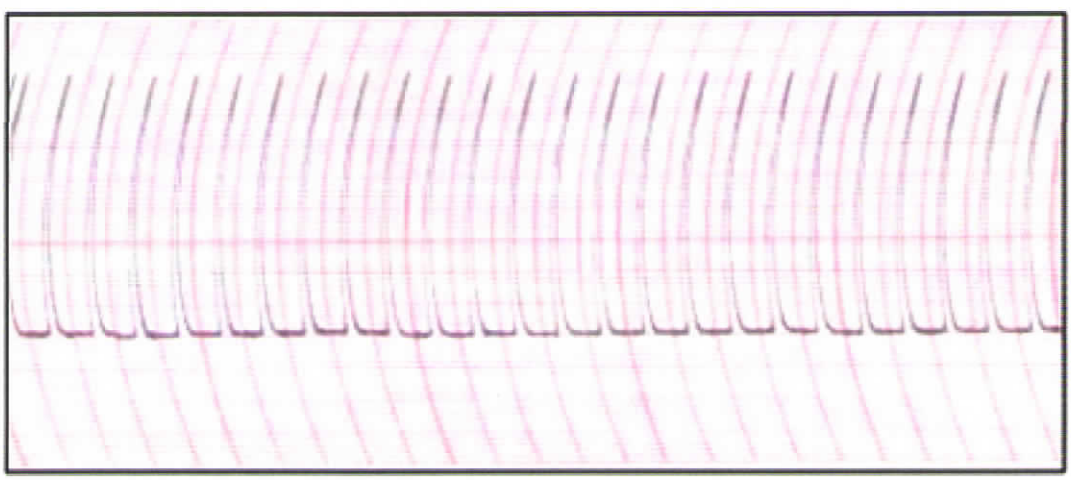


100 µM H_2O_2

Plate.III. Tracing of the effect of marginal Mg deficiency on force of contraction

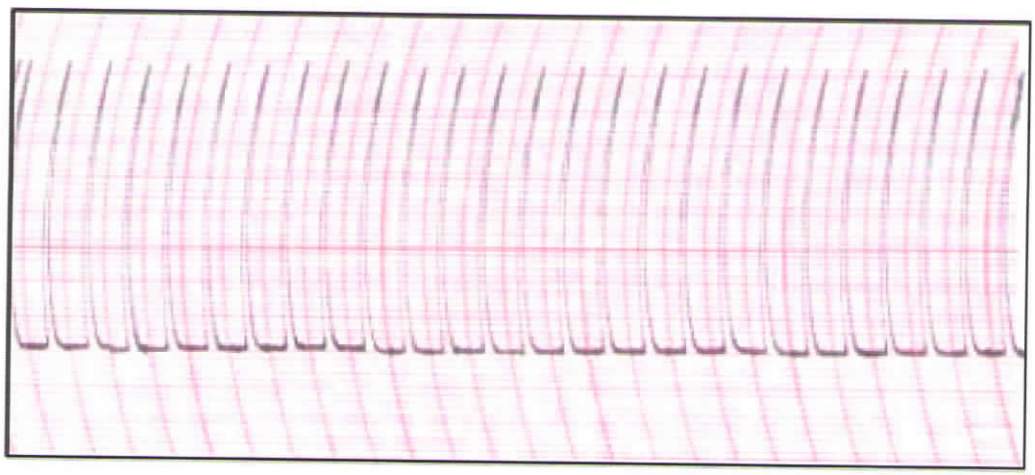


Control – 1.2 mM Mg

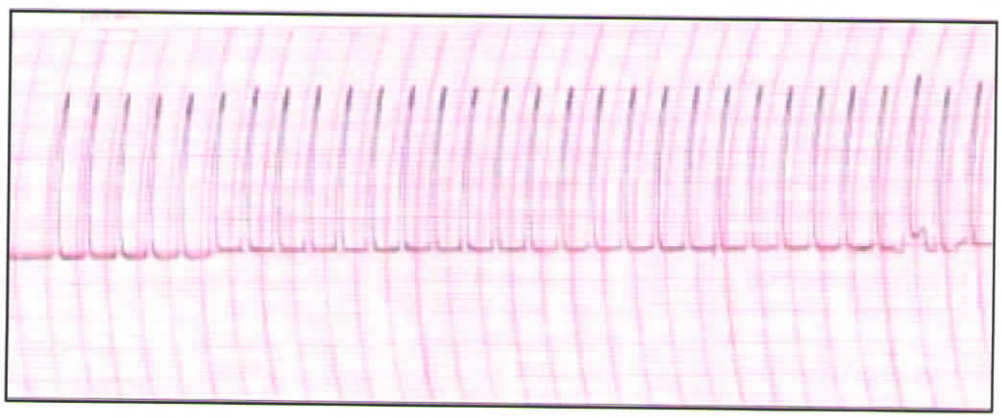


Marginal Mg deficiency – 0.48 mM Mg

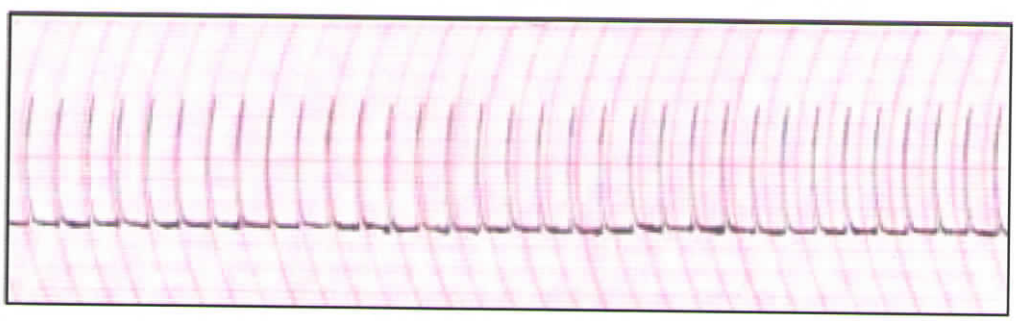
Plate.IV. Tracing of the effect of marginal Mg deficiency and HX+XO on force of contraction



0.48 mM Mg

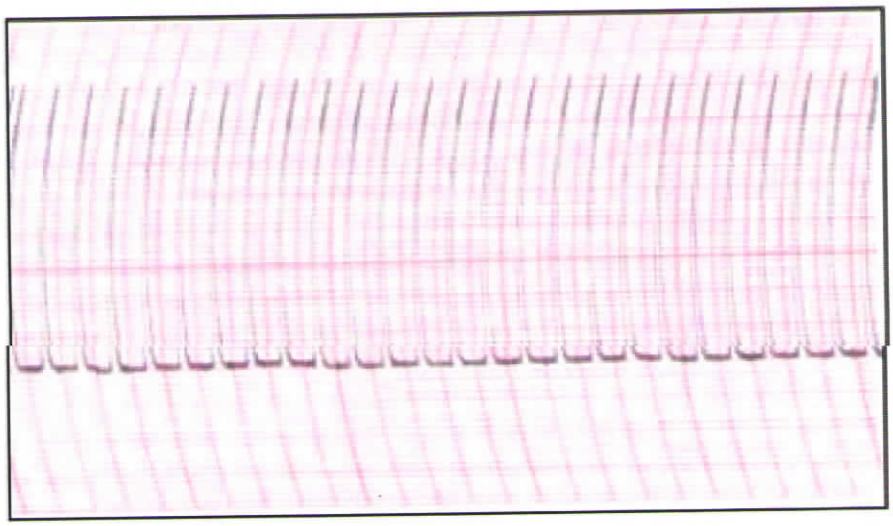


0.48 mM Mg+0.5 mM HX+0.02U XO

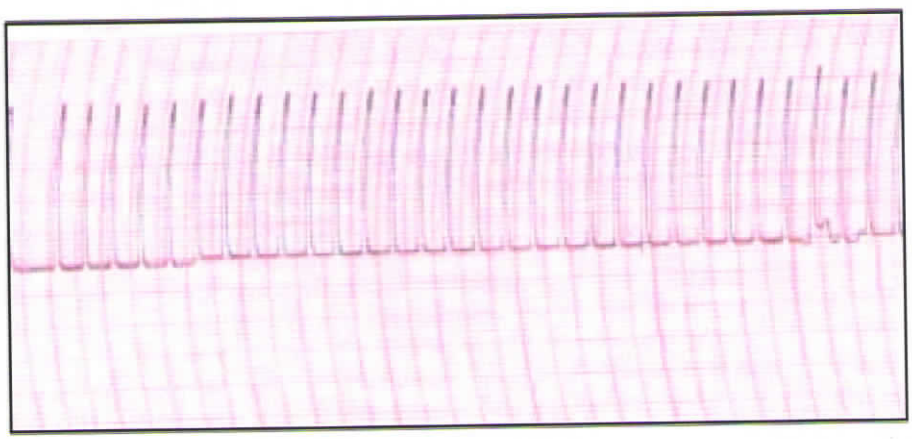


0.48 mM Mg+1 mM HX+0.04U XO

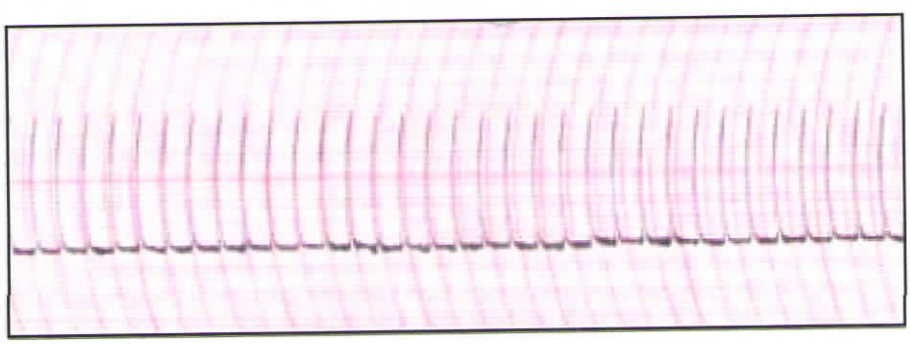
Plate.V. Tracing of the effect of marginal Mg deficiency and H_2O_2 on force of contraction



0.48 mM Mg



0.48 mM Mg + 50 µM H_2O_2



0.48 mM Mg + 100 µM H_2O_2

V. DISCUSSION

V.DISCUSSION

The response of heart to an altered environment is of consequence mainly when it affects the inotropic response. Contractile mechanics of mammalian heart is influenced by a number of intrinsic and extrinsic factors. Assessment of ventricular performance in terms of cardiac muscle mechanics helps to understand the functional response of the myocardium to specific physiologic and pathologic variations.

The effect of reactive oxygen species on cardiac muscle have been extensively investigated. Reactive oxygen species are constantly formed *in vivo*. Reactive oxygen species have been identified in the pathogenesis of various diseases such as hypertension, atherosclerosis, diabetes mellitus, heart failure etc. (Beck *et al*, 1991, Nakazono *et al*, 1991, Tesfamariam *et al*, 1992, Witztum, 1994). Experimental and clinical studies have suggested an increased production of ROS in the failing myocardium (Idle *et al*, 2000). A number of studies have highlighted the role of ROS in reperfusion injury. Reperfusion of ischemic myocardium results in generation of ROS from activated granulocytes, xanthine oxidase, arachidonic acid metabolism, autoxidation of catecholamines and electron transport chain in the mitochondria (Kukreja *et al*, 1992, Kaul *et al*, 1993, Valen *et al*, 1993).

The role of Mg as a regulator of myocardial mechanics and alteration of extracellular Mg as a cardiovascular risk factor has been recognized (Durlach *et al*, 1992b). Severe Mg deficiency in man is manifested by hyper

excitability and occasional behavioral disturbances. (Wacker *et al*, 1968). Tetany, convulsive seizures and other central nervous system disturbances have also been described (Burch *et al*, 1977). Symptomatic Mg deficiency is rare. Marginal deficiency of Mg due to insufficient intake is found to be more common. Dietary deficiency of Mg has been demonstrated to produce cardiac and arterial lesions in experimental animals (Heggtveit *et al*, 1969, Bloom *et al*, 1988). Large volumes of experimental and clinical studies are cited to support a relationship between Mg deficiency and increased vascular tone, vasospasm and hypertension (Altura and Altura, 1974). Marginal Mg deficiency is characterised by decrease in extracellular Mg^{2+} with the maintenance of tissue levels of magnesium (Kartha *et al*, 1998)

Several studies have reported enhanced stress sensitivity in Mg deficiency. When Mg deficiency exists stress increases the risk of cardiovascular damage including arrhythmias (Seelig, 1994). Pathology of dietary Mg deficiency has been related to free radical mechanisms (Weglicki *et al*, 1996). The defense mechanisms against oxidative stress have been shown to be compromised during Mg deficiency (Hsu *et al*, 1983, Herzog *et al*, 1994, Kumar *et al* 1997a). Magnesium deficiency as a predisposing factor in oxidative stress has also been reported (Freedman, 1992a, and 1992b). The present study was taken up to test the postulation that - marginal Mg deficiency augments the myocardial response to oxidative stress. The inotropic response of the myocardium to oxidative stress and associated

biochemical changes with possible involvement of SL and SR ion channels in the modulation of contractile variation was examined in isolated rat heart tissues in Mg sufficiency and marginal Mg deficiency.

V.1. MECHANICAL RESPONSE OF THE MYOCARDIUM TO ALTERATIONS IN EXTRACELLULAR MILIEU AND CORRESPONDING DIASTOLIC Ca LEVELS

Papillary muscle isolated from left ventricle of adult Sprague-Dawley rats of either sex have been used as the experimental model for recording the inotropic response. Since Ca^{2+} is the major ion mediating cardiac inotropy, diastolic Ca^{2+} level corresponding to various experimental interventions have also been assayed.

V.1.1. Negative inotropism of myocardium to ROS

Oxygen derived free radicals and their metabolites have been implicated in various types of myocardial injury. They have been the focus of intense investigation as potential mediators of myocardial ischemia-reperfusion injury (Kloner *et al*, 1989, Valen *et al*, 1993). These radicals and metabolites are thought to mainly consist of superoxide anion, hydrogen peroxide and hydroxyl radical. However, the culprit playing the most important role is still undetermined. There have been several reports in which the effect of various oxygen radicals were studied (Burton *et al*, 1984, Jackson *et al*, 1986, Miki *et al*, 1988).

A concentration dependent negative inotropic response of papillary muscle was observed in presence of SO anion generating system (HX+XO)

and H_2O_2 (figs. 7 and 11). The contractile impairment produced by 0.5mM HX+0.02U/ml XO was completely reversible in presence of the scavenging enzyme, SOD and in fresh medium (fig. 9). At higher concentrations an incomplete recovery was observed. In presence of H_2O_2, complete recovery in fresh medium as well as the protective effect of the scavenging enzyme, catalase were observed only upto 50 μM. At higher concentration of H_2O_2, 100 μM, the recovery was only partial (fig. 13). A significant difference in the time for contraction (t) and half relaxation ($t_{1/2}$) was observed only at 1.0mM HX+0.04U XO, with a decrease in time for contraction and an increase in time for half relaxation. Both time for contraction and half relaxation were reduced significantly at 100 μM H_2O_2 (figs. 10 and 14).

Negative inotropic response of the myocardium on exposure to ROS have been reported by a number of workers. Ytrehus *et al*, (1986) reported that oxygen radicals generated by HX+XO had a pronounced effect on the isolated perfused rat heart. An immediate increase in coronary flow was followed by a decrease in contractility. Furthermore these effects were found to be blocked by SOD and catalase.

Depression in contractility was observed by Burton and colleagues, using an isolated septum preparation with an $OH^{\bullet}$ radical generating system consisting of XO plus purine and iron loaded transferine. Left ventricular developed pressure was reduced during perfusion. The addition of SOD and catalase appreciably improved the function during perfusion with xanthine

oxidase and hypoxanthine indicating that the altered function is due to the generation of SO anions and/or H_2O_2. Electron microscopic study showed morphological alterations in hearts exposed even for 5' (Burton *et al*, 1984).

In a study by Mallet *et al* (2002), exposure of guinea-pig hearts to 100 μM H_2O_2 for 15' or 150 μM H_2O_2 for 10' produced profound irreversible contractile change and coronary hemodynamics.

Takemura *et al* (1994) reported that perfusion of rat hearts with 50 μM H_2O_2 caused no change in contractile function or any other biochemical variables within 15', but a higher dose of H_2O_2, 500μM, showed an abrupt loss of contractility which reached nearly zero after 5'. The heart rate and coronary flow were also depressed significantly.

Contrary to the above observations, Dekeulenear and coworkers (1995) have reported a positive inotropic effect of cat papillary muscle to ROS generated by electrolysis of physiological buffer solution. Preincubation of the muscles with catalase, but not SOD blocked the inotropic effect. This inotropic variation has been attributed to a partial damage of the endocardial surface and a brief release of endothelin from the endocardial endothelial cells.

A large number of studies implicate various aspects of ROS on myocardial performance. Though very few investigators have recorded the direct contractile response of myocardium to ROS, Idle *et al* (2000) have correlated the severity of heart failure to myocardial level of ROS. This

report is correlated with the study using the subcellular fractions that SO anion production was increased in heart failure mitochondria (Idle *et al*, 1999). Idle *et al* (2000) have also shown that SO anion is a primary radical that can lead to the formation of other ROS, such as H_2O_2 and hydroxy free radical. $OH^{\bullet}$ radicals have been suggested to be the predominant oxidant species causing cellular injury (Bolli *et al*, 1989)

Experiments by Blaustein *et al* (1986) have shown that a reduction in contractile force and rate of force development in adult LBN-F1 brown rats hearts were produced only after 60' of exposure to SO anion generating system. Oxygen radicals can exert its negative inotropic effect by decrease of myofilament Ca^{2+} sensitivity mediated by a decrease in reduced glutathione, a Ca^{2+} sensitizer or an increase in oxidised glutathione (Bauer *et al*, 1989, Ferrari *et al*, 1991, Gao *et al*, 1996). Decrease in myofilament sensitivity to Ca^{2+} may be one of the mechanisms by which a negative inotropic response is induced in oxidative stress.

Studies by Vaage *et al* (1997) have shown that the infusion of HX+XO increased the tissue levels of H_2O_2. Although H_2O_2 is not a free radical, it is an unstable intermediate in the univalent pathway reducing oxygen to water in which ROS are formed (Kukreja *et al*, 1992, Kaul *et al*, 1993). Josephson *et al* (1991) demonstrated that in presence of myocytes, H_2O_2 will result in $OH^{\bullet}$ radical generation due to the catalytic action of endogenous myocyte iron. The more reactive hydroxyl radicals produce

greater cellular injury which in turn results in the augmented negative inotropism. The role of H_2O_2 appears to be more likely signalling because it can cross intracellular membrane more readily than superoxide, and has shown to directly modify the regulatory domain of protein kinase C resulting in its activation (Gopalakrishna and Anderson, 1989). These reports suggest that the presence of one FR species can co-stimulate the production of other species. Hence the changes observed in this study may be the consequence of collective response to different FR species.

Due to the significant role of Ca^{2+} in muscle contractility the Ca^{2+} level of myocytes in presence of different ROS were determined. The diastolic level of Ca^{2+} was lower in the presence of both HX+XO and H_2O_2 (figs. 8 and 12). A number of studies have reported variation in Ca^{2+} on exposure to ROS, a study on indo-1-loaded embryonic chick cultured myocytes demonstrated that $[Ca^{2+}]_i$ actually decreased at the time of reoxygenation after subjection of the cells to hypoxic conditions (Quaife *et al*, 1991). Burton *et al* (1990) also demonstrated that cultured neonatal rat myocytes exposed to xanthine +xanthine oxidase, developed a loss of beating activity in association with a slight decrease or no change in diastolic $[Ca^{2+}]_i$. Increase in $[Ca^{2+}]_i$ was consequent to bleb formation and contracture. Bleb formation and contracture of myocytes were observed after an exposure time of more than 1h in the present study which may also be associated with an $[Ca^{2+}]_i$ overload. In fetal mouse myocytes, Nakamura *et al* (1993) have

observed no increase in diastolic $[Ca^{2+}]_i$ at the time the myocytes developed contractile dysfunction, but a significant increase of the diastolic $[Ca^{2+}]_i$ and concomitant hypercontraction occurred later.

A great deal of attention has been focused on intracellular calcium overload as an important mediator of reperfusion injury in cardiac tissue. It has been proposed that oxygen derived free radicals and oxidants induce intracellular calcium overload (Allshire *et al*, 1987, Jennifer *et al*, 1989, Klein *et al*, 1989, Weiss *et al*, 1990, Josephson *et al*, 1991, Kaneko *et al*, 1991c and1994,). Dramatic increase in $[Ca^{2+}]_i$ associated with cellular injury was observed in adult rat isolated myocytes undergoing reoxygenation after anoxic exposure (Allshire *et al*, 1987). Measurements with calcium sensitive fluorescent dye also demonstrated increase in both systolic and diastolic $[Ca^{2+}]_i$ levels which were concomitant with H_2O_2 and $OH^{\bullet}$ induced contractile dysfunction (Josephson *et al*, 1991).

Relating the observations of this study to earlier reports, the negative inotropic effect of ROS is a consistent finding. A number of mechanisms have been identified with the functional damage such as alterations in signal transduction pathways, protein synthesis, myofilament sensitivity to Ca^{2+}, DNA damage etc. The decrease in diastolic Ca^{2+} is supported by a number of studies. Reports of increased intracellular Ca may be the consequence of Ca overload following cell injury. A study of Ca^{2+} transients is expected to provide a better correlation between the contractile variation and $[Ca^{2+}]_i$

levels. The role of ion channels influencing Ca^{2+} transients and changes in energy metabolism have been investigated later.

An interesting observation of this study is that the scavenging enzymes exerted their protective effect only when the muscles were pretreated with the enzymes, ie; before induction of contractile variation by ROS. Post treatment of the muscle with the scavengers augmented the inotropic effect. This shows that pretreatment of the muscle with the scavenging enzymes is essential for producing a protective effect from the ROS, and post treatment will have a paradoxic effect. This is in agreement with the postulation of Halliwell (2000) that administration of a powerful antioxidant after oxidative damage has been started could promote the damage. The depletion of antioxidants during ischemia-reperfusion has been postulated to be secondary to the action of ROS. Roth *et al* (1987) found that pretreatment with synthetic antioxidants inhibited the depletion of endogenous antioxidants during reperfusion. A pioneering study by Dhalla *et al* (2000) have shown an increased formation of ROS and/or decreased antioxidant reserve in cardiac and vascular myocytes due to oxidative injury.

V.1.2. Positive inotropism of myocardium to marginal Mg deficiency

The inotropic response to variation in $[Mg^{2+}]_o$ was studied by exposing the papillary muscle to different levels of Mg ranging from 1.4 mM to 0.32 mM. A positive inotropy with decrease in $[Mg^{2+}]_o$ attaining a peak at 0.48 mM was observed (fig. 15). This result is in agreement with the

previous report on the contractile response of myocytes (Nair and Nair, 2000). The serum Mg levels of animals on Mg deficient diet was found to be 0.45 mM (Kumar *et al*, 1997a). Alterations in extracellular Mg level has been reported to exert profound effects on cardiac contractility and excitability (Fry et al, 1993). It has also been reported that the cardiac output of rats on Mg deficient diet was higher than that in rats on a normal diet (Nishiyama et al, 1990). The time for half relaxation was reduced with decrease in $[Mg^{2+}]_o$ (fig. 17).

Concentration of diastolic $[Ca^{2+}]_i$ also showed a direct correlation with the contractile response (fig. 16), suggesting that the inotropic response is mediated by a rise in basal calcium level. Decrease in relaxation time with rise in basal level of Ca can be an indicator of incomplete relaxation. As observed in this study the cytosolic Ca^{2+} levels from myocytes of mature New Zealand rabbits was found to increase at 0.5mM $[Mg^{2+}]_o$ (Cyran *et al*, 1992). A direct blocking of Ca influx by Mg during the slow current phase of activity was described in giant squid neural axon by Baker *et al* (1970) and in motor nerve endings by Katz *et al* (1969). Interaction of Mg with a number of subcellular organelles explain the mechanical response of heart to variation in cellular magnesium. Within the muscle cell Mg appears to : (a) inhibit release of Ca from SR in response to a sudden influx of extracellular Ca which normally triggers this release (Dunnett *et al*, 1978). (b) Actually drive Ca into SR by stimulation of Ca ATPase enzyme activity (Stephenson

and Podolsky, 1977). (c) Compete with Ca at certain binding sites on troponin C and myosin (Podolsky and Constantin, 1964). (d) Inhibit the ability of Ca to stimulate myocardial tension (Holyrode *et al*, 1980). (e) Distribute itself within the mitochondria and regulate the amount of cytosolic Mg for the specific purpose of interacting with cytosolic Ca (Sordahl, 1975). (f) Reduce the development of muscle tension (Shine, 1979). (g) Act as non competitive inhibitor of IP_3 gated Ca channels (Berridge and Irving, 1989, Volpe *et al*, 1993, Bezproavanny *et al*, 1995).

$[Mg^{2+}]_o$ is said to exert its inhibitory effect on Ca influx, due to its smaller ionic radii (Mg^{2+} -0.6 A^0 and Ca^{2+} 0.95 A^0) and it is known to compete for the same site on the SL with Ca^{2+} (da Salva and Williams, 1991). Mg^{2+} is considered as the physiological Ca antagonist (Iseri, 1984) and this may explain the negative inotropy at higher concentration of $[Mg^{2+}]_o$ The relaxation of the inhibitory effect at 0.48 mM $[Mg^{2+}]_o$ may be responsible for the rise in Ca^{2+} level and positive inotropy. Katholi *et al* (1979) studied the dual dependency of heart cells on both Ca^{2+} and Mg^{2+} for electrical stability. According to them Ca^{2+} participates in the generation of action potential, promoting electrical stability and initiating myocardial contraction while Mg^{2+} has importance as an activator of cation transport through the sarcolemma.

In a large number of studies $[Mg^{2+}]_o$ is shown to exert negative inotropic and chronotropic effects in cardiac tissue (Shine and Douglas,

1974, Fabiato and Fabiato, 1975, Iseri, 1984, Friedman *et al*, 1987, Hall *et al*, 1992, Silverman *et al*, 1994), although there is paradoxical evidence of positive inotropic effects of moderate elevations in $[Mg^{2+}]_o$ (James *et al*, 1987, Barbour *et al*, 1992). In cardiac muscle, Mg influences tension development (Shine, 1979). The ion has been found to impart negative inotropic effect in ventricular muscle owing to the competitive antagonism against Ca^{2+} (Hall *et al*, 1992). The inverse relation between extracellular $[Mg^{2+}]$ and contractile force may therefore be related to Ca^{2+} availability.

V.1.3. Augmentation of negative inotropism due to ROS in Mg deficiency

Eventhough the separate effect of ROS and Mg on cardiovascular system has been studied extensively, the synergistic effect of these bio molecules have got relatively little attention. In the presence of HX+XO and H_2O_2 an augmentation of the negative inotropism was observed in Mg deficiency (fig. 18 and 22). Diastolic $[Ca^{2+}]_i$ level correlated with the contractile variation (figs. 19 and 23). Recovery of contraction of the muscle in ROS free medium as well as in presence of the scavenging enzymes was relatively lower in Mg deficiency when compared to sufficiency (figs. 20,24), suggesting the presence of irreversible muscle damage. Time for contraction was lower with both the ROS, but the time for half relaxation was variable (figs. 21 and 25). Differential response to variation in extracellular Mg was not apparent for these parameters.

Other reports on the inotropic changes in oxidative stress associated with Mg deficiency are not available. In a study conducted in the laboratory it was observed that the negative inotropic effect of Ce was increased in Mg deficiency (Manju and Nair, 2003a). The Ce induced inotropic changes were found to be mediated by ROS (Manju *et al*, 2003b).

Thus it can be inferred from this study that **"marginal Mg deficiency augments the negative inotropic response to oxidative stress in rat papillary muscle".**

In an attempt to elucidate the mechanism responsible for the inotropic changes, the possible role of ion channels and pumps in the mediation of mechanical change and the biochemical variables influencing mechanical function were assessed.

V.2. ION CHANNELS AND PUMPS INFLUENCING THE INOTROPIC CHANGES

Since mechanical variations are generally mediated by variation in ion transients, the channels and pumps which are likely to produce variation in intracellular Ca level was assessed. Competitive channel blockers were used to assess the functional contribution of different channels to varying experimental conditions. The channel specific antagonist was added to the contracting papillary muscle followed by different experimental conditions, and effect of the ion channel was assessed based on the contractile variation produced by the muscle.

V.2.1. Channels and pumps influenced by ROS

Reactive oxygen species can induce a negative inotropic response in papillary muscle. The variation in response induced by ROS in the presence of channel inhibitors are shown in figs. 26.and 27. The negative inotropic response induced by HX+XO was significantly less (neutralized) in presence of SL L-type Ca channel blocker verapamil. Kaneko *et al* (1991c) also have reported an inhibition of SL Ca pump activity in isolated sarcolemmal membrane preparation and this inhibition was found to be time dependent. Interaction with other channels may be responsible for this observation.

The response to H_2O_2 was significantly enhanced in presence of SL L-type Ca channel blocker verapamil. Kaneko et al have also shown a reduction in the number of Ca channels in the cell membrane by oxygen free radicals which contributes towards the decreased voltage dependent Ca influx in the cardiac cell (Kaneko *et al*, 1989)

The mechanical response to HX+XO and H_2O_2 was not significantly affected by the presence of T type Ca channel blocker, indicating that the function of this channel is unaffected by reactive oxygen species.

The negative inotropic response produced by SO anion generator was augmented in presence of Na K ATPase inhibitor ouabain. But the response to H_2O_2 was not significantly affected by the presence of ouabain Reduced activity of Na K ATPase during ischemia reperfusion by oxygen free radicals have been reported by Kim and Akera (1987). Oxidative modification of

Na K ATPase from brain and kidney have been reported by Kurella *et al* (1999). Gonzalez *et al* (1996) have inferred that the function of Na K ATPase is increased in alveolar type II cells by H_2O_2. Reports also suggest that SO anion generated from xanthine oxidase action on xanthine had no significant effect on Na K ATPase activity, whereas H_2O_2 produced an inhibitory effect on Na K ATPase activity (Kukreja *et al*, 1990). Studies in SL membrane led Vinnikova *et al* (1992) to the conclusion that inhibition of Na pump by singlet oxygen and further Ca^{2+} overload ultimately leads to cell injury. Inhibition of Na K ATPase is associated with a positive inotropic effect, but it has been reported that superoxide anions also block the Na Ca exchanger in addition to Na K ATPase (Xie *et al*, 1990) and that may be responsible for the augmented negative inotropic response.

The negative inotropic response to ROS was reduced in presence of SR Ca uptake inhibitor caffeine. Kanekao *et al* (1989) have also shown that H_2O_2 inhibited both ATP dependent Ca accumulation and Ca stimulated ATPase activity in a dose dependent manner. On the otherhand Rowe *et al* (1983) have reported that H_2O_2 significantly depressed the Ca uptake by SR without any change in Ca ATPase activity. ATP dependent Ca^{2+} accumulation and Ca^{2+} stimulated ATPase activity were also reduced by hydroxyl radicals (Tien *et al*, 1982, Reeves *et al*, 1986). Reduction in inotropic response to HX+XO by SR Ca release channel blocker ryanodine was not statistically significant. The negative inotropic response to H_2O_2 was

increased in presence of ryanodine. Though it is difficult to provide an explanation for this finding, one interesting observation is that, the negative inotropic response to H_2O_2 is augmented in the presence of L type blocker as well as the Ca release inhibitor ryanodine and decreased by HX+XO. It is therefore apparent that the inotropic variations induced by HX+XO and H_2O_2 may be mediated by the functioning of a number of channels with possible difference in intracellular mechanisms. Inhibition of CaATPase activity by both the ROS may be the consequence of insufficient availability of ATP.

Changes in the functioning of SR channels and pumps can be the direct effect of ROS or indirectly influenced by the functioning of the SL channels. Decrease in the time for contraction accompanied by decrease in force of contraction may be attributed to decrease in the influx of Ca^{2+} and the prolongation of relaxation time can be the consequence of inefficient functioning of Ca^{2+} pump.

V.2.2. Channels and pumps modulated by Mg deficiency

Magnesium can modulate the functioning of ion channels located both in the SL and SR either by acting as an antagonist to Ca^{2+} or as an activator of the enzyme systems which use ATP (Agus *et al*, 1989, White and Hartzell, 1989). Hence the inotropic response of papillary muscle to variation in $[Mg^{2+}]_o$ was recorded in presence of different ion channel modulators (fig. 28).

Magnesium is known to influence the functioning of voltage gated Ca^{2+} channels. The positive inotropic effect of Mg deficiency was neutralized by verapamil which suggests that the increase in contraction in Mg deficiency is mediated by increased Ca^{2+} influx through the SL L type Ca channel. The negative inotropic response produced by T type Ca channel blocker, $NiCl_2$ was greater in Mg deficiency when compared to sufficiency, which suggests that the activity of the T type channels may also be enhanced in Mg deficiency.

Ouabain is a digitalis cardiac glycoside which is known to produce positive inotropy by inhibiton of Na K ATPase. The positive inotropy in presence of ouabain was found to be augmented in Mg deficiency. Inhibiton of Na K ATPase can cause reversal of Na Ca exchanger (Ahmad and Bloom, 1989). Low levels of Mg has been found to inhibit Na K ATPase activity (Bara *et al*, 1993). Augmented response to ouabain in Mg deficiency may be due to additive effect.

Ryanodine sensitive SR-Ca release channel functions are reported to be sensitive to Mg^{2+}. Mg^{2+} is a critical physiological determinant of the dynamic behavior of the ryanodine receptor channel, which is expected to profoundly influence the fidelity of coupling between L type Ca^{2+} channels and ryanodine receptors in heart cells (Zahradnikova *et al*, 2003). Inclusion of 3 mM free Mg shifted the Ca dependence of Ca efflux rate in cardiac SR vesicles to higher $[Ca^{2+}]$, and intracellular Mg when lower than 3 mM in

cardiac muscle would increase the Ca sensitivity of the release channel (Meissner and Henderson, 1987). They also suggested that Ca uptake in cardiac SR vesicles can be dramatically increased by inclusion of agents like Mg^{2+} and ruthenium red which block the Ca release channel. Fabiato and Fabiato (1978) speculated that variation in $[Ca^{2+}]_i$ due to $[Mg^{2+}]_o$ depletion can also affect Ca uptake and Ca release by SR. The force of contraction was found to be reduced significantly in presence of ryanodine and decrease in $[Mg]_o$ level augmented this response, but the difference was not statistically significant. The positive inotropic response produced by Mg deficiency was attenuated by caffeine, but as observed for ryanodine, the difference was not statistically significant.

The conclusion from these observations is that the positive inotropic response in Mg deficiency is mediated by increased Ca^{2+} influx through the voltage gated Ca^{2+} channels as well as by the inhibition of Na K ATPase possibly accompanied by reversal of Na Ca exchanger.

V.2.3. Channels and pumps modulated by ROS in Mg deficiency

The negative inotropic response in presence of ROS and positive inotropic response in Mg deficiency have been shown be to influenced by the SL and SR channels and pumps which modulate the $[Ca^{2+}]_i$ transients. Augmentation of negative inotropic response of ROS in Mg deficiency indicates the presence of an interactive effect.

An interaction between Mg deficiency and HX+XO was observed for Na K pump, with HX+XO exhibiting a reduction in the negative inotropic response when associated with Mg deficiency. The inotropic variation in the presence of the other channel modulators are found to be statistically insignificant (fig. 29). As observed for HX+XO, H_2O_2 also showed the same response to ouabain, but the difference was not statistically significant. The negative inotropic response to H_2O_2 was significantly higher in Mg deficiency in the presence of SL - T type Ca channel blocker, $NiCl_2$ and SR – Ca pump antagonist caffiene. The other inotropic variations were not statistically significant (fig. 30). An interaction between Mg deficiency and ROS is apparent for the functioning of both the SR Ca pump and SL Na pump with the values reaching statistical significance with H_2O_2 in former and HX+XO in latter. Due to the requirement for ATP the functioning of the pumps may be influenced by variation in energy metabolism.

V.3. RELATIONSHIP BETWEEN FUNCTIONAL RESPONSE AND METABOLIC PARAMETERS

In an attempt to correlate the inotropic changes with metabolic effects of altered extacellular milieu in mammalian myocardium, the following aspects have been studied. a) Peroxidation of lipid bilayer, b) Release of lactate dehydrogenase, and c) high energy phosphate compounds.

V.3.1. Lipid peroxidation

In general, membrane lipid peroxidation requires breakdown in normal balance between oxidative stress and the mutilayered antioxidant defenses of living tissue (Babbs *et al*, 1990). Reactive oxygen species can react with polyunsaturated fatty acids resulting in the formation of lipid peroxides and hydroperoxides. The latter substance can initiate chain-propagation reactions that can damage an extensive section of membrane and result in the disruption of cell integrity (Fridovich, 1978, Del Maestro, 1980). Lipid peroxidation is measured by the formation of thiobarbituric acid reactive substances like malone dialdehyde. Malone dialdehyde is one of the low molecular weight end products formed via the decomposition of certain primary and secondary lipid peroxidation products and therefore it can be considered as a marker for lipid peroxidation. Lipid peroxidation can damage plasma membranes or organellar membranes, resulting in the impairment of membrane function and disruption of cell integrity (Halliwell and Gutteridge, 1984). Lipid peroxidation has been reported to decrease Ca^{2+} ATPase activity in SR isolated from rabbit hearts. The release of products of lipid peroxidation such as arachidonic acid into the extracellular space may result in the subsequent formation of prostaglandins and endoperoxides which can further aggravate the injury process (Burton *et al*, 1984). Studies by Vaage *et al* (1997) revealed ROS induced cardiac injury and associated functional damage and lipid peroxidation with depletion of endogenous antioxidants.

Peroxidation of biological membranes caused by ROS have been studied extensively. In this study also both HX+XO and H_2O_2 produced a significant enhancement in the level of TBARS; the effect of H_2O_2 being slightly higher when compared to HX+XO (fig. 31). Pool - Wilson (1983) reported that oxygen radicals will easily react with unsaturated lipids and sulphydryl groups in proteins and other prominent constituents of the cellular membrane. This can lead to alteration or destruction of membrane function and integrity which later leads to calcium paradox. ROS has also been reported to cause lipid peroxidation of membrane phospholipids which ultimately leads to myocyte structural damage (Idle *et al*, 1999) and apoptosis (Cheng *et al*, 1995, Siwik et al, 1999). Studies examining hypoxic and ischemic injury have indicated that the cellular generation of free radicals may be involved in damage to plasma and mitochondrial membrane (Del Maestro, 1980, Guarnieri *et al* (1980). Guarnieri et al (1978) have shown an increase in lipid peroxidation, evaluated as an increase in MDA in hearts made hypoxic and reoxygenated. Decrease in both SOD and glutathione peroxidase activity were found in these hearts, indicating a reduced cellular defense mechanism against free-radical induced damage during hypoxia. Khalid *et al*, (1993) have shown the presence of MDA release in cultured adult cardiomyocytes subjected to anoxia and reoxygenation, whereas Toraason *et al* (1994) reported increased TBARS release and LDH leakage from cultured neonatal cardiomyocytes due to H_2O_2 in a concentration

dependent manner. Continuous suffusion with H_2O_2 first altered the configuration of $[Ca^{2+}]_i$ transients with an ultimate $[Ca^{2+}]_i$ overload.

Several studies have shown an increase in MDA level in Mg deficient situations. Cerebral ischemia-reperfusion injury leads to an increase in brain tissue MDA level, and it is reported that magnesium sulphate supresses the increment and associated ECG changes (Barishkaner *et al*, 2003). Gunther and Hollriegl (1989) have reported that young rats on Mg deficient diet for four weeks resulted in an increase in liver mitochondrial MDA, whereas the heart and kidney levels remained the same as that of the control. The modification of lipid metabolism during experimental Mg deficiency have been characterised by Rayssiguier and Guex (1986) and Rayssiguier *et al*, (1993b). Severe Mg deficiency in weanling rats produced marked hypertriglyceridemia and a decrease in the percentage of cholesterol transport by high density lipoprotein. A marked reduction in plasma activity of lecithine cholesterol acyl transferase and a significant decrease in esterified/total plasma cholesterol ratio have also been reported. Mahfouz and Kummerow (1989) reported that experimental Mg deficiency induced in rats by feeding Mg deficient diet for 23 days resulted in an increment in the level of serum TBARS. The increase was attributed to the increased cytosolic Ca^{2+} in Mg^{2+} deficiency. Rock *et al* (1995) has suggested a free radical mediated process of tissue injury in Mg deficiency. The study was carried out in rat skeletal muscle. Rats fed Mg deficient diet showed an ultrastructural change

associated with mitochondrial swelling and disorganization of sarcoplasmic reticulum. Greater concentration of TBARS and lower concentration of thiol groups were found in skeletel muscle of Mg deficient rats. Only few reports are available on myocardial level of MDA in Mg deficiency. In the present study also the extent of lipid peroxidation produced in the myocardial tissue in Mg deficiency was slightly higher than that in Mg sufficiency, but the difference was not statistically significant (fig. 32).

When Mg deficiency was associated with oxidative stress an augmented production of TBARS was observed. The level of MDA in presence of H_2O_2 was slightly higher than that in presence of HX+XO (fig. 33). These results indicate that the damage of membrane bilayer can be augmented in presence of ROS when associated with Mg deficiency. The membrane changes mediated by lipid peroxidation can induce inotropic changes.

V.3.2. Tissue injury

Lactate dehydrogenase was assayed as the marker for tissue injury. Elevated LDH release is an indicator of SL damage and hence used widely as the marker of tissue injury. In ischemic myocardium, the pyruvate is converted to lactate in a reaction catalysed by LDH. Hence an increased level of LDH in peripheral circulation confirms the occurrence of myocardial infarction. An increase in tissue lactate was demonstrated in cerebral

ischemia-reperfusion injury, and magnesium sulphate suppresses the increase of lactate concentration (Barishkaner *et al*, 2003).

Coronary effluent samples were used to determine LDH release using the rate of decline of NADH absorbance. Myocardial LDH release was found to be high in HX+XO as well as H_2O_2 treated myocardium (fig. 34) indicating the possibility of tissue damage. The level of LDH reported in this study is comparable to that reported by Taylor *et al* (2003). Hydroxyl radical induced release of LDH during anoxia and reoxygenation of cultured adult cardiomyocytes have been reported by Khalid *et al* (1993). Magnesium deficiency also produced an enhancement in the release of LDH (fig. 35). When oxidative stress was given in combination with Mg deficiency an augmentation in the release of LDH was observed as compared to control, indicating the additive effect of Mg deficiency and ROS on tissue injury (fig. 36). Enhanced release of LDH may be correlated with the incomplete recovery of contraction at higher concentration of ROS, with additive effect in Mg deficiency.

V.3.3. High energy phosphate metabolism

Under normal conditions myocardial metabolism is aerobic; fatty acids, ketone bodies, pyruvate and lactate are oxidised to CO_2 and H_2O with release of energy which is transferred to ATP. Energy is stored as CP formed from ATP by the enzyme creatine kinase. The CP is used to maintain the

level of cellular ATP to drive the contractile process of the myocardium involving interaction of proteins, actin and myosin.

V.3.3.1. ATP content

Early physiologists recognised that the heart required energy for contraction and external fuels ("substrates") for provision of energy. The general principle was to control the environment of the heart exactly by isolating it and then to re-examine its requirements for fuels and oxygen. Hence modified Langendorff perfusion technique was used for studying cardiac metabolism (Opie, 1992). The direct participation of ATP in the process of contraction was most unequivocally demonstrated by Cain and Davis (1962).

Tissue ATP content is the measure of energy status and provides valuable information regarding the role of energy impairment and contractile dysfunction. Several studies pointed out the contractile dysfunction by oxygen derived free radicals and their metabolites (Burton *et al*, 1984, Jackson *et al*, 1986, Ytrehus *et al*, 1986, 1987, Miki *et al*, 1988). In this study, decrease in contractile force due to HX+XO and H_2O_2 was accompanied by a decrease in ATP levels (fig. 37). Oxygen derived free radicals and their metabolites may cause enzyme inactivation either directly or indirectly. Hydrogen peroxide can cause an inhibition of ATP synthesis through inactivation of enzymes necessary for glycolysis and oxidative phosphorylation (Spector *et al*, 1988, Goldhaker *et al*, 1989). Thus functional

deterioration may be linked to ATP reduction by the above mechanism. Josephson *et al* (1991) reported myocyte contracture in correlation with ATP depletion due to H_2O_2 in rats. The relationship between myocardial ATP content and cardiac function during ischemia has been demonstrated by Takeuchi *et al* (1992) who showed that ATP was dephosphorylated to adenosine and further degraded to uric acid. Maulik et al (1997) have shown a significant depletion in myocardial ATP content during ischemia and it was not replenished following reperfusion. Takemura et al (1994) have shown a dose dependent reduction in contractility associated with ATP reduction and structural damage by $OH^{\bullet}$ free radical in adult rat hearts. Nakamura *et al* (1993) reported that ATP depleted myocytes were more susceptible than normal cells to oxygen radical induced toxicity. A decrease in Ca handling activity of the SR and mitochondria in ATP depleted cells contribute to the high sensitivity to oxygen radicals. Partial inactivation of the enzymes of glycolysis, TCA cycle, and HMP shunt by H_2O_2 have been reported by Chathem and Janero (Chathem *et al*, 1989, Janero *et al*, 1994). Such changes may be responsible for the decrease in ATP and associated functional alterations observed in this study.

In Mg deficiency the ATP content of myocardium was found to be maintained comparable to the control (fig. 38) with a significant reduction in the level of CP. It was previously reported that in skeletal muscle the contractile machinery was impaired when the intracellular CP was depleted

while the ATP concentration remained practically unchanged, and the action potential could still be elicited (Seraydarian *et al*, 1961). It has been suggested that creatine phosphokinase plays an important regulatory role in oxidative phosphorylation and thus in transfer and the stocking of energy as CP, and it is possible that creatine regulates creatine phosphokinase activity (Vial *et al*, 1972). Creatine might act as an effector molecule in the control of muscular activity not only in terms of myosin synthesis, as suggested by Ingwall *et al* (1972) but also in terms of stimulating mitochondrial creatine kinase in the regulation of oxidative phosphorylation in a feed back control system (Seraydarian *et al*, 1974). A number of studies have shown a direct correlation between $[Mg^{2+}]_i$ and ATP level as well as mitochondrial Ca^{2+} transport (Saks *et al*, 1975, Garfinkel *et al*, 1986, Ferrari *et al*,1993, Tsukube *et al*, 1994). Studies by Savabi *et al* (1988) have shown that prolonged dietary depletion of Mg leads to reduced atrial contractile activity, elevated creatine phosphate, reduced intracellular inorganic phosphate and adenine nucleotides in rats. Intraperitonial administration of $MgCl_2$ to Mg depleted rats resulted in complete recovery of contractile force without changes in the level of inorganic phosphates and adenine nucleotide. According to the author the reduced intracellular level of high energy phosphate cannot therefore be responsible for the impaired contractility seen in Mg depleted heart muscle (Savabi *et al*, 1988). These results can be correlated with the observations of the present study where we have seen a Ca^{2+} mediated

mechanism for the positive inotropy in Mg deficiency without an alteration in the level of ATP. Headrick *et al* (1998) have shown an improved myocardial energy metabolism with high levels of $[Mg^{2+}]_o$ which is independent of $[Mg^{2+}]_i$, supporting an extracellular locus of action. According to Li *et al* (1993a, 1993b) $[Mg^{2+}]_o$ is not a limiting factor in ATP metabolism after energy depletion with chemical hypoxia in opposum kidney cells. An augmentation in the reduction of ATP content was observed when Mg deficiency was accompanied by free radical stress (fig. 39), which further confirms the augmented response of myocardium to FR stress in marginal Mg deficiency.

V.3.3.2. Subcellular distribution of ATP

Physical compartmentalisation of ATP between mitochondria and cytosol is well established. ATP made in the mitochondrial compartment has to be transported to the cytosolic compartment for use. The adenine nucleotide transport system, the ATP-ADP translocase has a significant role in ATP transport (Atlante *et al*, 1998). Opie (1992) has proposed that ATP, having been extruded from the mitochondria by the translocase, is immediately converted to phosphocreatine by creatine kinase situated outside the mitochondria.

Inorder to check whether the synthesis and/transport of ATP was affected by the stress situations – free radical stress, Mg deficiency or both – mitochondrial to cytoplasmic ratio of ATP was determined.

In ROS treated (both HX+XO and H_2O_2) myocardium a significant increase in the mitochondrial to cytosolic ratio of ATP was observed (fig. 41). The total tissue ATP as well as the mitochondrial ATP was reduced significantly in these experimental groups. These results indicate the possibility of impaired synthesis as well as transport of ATP in the mitochondria.

In Mg deficiency though the total ATP level was maintained, the mitochondrial to cytosolic ratio was higher when compared to control (fig .42). Increased consumption of ATP due to positive inotropy in Mg deficiency can also decrease the content of cytosolic ATP.

When the ROS acted synergistically with Mg deficiency the mitochondrial to cytosolic ratio of ATP was further enhanced (fig. 43). The results indicate that in addition to a decrease in total ATP the available ATP is significantly compromised due to insufficient subcellular transport. Reduction in available ATP can affect cardiac contractility by multiple mechanisms.

V.3.3.3. Myocardial creatine phosphate

Since CP is also a measure of energy status, the decrease in contractile force due to HX+XO and H_2O_2 can be correlated with reduction in the tissue level of creatine phosphate (fig. 44). In marginal Mg deficiency myocardial content of ATP was maintained unaltered with a significant reduction in tissue CP (fig. 45) An elevation in CP and reduced inorganic phosphate

without modifying [ATP] was demonstrated by Headrick *et al* (1998) with an increase of $[Mg]_o$ from 1.2mM to 8.0 mM. It is possible that increased ATP utilisation associated with positive inotropic effect of Mg deficiency may limit the conversion to creatine phosphate.

The occurrence of CP in myocardial tissues is as an energy reservoir. It is the effector molecule in the maintenance of functional levels of ATP (Seraydarian *et al*, 1974). Creatine phosphate does not contribute directly to endorgonic reactions, and yet the depletion of CP correlates well with the cessation of contractile activity. There is also a correlation between the depletion of CP and the cessation of beating of cardiac cells in culture when glycolysis and oxidative phosphorylation are inhibited (Seraydarian *et al*, 1969). It has been suggested that the early cessation of contractile activity in ischemic myocardial cells in the presence of apparently adequate ATP stores might be due to a reduction in the rate of regeneration of a small pool of extramitochondrial ATP available for muscle contraction and other ATP requiring process such as ion transport (Gudbjarnason *et al*, 1970). It is suggested that creatine is involved in the control of supplying the necessary energy for contractile process and in the transfer of energy from the site of synthesis to site of utilization. Under normal physiological conditions an increased production of creatine would stimulate the mitochondrial creatine kinase; the phosphorylation of creatine to CP would provide mitochondrial ADP to stimulate the rate of oxidative phosphorylation. The CP thus

generated would reactivate myosine – ATP. It has also been demonstrated that inhibition of either glycolysis or oxidative phosphorylation did not alter significantly the concentration of ATP in cultured heart cells, but caused a decrease in the intracellular concentration of CP (Serraydarian *et al*, 1972 and 1969). Decrease in CP has also been reported in acute HF and ischemia (Maulik *et al*, 1997, Vogt and Kubler, 1998). Similar to the high energy phosphate compound ATP, when oxidative stress was given in combination with Mg deficiency an augmentation in the reduction of CP was observed as compared to control (fig .46) indicating the additive effect of Mg deficiency and ROS on energy metabolism.

Relating the inotropic changes to variation in energy metabolism and the functioning of ion channels, it is apparent that positive inotropic response in Mg deficiency is mediated by increased Ca^{2+} influx through the voltage gated Ca^{2+} channels and also possibly through the Na Ca exchanger. The negative inotropic response in oxidative stress is likely to be mediated by a decrease in the energy available for contraction, which can also influence the functioning of energy dependent ion pumps. Membrane changes due to lipid peroxidation can also affect ion transients. A synergistic action is effected when Mg deficiency is associated with oxidative stress. Enhanced tissue injury, lipid peroxidation and reduction in available energy appears to be the mediators of the augmented negative inotropic effect.

VI. SUMMARY AND CONCLUSION

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VI.1. SUMMARY

Cardiac function is known to be regulated by a number of factors. Variation in the extracellular milieu modulate myocardial contractility in several ways, including modification of membrane proteins as well as regulatory and contractile components of the cell.

Pathogenesis of heart diseases are believed to be mediated at least in part from the development of oxidative stress resulting from the generation of ROS and reduced antioxidant defense system. Conditions which elicit inflammatory responses such as hemostasis and myocarditis can result in local production of large quantities of reactive oxygen species. Free radicals are important in the maintenance of normal physiological function; however, an increased production of these toxic biomolecules through an uncontrolled chain reaction is potentially deleterious. Though in normal conditions there is a balance between FRs and their quenchers the antioxidants; under acute as well as chronic stress conditions, the oxidant status of the heart may be adversely affected. Myocardial response to oxidative stress can be modified further by variation in the extracellular milieu such as magnesium insufficiency.

Hypomagnesemia has been positively correlated with the prevalence of cardiovascular disorders in regions with soft water (Durlach *et al*, 1989). The assessment of the effect of suboptimal levels of Mg on cardiac function assumes importance in Kerala, where the Mg content of drinking water is low (<5mg /L). Experimental and clinical studies indicate the possibility of a marginal decrease in

extracellular Mg compared to those with sufficient intake. Magnesium deficiency has not received the attention it deserves probably due to the absence of clinical symptoms. Magnesium deficiency concomitant with oxidative stress may be of clinical significance, leading to arrhythmic, hemodynamic and ischemic changes in heart. Chronic Mg deficiency is accompanied by increased free radical generation. Several studies have reported the enhanced stress sensitivity in Mg deficiency. When Mg deficiency exists, stress increases the risk of cardiovascular damage including arrhythmias.

The focus of this investigation has been to examine the functional response of the myocardium to oxidative stress when associated with Mg insufficiency, based on the hypothesis that "marginal magnesium deficiency augments the myocardial response to oxidative stress." Mechanical and biochemical response of the myocardium to oxidative stress and marginal magnesium deficiency was examined independently for these two conditions followed by the synergistic effects.

Studies were carried out in isolated heart preparations of adult Sprague - Dawley rats. Papillary muscle isolated from left ventricle served as the model for recording the inotropic response. In an attempt to correlate the contractile changes with changes in $[Ca^{2+}]_i$, the diastolic levels of $[Ca^{2+}]_i$ was assessed by the dual wave length excitation method with the help of ion sensitive flurochrome, fura - 2 AM. Diastolic Ca^{2+} levels were assessed in isolated cardiomyocytes. As Ca^{2+} is the major ion modulating the cardiac mechanics, the influence of ROS and $[Mg]_o$ on channels and pumps likely to mediate Ca^{2+} transients were assessed using channel specific

inhibitors. Biochemical variables were examined in the ventricular tissue of Langendorff perfused isolated rat hearts. Lipid peroxidation and tissue damage were assessed by the quantitation of MDA and LDH respectively. As energy metabolism has a significant influence on myocardial mechanics, high energy phosphate compounds, ATP and CP were determined.

The response to different treatments were recorded as percentage change from baseline and presented as mean ± SD. Significance of difference between means was determined by Student's t test. $p < 0.05$ was selected to indicate statistical significance.

Major findings

1. Inotropic response of papillary muscle to two commonly used generators of ROS: (HX+XO) and H_2O_2, showed a significant reduction at pathophysiological concentrations. The reduction in contractile force was found to be directly proportional to the concentration of reactive oxygen species. The diastolic Ca^{2+} also showed the same pattern as that of contractile force.

2. At lower concentrations 0.5 mM HX+0.02 U/ml XO and 50 μM H_2O_2, the contractile variations produced by the ROS were found to be completely reversible whereas at higher concentrations 1 mM HX+0.04 U/ml XO and 100 μM H_2O_2 partial reversibility was observed in fresh medium.

3. The scavenging enzymes SOD and catalase produced a protective effect against the ROS only when the enzyme supplementation preceded the induction of variation by

the reactive oxygen species. It is interesting to note that addition of antioxidants after exposure to ROS had a paradoxical effect.

4. Decrease in $[Mg^{2+}]_o$ showed a positive inotropic response with a peak at 0.48 mM. Diastolic Ca^{2+} also showed an increase at this concentration.

5. The negative inotropy of papillary muscle produced by ROS was found to be augmented in Mg deficiency, indicating the presence of an interaction between oxidative stress and Mg deficiency.

6. Sarcolemmal L and T type Ca channels and Na K ATPase were found to be significantly influenced by the SO anion generator HX+XO. Comparable to SO anion generator, contractile variation in H_2O_2 was also influenced by SL L type and T type Ca channels, but not by Na K ATPase inhibitor. Ryanodine receptors were significantly influenced by H_2O_2.

7. The positive inotropic response in Mg^{2+} deficiency was mediated by voltage gated Ca channels, with Mg^{2+} acting as SL Ca channel blocker. Na K ATPase was also found to be inhibited by Mg^{2+}.

8. An interaction between HX+XO and Mg^{2+} deficiency was observed only for Na K ATPase, whereas an interaction between H_2O_2 and Mg^{2+} was observed for SL T type Ca channel and SR Ca pump.

9. Lipid peroxidation was significantly enhanced on exposure to both the ROS, HX+XO and H_2O_2 as indicated by the increase in MDA. The level of MDA was not affected by Mg deficiency but, when Mg deficiency was associated with oxidative

stress, an augmentation in the production of MDA was observed both in the presence of HX+XO and H_2O_2.

10. Magnesium insufficiency and exposure to ROS was associated with enhanced release of LDH, a marker of tissue injury. When Mg deficiency was combined with oxidative stress, the release of LDH was higher than the individual stress situations, suggestive of an interaction.

11. In presence of HX+XO and H_2O_2 a significant reduction in the level of high energy phosphate compounds, ATP and CP were observed, but in Mg deficiency the ATP content of the myocardium was comparable to the control value with a significant reduction in the level of CP. When the two stress situations acted synergistically, an augmentation in the reduction of high energy phosphate compounds were observed with possible reduction in the production and mitochondrial transport, thereby reducing drastically the available ATP for myocardial contraction.

VI.2. CONCLUSION

This study has shown conclusively that, ROS and Mg insufficiency in pathophysiological concentrations modulate myocardial mechanics. The negative inotropic response to ROS was mainly mediated through a reduction in high energy phosphates and alteration in the response of ion channels requiring ATP for their function, where as the functional alteration produced in marginal Mg deficiency was mediated through voltage gated Ca^{2+} channels. When these two stress situations acted synergistically, functional and biochemical changes in the myocardium was

significantly enhanced. Decrease of high energy phosphate compounds were the major regulators of inotropic response. Magnesium deficiency and oxidative stress was associated with a significant increase in lipid peroxidation and tissue injury. The experimental data therefore supports the contention that, marginal Mg deficiency augments the mechanical and biochemical response of the myocardium to oxidative stress.

VI.3. FUTURE DIRECTIONS

The observations of this study envisages the need for further investigations into the molecular and electrophysiological mechanisms leading to the functional changes.

1. The inotropic changes have been correlated with diastolic $[Ca^{2+}]_i$ in this study using ion sensitive fluorochromes. A better understanding of the molecular mechanism will be possible if the Ca^{2+} transients are studied.
2. Apart from the SL ion channels and pumps studied, the other channels have also to be studied. One of the ion ~~channels~~ exchangers that needs detailed examination is the Na Ca exchanger. The possible role of this channel could not be examined due to the non availability of the specific inhibitor that prevents the reversal of Ca^{2+} flow.
3. Electrophysiologic studies using patch clamp technique will help to understand the role of individual channels in the modulation of action potential, eliminating the influence of the other channels.
4. A better understanding of the inotropic variations will be possible by identifying the intracellular signalling cascade activated or deactivated by the biomolecules ROS and magnesium.

5. The observation that supplementation of antioxidants before the introduction of oxidative stress has a protective effect, needs further examination. This finding exemplies the importance of maintaining a basal antioxidant defense. This also suggests the possible harmful effects of unconditional antioxidant supplementation.

VII. BIBLIOGRAPHY

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