

**BIOCHEMICAL CORRELATES OF CARDIAC  
FIBROGENESIS IN RESPONSE TO MAGNESIUM  
DEFICIENCY AND CERIU**

A THESIS PRESENTED

BY

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(DIVISION OF CELLULAR AND MOLECULAR CARDIOLOGY)

IN PARTIAL FULFILMENT OF THE REQUIREMENTS  
FOR THE DEGREE OF  
**DOCTOR OF PHILOSOPHY**  
IN THE SUBJECT OF BIOCHEMISTRY

TO



**SREE CHITRA TIRUNAL INSTITUTE  
FOR  
MEDICAL SCIENCES AND TECHNOLOGY  
THIRUVANANTHAPURAM**

FEBRUARY 1997

*Dedicated to my  
beloved parents*

## CERTIFICATE

I, B. PRAKASH KUMAR, hereby certify that I had personally carried out the work depicted in the thesis entitled "BIOCHEMICAL CORRELATES OF CARDIAC FIBROGENESIS IN RESPONSE TO MAGNESIUM DEFICIENCY AND CERIUM", except where external help was sought and is acknowledged.



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## **DECLARATION**

This is to certify that Mr. B. PRAKASH KUMAR 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. The work relating to his thesis entitled "BIOCHEMICAL CORRELATES OF CARDIAC FIBROGENESIS IN RESPONSE TO MAGNESIUM DEFICIENCY AND CERIUM" was carried out under my direct supervision.



**( K. SHIVAKUMAR )**

Guide

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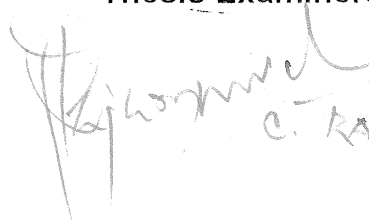
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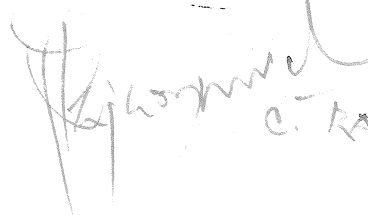
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## ABBREVIATIONS

apo-B	-	Apoprotein B
ASO	-	Anti - streptolysin O
ATP	-	Adenosine triphosphate
cpm	-	Counts per minute
DCM	-	Dilated cardiomyopathy
DNA	-	Deoxyribonucleic acid
dpm	-	Disintegrations per minute
DPX	-	Dibutyl phthalate xylene
EMF	-	Endomyocardial fibrosis
EMD	-	Endomyocardial disease
EDTA	-	Ethylenediaminetetraacetic acid
HDL	-	High density lipoprotein
ICP-MS	-	Inductively coupled plasma - mass spectrometer
IgA	-	Immunoglobulin A
IgG	-	Immunoglobulin G
IL - 1	-	Interleukin - 1
IL - 6	-	Interleukin - 6
kg	-	kilogram
LDL	-	Low density lipoprotein
MDA	-	malondialdehyde

mg	-	milligram
mCi	-	millicurie
ml	-	millilitre
mEq	-	milli equivalent
mmol	-	millimol
NADH	-	Nicotinamide adenine dinucleotide [reduced]
ng	-	nanogram
nmol	-	nanomol
pmol	-	picomol
L-phe	-	L-phenylalanine
PPO	-	2, 5 - Diphenyl oxazole
POPOP	-	1, 4 - Bis (5- Phenyl oxazol - 2 yl) benzene
RHD	-	Rheumatic heart disease
RNA	-	Ribonucleic acid
SOD	-	Superoxide dismutase
SD	-	Standard deviation
TCA	-	Trichloroacetic acid
tRNA	-	Transfer ribonucleic acid
TBARS	-	Thiobarbituric acid - reactive substances
TNF - $\alpha$	-	Tumor necrosis factor - $\alpha$
$\mu$ g	-	microgram
$\mu$ Ci	-	microcurie
$\mu$ M	-	micromolar

Although the prevailing paradigm is that heart disease is largely explicable in terms of muscle cells and their contractile proteins, one should not lose sight of the important role of the extracellular matrix in the structure and function of the myocardium.

Am. J. Pathol. (1996) **148**, 1639

# **INTRODUCTION**

The heart is a muscular pump composed of the parenchyma and the stroma. While parenchyma consists of highly differentiated cells (like the cardiac myocytes) having specific functions, the stroma is made of pluripotent, highly undifferentiated cells like cardiac fibroblasts, endothelial cells and smooth muscle cells and a structural protein network formed largely by fibrillar collagen (1). Much attention has been focussed on the parenchyma, alterations in which were presumed responsible for organ failure. This was because structural protein turnover was assumed to be too slow to be of any pathophysiologic consequence. However, the introduction of the flooding-dose technique (2) in protein turnover studies and its subsequent application to collagen turnover (3) have shown that collagen turnover is dynamic. It has now become clear that in many pathologic states, continued accumulation of the stroma impairs organ function. The regulation of stroma, and collagen turnover in turn, is therefore attracting considerable attention in recent years (1). Cardiac collagen synthesis is sensitive to a variety of pathophysiologic stimuli. A direct response of the cardiac tissue to parenchymal cell necrosis is the formation of a scar tissue following deposition of collagen. Tissue repair by scar synthesis is no more than a second best solution to the

problem created by interrupting tissue integrity. The scar that replaces the complex tissue during healing may bring about deformities and functional aberration due to formation of contractures and fibrous adhesions. The abnormal accumulation of fibrous tissue, or fibrosis, creates a pathological remodeling of the myocardium. The progressive and extensive nature of remodeling negates what is initially an adaptive response and may eventuate into a pathologic state with muscle entrapment, cell loss and abnormal diastolic stiffness and systolic wall stress of the myocardium. A classic example of such abnormal accumulation of fibrous tissue leading eventually to cardiac failure is endomyocardial fibrosis (EMF).

### **Endomyocardial Fibrosis**

EMF is a restrictive cardiomyopathy characterised by fibrotic thickening of the endocardium and inner third of the ventricular myocardium. A striking feature is the excessive accumulation of extracellular matrix components in the endocardium and subendocardial myocardium. Ever since its recognition by J.N.P. Davies and J.D. Ball (4) as a distinct clinical and pathologic entity, studies on causative factors such as vitamin E deficiency, excessive serotonin from consumption

bananas, viral and parasitic infection, hyper-eosinophilia etc. have not so far conclusively demonstrated the aetiologic link. Epidemiologic studies (5) reveal that out of the 799 cases reported in the last two decades, 730 originated from countries within  $15^{\circ}$  of the equator. Given the predominant tropical distribution, it would appear likely that there exist geochemical factors, common to the equatorial region, whose identification should provide the key to the aetiology of tropical EMF.

Any hypothesis on the causation of EMF should take into consideration the following facts:

1. It affects the lower socio-economic group that is relatively susceptible to nutritional deficiencies.

2. Its prevalence in regions around the equator where there are abundant deposits of rare earth elements like Ce in the soil; and

3. Presence of higher levels of Ce and lower levels of Mg in the cardiac tissue of patients, compared to healthy control (6,7).

Based on these, a geochemical hypothesis has been proposed that tropical EMF could be the cardiac expression of Ce toxicity in conjunction with Mg deficiency (8).

While the importance of  $Mg^{2+}$  as a metabolic cofactor and regulatory cation in cardiac cell is recognized (9), the molecular basis of cardiac muscle damage and fibrosis associated with Mg deficiency (10) is not clear. Further, although rare earth elements have attracted considerable attention in recent years and are known to initiate pathobiologic processes in humans (11), the influence of these elements on metabolic processes in the heart has received little attention. Despite stray reports on the localization of Ce in the intracellular milieu, its affinity for DNA and its cardiotoxicity (12), tripositive lanthanides are generally believed to be poor permeators of the sarcolemmal membrane. The physiological properties of lanthanides are largely explained on the basis of their attachment to the outside of the cell membrane, with resulting disturbances in cellular transport of cations (13). Moreover, lack of correlation between their effects *in vitro* and *in vivo* has been reported (13). Hence, the influence of these elements on metabolic processes in the heart calls for further exploration in the

context of the geochemical hypothesis on the causation of tropical EMF.

### **The objectives of this study were**

1. To ascertain whether a combination of Mg deficiency and Ce would produce fibrotic changes in the heart.
2. If so, to establish the biochemical correlates of cardiac fibrogenesis promoted by Mg deficiency and Ce, focussing on changes in collagen metabolism.

In keeping with the objectives, this study consisted of a long-term experiment and several short-term experiments on rats.

1. Long-term experiment: The aim was to look for histological changes in the heart in response to Mg deficiency and Ce.
2. Short-term experiments: The aim of these experiments was to investigate the biochemical basis of cardiac muscle damage and fibrosis induced by Mg deficiency and Ce.

### **Findings in a nutshell**

1. This work has generated histological evidence of endomyocardial fibrosis produced by a combination of Mg deficiency and Ce.

2. The work has also established the molecular basis of cardiac fibrosis in response to Mg deficiency and Ce.

a. The observed changes in parameters of oxidative stress, collagen metabolism and fibroblast proliferation associated with Mg deficiency suggest that cardiac fibrogenesis in response to Mg deficiency may be reparative in nature, upon oxidative damage to the myocardium.

b. The results suggest a mechanism of myocardial damage in Ce-treated rats based on oxidative injury, increased fibroblast proliferation and alterations in collagen turnover.

### **Significance of the study**

The findings presented in this thesis are consistent with the geochemical hypothesis on the causation of tropical EMF. Further, the underlying theme of these investigations is the reparative response of the myocardium with accent on fibroblast proliferation and collagen turnover, which is an emerging theme of importance in molecular cardiology.

# **REVIEW OF LITERATURE**

## **INTRODUCTION**

The heart consists of the parenchyma and the stroma. The parenchyma is made up of highly differentiated cells (like cardiac myocytes) carrying out specialized functions while the stroma includes pluripotent, highly undifferentiated cells (like cardiac fibroblasts) and a structural protein network formed largely by fibrillar collagen (1). Structural protein turnover was assumed to be too slow to be of any pathophysiologic consequence and, therefore, most of the research carried out to understand the pathogenesis of heart failure was focussed on alterations in cardiac myocyte function. However, there is now considerable evidence to show that, continued accumulation of stroma impairs organ function. Thus, the regulation of stroma, and collagen turnover in turn, have attracted much attention in recent years (1).

The fibrillar collagen network serves several functions.

- a. Provides a scaffolding that supports muscle cells and blood vessels (14)
- b. Acts as a lateral connection between cells and muscle bundles to govern architecture (14,15) while co ordinating the delivery of force, generated by the myocytes, to the ventricular chamber (16)
- c. Provides tensile strength and resistance (17)

- d. Is an important determinant of diastolic and systolic myocardial stiffness (18,19), and
- e. Serves to resist myocardial deformation, maintain shape and wall thickness and prevent ventricular aneurysm and rupture (20, 21).

The normal regulation of collagen turnover guarantees that collagen concentration in the myocardium remains within normal limits under a variety of conditions. A remarkably ordered series of transcriptional, post-transcriptional and post-translational events mark the biosynthesis of collagen and the formation of collagen fibrils (22). Using the *in situ* hybridization technique in cultured cells and whole heart tissue, Eghbali *et al* (23, 24) demonstrated that the fibroblasts and not myocytes have the message for fibrillar collagen. Even though fibroblasts represent 70 % of the total cell population in the heart, these cells are normally quiescent (25) but play a pivotal role in tissue repair. Fibroblasts remain quiescent, out of the cell cycle or G<sub>0</sub> state, until recruited back into the cell cycle when they can be stimulated to proliferate.

Fibroblasts found in adult tissue retain pluripotentiality and hence exhibit marked diversity in their function, a trait related to distinct phenotypic sub-types (26 - 28). Fibroblast-

like cells appear at the site of cardiac myocyte necrosis (29). These cells are larger than usual fibroblasts, and, in addition, have acquired  $\alpha$ -smooth muscle actin microfilaments during phenotypic transformation from presumably quiescent fibroblasts of the interstitium. Contractile fibroblasts involved in tissue repair are termed myofibroblasts (30). Myofibroblasts, which play a central role in connective tissue formation and matrix remodeling, are gradually reduced in number at the site of repair (29). Fibrocytes, smaller fibroblasts, are another type of fibroblast-like cells which are seen at the site of tissue repair. Fibroblast-like cells, found within the connective tissue of the adventitia surrounding intramyocardial arteries referred to as pericytes, ultimately account for the perivascular accumulation of fibrillar collagen (31). Valvular interstitial cells are another class of fibroblast-like cells which are residents of valve leaflets where collagen turnover is normally high.

In response to cell injury, or following invasion by foreign chemicals, bacterial toxin or protein, tissues initiate a cascade of responses designed first to contain the assault and then heal the affected tissue. The process of wound healing involves various phases characterised as exudation, inflammation and fibrogenesis.

**Schematic representation of various phases  
of wound healing response (from ref : 1)**

Exudation	Vasodilation	Hyperpermeability	Interstitial edema
	Macrophages	Inflammatory cells	
Inflammation	Endothelial cells	Fibroblast proliferation & myofibroblasts	Granulation tissue
		Collagen synthesis & Fibrillar collagen accumulation	Fibrous tissue
Fibrogenesis			

The fibrillar collagen matrix of the cardiac interstitium is an active participant in the process of remodeling in response to parenchymal cell necrosis. This reparative fibrosis or scarring, has been recognized as an adaptive process that preserves the structural integrity of an organ following the loss of parenchyma. It can occur within a macroscopic segment of the myocardium following myocardial infarction or at a microscopic level in response to discrete myocyte necrosis (32). Fibrous replacement may occur in the absence of cell loss. This reactive fibrosis or collagenosis distorts tissue structure, increases tissue stiffness and electrically isolates

myocytes from each other (33). The accumulation of collagen in the scar and remodeling are both dependent on collagen breakdown as well as collagen synthesis (34).

The progressive accumulation of fibrous tissue is characteristic of endomyocardial fibrosis.

# **ENDOMYOCARDIAL FIBROSIS**



The first awareness of endomyocardial fibrosis (EMF) is ascribed to Arthur Williams (Foundation Professor of Medicine at Makerere University). He published an account of a heart disease in Uganda (35) describing two cases of mitral incompetence and cardiac failure, with necropsy findings of large patches of fibrosis affecting the ventricular walls. He ascribed the disorder to syphilitic myocarditis. Eight years later, in 1946, Bedford and Konstam (36) observed that some of the West African soldiers who died of heart failure had "extensive subendocardial fibrosis and necrosis without appreciable inflammation". The significance of these lesions was recognized by J.N. P. Davies (4) who described in detail the pathological features of the disease and designated the dense fibrous plaque of the mural endocardium as "Endomyocardial fibrosis" (EMF). Within two decades of the recognition of EMF, identical lesions were reported from South India and Brazil and, the geographical distribution of the disease in other tropical regions also became apparent (37-39).

### **Prevalence**

EMF has a marked preference for the tropics and is highest in Uganda, Nigeria, the Ivory Coast, South India and

Brazil- countries which are located within 15° of the equator. 15% of all deaths due to heart failure in Uganda (40), 20 % of cases of heart failure in patients aged below 40 years in Ivory Coast (41), 22 % of all acquired heart diseases in children in Western Nigeria (42) and 2.5 % of all cardiac patients under 40 years of age in Kerala (42) have been accounted for by EMF.

In India, prevalence of EMF is highest in the coastal areas of Kerala with very few cases being reported from Northern India. In Nigeria, it is endemic in the South- West but rare in the hot, dry north. In Uganda, occurrence of EMF is in South- West Rwanda, Burundi and Ankole (42).

### **Pathological features**

Massive and occasionally calcified thickening of the endocardium in one or both ventricles of the heart is a distinctive feature of EMF (4,43- 45). Though typically small, the size and weight of the heart may vary over a range from atrophy to hypertrophy (4). A massive collection of serous fluid is observed in otherwise normal pericardium when disease affects the right ventricle . Characteristic external feature of right ventricular disease is a depressed notch over the retracted apex of the right ventricle (43 - 45). Right atrium is markedly dilated and filled with massive thrombus; right

ventricular cavity is reduced in size. Diffuse and extensive endocardial fibrosis involving both inflow and outflow portions of the right ventricle reduces the papillary muscles to fibrotic stumps. The apical regions are entirely obliterated by a plug of thick fibrous tissue and superimposed organising thrombus (4, 43-45) when the disease affects the left ventricle. The endocardial thickening may involve the entire flow region and the posterior cusp of the mitral valve is often seen plastered to the underlying mural endocardium. Endocardial fibrosis may not always be diffuse and extensive. Isolated patches of fibrosis with intervening normal areas are seen in the ventricles over the endocardium of the apex, base of papillary muscle, beneath the posterior cusp of the mitral valve or other regions of the inflow tract (43, 44).

Microscopically, two zones are seen in the thickened endocardium: the superficial zone composed of a layer of fibrin and dense connective tissue components, mainly bundles of collagen fibrils and less elastic fibres (46). Alcian blue stain also reveals acid mucopolysaccharide in the fibrous tissue (43). Numerous vascular channels and chronic inflammatory cells which are predominantly lymphocytes (eosinophils are not common), strands of vascularised fibrous septae, often containing dilated lymphatic channels extending into the

myocardium are seen in the deeper zone (46). Extensive myofibrillar lysis is not evident. Foci of aggregates of lymphocytes, perivascular and intermyofibre fibrosis as well as microscopic scars are also seen in the myocardium (42).

Intramyocardial coronary artery lesions which include medial degeneration, medial fibrosis, organizing fibrin deposits on the intima and recanalized thrombus in the lumen have been found in EMF. Neither aortic nor epicardial coronary artery lesions are found in EMF. Extra cardiac lesions are only those consequent to cardiac failure (47).

### **Pathogenesis**

Endomyocardial disease (EMD) associated with hyper eosinophilia was first described by W. Loeffler in 1936 and since then the clinical and morphologic features as well as the mechanisms of eosinophil-mediated cardiac injury have been extensively investigated (48). Olsen described 3 phases in the morphologic evolution of eosinophilic endomyocardial fibrosis (49). The first phase seen within the first five weeks is that of acute myocarditis during which there is eosinophilic infiltration of the myocardium, necrosis of the subendocardial myofibres and vasculitis. The second phase after illness of about 5 weeks, is associated with thrombus deposition on

damaged and thickened ventricular endocardium. Inflammatory activity is reduced at this stage. The morphology of the final phase is similar to that of classical EMF, in which the endocardium is thickened at specific sites. Inflammatory cells may be present but are scanty.

### Staging of pathomorphologic changes in Endomyocardial fibrosis (from ref. 48)

		Active myocarditis
Onset	Necrotic phase	Inflammatory infiltrate Eosinophils
		Endocardial thickening
5 weeks	Thrombotic phase	Thrombosis Inflammatory cells are less
		Endocardium
10 weeks	Fibrotic phase	replaced by collagen, superficial thrombosis.

Identical pathologic features of EMF and the late stage of EMD prompted Olsen & Spry to propose the unitarian view

that the two entities are different ends of a spectrum of cardiac response to injury by eosinophils (50).

There are also other views on how the lesions evolve. Davies, in his early reports, mentioned that the vacuolar degeneration in subendocardial myocytes is similar to changes in hypokalemia and suggested an involvement of metabolic factors (51). Selye (52) has observed EMF-like lesions in animals subjected to chronic mineralocorticoid excess. Another opinion attributes EMF to a replacement process in mural endocardium secondary to subendocardial atrophy due to intra-myocardial coronary arterial changes. However, Andrade and Taxeira concluded from their studies that arterial lesions are caused by the same factors that lead to endocardial and myocardial damage. They draw attention to the similarity of the lesions to those associated with Mg-deficiency in animals (47).

A generally accepted view is that the lesion starts as an acute endomyocarditis with overlying fibrin deposits which heal with incorporation of thrombus into a fibrosed and contracted ventricle. There is little similarity between EMF and cardiac damage secondary to toxic and infective processes which commonly result in dilated cardiomyopathy.

The functional abnormalities in EMF resemble those in interstitial heart disease (42). Recent observations on biopsies from contralateral ventricles when there is univentricular involvement suggest that EMF is possibly a reactive and progressive interstitial fibrosis (53).

### **Aetiology of endomyocardial fibrosis**

Various factors, nutritional deficiency, serotonin from the consumption of bananas and plantains, vitamin E deficiency, obstruction of cardiac lymphatics, variants of rheumatic heart disease and dilated cardiomyopathy, hypersensitivity reaction to malaria and hyper-eosinophilia have been considered and investigated in the search for the aetiology of endomyocardial fibrosis.

#### **I. Nutrition**

Although EMF appears to be a disease predominantly affecting the poorer socio-economic groups, its absence from many areas where malnutrition is common and its sporadic occurrence in well-nourished Europeans who have lived in endemic areas, suggest that malnutrition is unlikely to be a primary factor.

**a. Serotonin** : Excess of serotonin (5 - hydroxy tryptamine) in the blood, as in carcinoid syndrome, produces a cardiac lesion

which some workers consider to be similar to EMF (54). Plantains and bananas which contain high levels of serotonin are consumed in large quantities in communities where EMF is common (55). It was therefore suggested by Arnot (56) that EMF might be due to excessive consumption of plantains. This view was further reinforced by observations of cardiac lesions in guinea pigs fed a plantain diet (57).

It is difficult to substantiate this theory on epidemiological grounds. EMF is rare in some communities, such as West Indians, which also consume large quantities of plantains and bananas. Further, experimental studies by Antia *et al* (58) and Ojo (59) have shown conclusively that serotonin has no part to play in the aetiology of EMF.

**b. Vitamin E Deficiency:** The role of Vitamin E is to prevent oxidation of tissue lipids. Deficiency of Vitamin E, therefore, leads to auto-oxidation of unsaturated lipids with deposition of ceroid bodies in the tissues (60). These ceroid bodies later become fibrosed and in the endocardium this leads to endocardial fibrosis. In a study conducted by measuring vitamin E status of patients with EMF and patients with cardiac failure due to other causes by Jaiyesimi *et al* (61), it was found that none of the patients was vitamin E deficient, thus rejecting

the hypothesis of Vitamin E deficiency as a causative factor of tropical EMF.

## **II. Obstruction of cardiac lymphatics**

Miller *et al* (62) suggested obstruction of cardiac lymphatics as an aetiological factor for EMF. This was based on surgical blockage of cardiac lymphatics in dogs which resulted in ventricular subendocardial haemorrhage and endocardial thickening due to increase in fibrous and elastic tissue in many animals. These findings have, however, not been confirmed as a cause of EMF.

## **III. EMF and Rheumatic heart disease**

The possibility of EMF being an expression of the rheumatic process was first put forward from Nigeria by Abraham (63) who observed an increase in anti-streptolysin O (ASO) titres during the acute condition in some patients. Later work on EMF by Abraham and Brigden (64) pointed out the co-existence of EMF and RHD in the same heart. Both RHD and EMF appear to be disorders of cardiac connective tissue based on a hyper-sensitivity mechanism. However, the pathologic lesions are strikingly dissimilar and there are many communities in which RHD is common but EMF is rare (42).

#### **IV. Immunological Aspects**

Van der Geld *et al* (65) in 1966 demonstrated the presence of bound IgG in the sarcolemmal and sub-sarcolemmal sites in myofibers and, to a lesser extent in the endocardium of patients with EMF. This finding is, however, not specific for EMF because similar deposits have been found in rheumatic hearts (66) and in patients with DCM (67).

Patients with EMF have also been shown to have antibodies to cardiac tissue but their presence is not specific for EMF, since elevated levels of antibodies IgG, IgA have also been found in DCM & RHD (68,69).

#### **V. Degranulated Eosinophils, hyper-eosinophilia and parasitic infection**

Brockington and Olsen (70) in 1973 conducted a retrospective study of 30 cases of Loeffler's endomyocardial disease and compared their findings with those of 6 non-tropical cases of EMF without eosinophilia and 26 patients with EMF from Nigeria, Uganda, Brazil and Europe. They concluded that both Loeffler's endomyocardial disease and EMF belonged to the same disease process, the origin of which could be traced to the presence of eosinophils in the myocardium.

Emphasising the prevalence of EMF in regions with latosolic soils with abundant deposits of monazite - rich minerals and the striking preference for malnourished children, a geochemical basis for the disease was recently suggested (6,7). The hypothesis that EMF could be the cardiac expression of Ce toxicity in conjunction with Mg deficiency (8) is based on elemental analysis of endomyocardial samples from EMF patients. While there was no significant difference between the patients and the controls with respect to the level of K, Fe, Si, Al and Mn, the samples from patients had a significantly lower level of Mg and higher levels of Na, Ca and Th (6). It was argued that alteration in Na and Ca levels could be due to salt retention and reciprocal response of Ca to Mg deficiency, as occurs in a variety of myocardial diseases. Among the monazite elements present in latosolic soils in these places which are noted for the high incidence of EMF, levels of Ce were found to be higher than those of Th (7). Subsequent studies showed that Ce levels in cardiac tissue samples from patients were higher than those of Th (7). Findings from epidemiologic studies on geographical distribution of EMF in Southern Kerala are consistent with the geochemical hypothesis. The co- existence of high density of occurrence of endomyocardial fibrosis and deposits of monazite elements (72)

support the geochemical hypothesis. The elevation in the levels of Ce which has no known physiologic role may point to a possible link between the disease and the monazite element.

**MAGNESIUM**

## Magnesium Metabolism

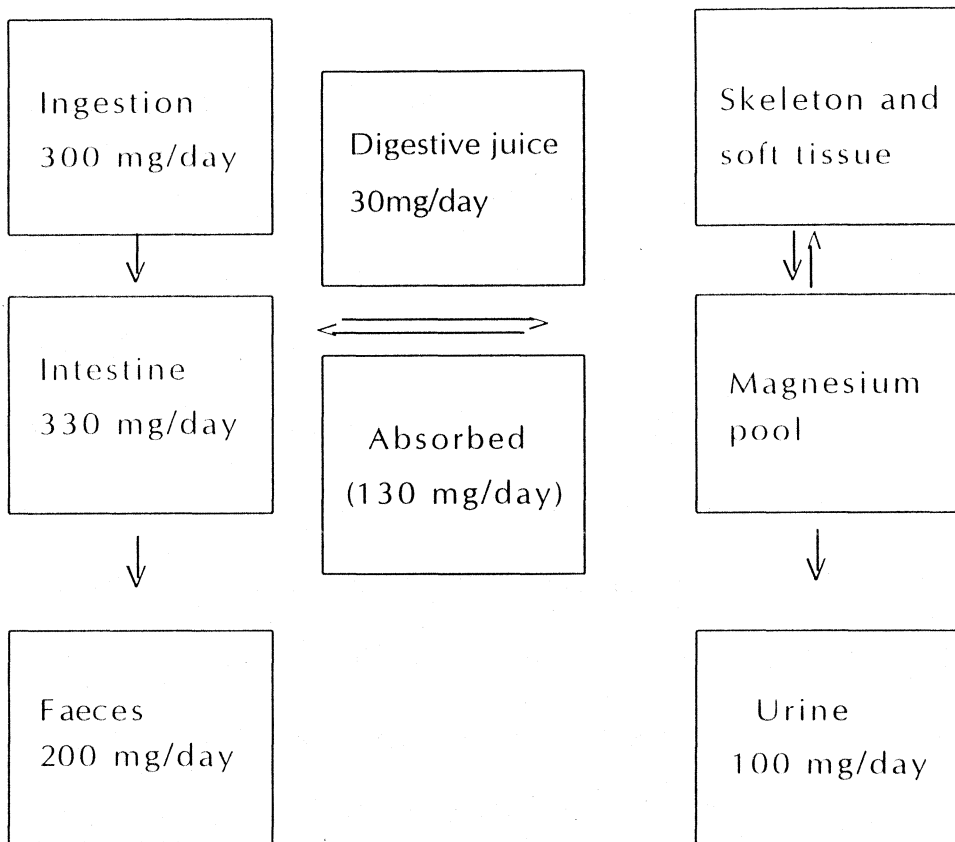
Magnesium (Mg) is the second most abundant intracellular cation (second only to potassium), and the fourth abundant total cation in the body. In addition to its central function in photosynthesis, Mg participates in many of the biochemical reactions that take place in the body, particularly in processes involving the formation and utilization of ATP. Mg is involved in more than 300 enzymatic steps, in several metabolic pathways. However, only recently has the regulatory role of the element been recognized in the wake of the discovery that intracellular free Mg,  $[Mg^{2+}]_i$ , is in the submillimolar range and that several intracellular systems have  $K_m$  values for Mg within this range (9).

## Homeostatic Mechanism

Dietary Mg is often better absorbed in the ileum and colon than in the jejunum. The percentage absorption of ingested Mg by individuals is influenced by its dietary concentration and by the presence of inhibiting or promoting dietary components. The homeostasis of the individual with respect to mineral balance depends on the amount ingested, intestinal and renal absorption and excretions. Mg homeostasis and distribution of Mg in various compartments

of an apparently healthy adult is given below:

### Magnesium homeostasis in man (from ref: 73)



### Magnesium deficiency

Given the importance of Mg in a wide range of fundamental cellular reactions, it is not surprising that deficiency of Mg in an organism may lead to serious biochemical and symptomatic clinical changes. A nutritional deficiency can be defined as a condition that develops when the intake of dietary substance is at a level lower than that

necessary to maintain optimum health. It follows that an individual may appear healthy but actually be at increased risk of developing some pathologic condition by virtue of inadequate dietary intake of a nutrient (74).

### **Assessing Magnesium Nutriture**

The diagnosis of magnesium deficiency is complicated by methodologic problems. Because of slow equilibration with intracellular stores, the serum magnesium level does not necessarily correlate with total body stores (75) and, therefore, Mg deficiency can be difficult to diagnose, especially when even severe deficiency may be asymptomatic. Attempts have, therefore, been made to determine the utility of other measures of tissue magnesium. While Alfrey *et al* (76) demonstrated a significant correlation between serum and bone magnesium levels, others have tried to utilize lymphocyte, erythrocyte, and muscle levels as tissue measures of magnesium balance, but without good correlation being demonstrated (76). The most reliable method of evaluating a patient's Mg status is determination of 24 hr urinary output before and after a parenteral magnesium load, and evaluating the percentage retention in terms of renal function and serum magnesium levels (77). Retention of greater than 50 % of administered dose

generally indicated a Mg deficiency, and retention of less than 20 % indicated that a deficiency was unlikely.

In general, severe deficiency is accompanied by decreased serum levels, referred to as hypomagnesemia, but low serum magnesium levels may not always indicate a cellular deficiency (75). Suspicion of Mg depletion should exist regardless of measured serum level in those conditions that seem to either cause or be accompanied by Mg deficiency.

### **Occurrence of hypomagnesemia**

A series of clinical reports in the early 1960's helped focus attention on the occurrence of hypomagnesemia (78). Congenital primary hypomagnesemia, familial hypomagnesemia and hypomagnesemia associated with hypocalcemia, hypokalemia and hyponatremia have been reported (79). Studies carried out in the US show that the overall incidence of hypomagnesemia in hospitalized patients ranges from 7 to 52 % (79). An inverse relationship between water hardness (Mg content of water) and incidence of coronary artery disease has received much attention recently (77).

## **Causes of hypomagnesemia**

Causes of hypomagnesemia are many and are divided into four general categories :

- a. Gastro-intestinal factors which include alcoholism, malnutrition, diarrhoea, malabsorption etc.
- b. Renal loss of Mg associated with some diuretics and antibiotics (ticarcillin, gentamicin, cisplatin) & acute tubular necrosis .
- c. Endocrine disorders with renal wasting of Mg include diabetic ketoacidosis, hyperparathyroidism, hyperaldosteronism etc.
- d. Excessive lactation, transfusion and acute intermittent porphyria are included in the miscellaneous category (80).
- e. Hypomagnesemia may also occur with trauma, strain and overhydration in hypoalbuminemia (81).

## **Clinical Symptoms of hypomagnesemia**

Severe hypomagnesemia associated with a significant deficit may remain undetected clinically as it may be masked by other disease states (82). Muscular twitching and tremor of any or all muscles including tongue, muscle wasting and muscle weakness, convulsion, mild to severe delirium

primarily an anti-platelet effect. Born and Cross (83) have noted that supranormal concentration of Mg significantly inhibited platelet aggregation. Adams and Mitchell (84) in their studies reported that Mg had a retarding effect on the formation of white thrombi in the injured rabbit artery, implying the platelet inhibiting effect of Mg.

#### **b. Increased severity of atherosclerosis**

Elevated levels of cholesterol, triglycerides and low density lipoprotein (LDL) and diminished levels of high density lipoprotein (HDL) have been observed in Mg-deficient rat (85, 86). A 27 % reduction in triglyceride, very low density lipoprotein, a significant reduction in apo-B and an elevation in plasma HDL have been observed in humans after three months of Mg therapy (87). At least two animal studies have suggested that increased dietary Mg retards atherosclerotic changes (88, 89). These data point to the fact that profound Mg deficiency results in significant perturbation in plasma lipids that would favour atherogenesis.

#### **c. Higher coronary artery tone or tendency towards vasospasm**

Coronary artery spasm represents an ischemic process that may be amenable to Mg therapy. Mg deficiency potentiates vasospasm and intravenous Mg relieves vasospasm

in a variety of clinical situations (77). Although there is a relative wealth of *in vitro* data supporting hypomagnesemia as a cause of vasospasm, the degree of Mg deficiency in many of these studies is well below the physiologic range. Chadda and Schultz (90) in their studies have reported that dogs fed a Mg-free diet were more likely to develop electrocardiographic evidence of coronary artery spasm. Recent studies on the effect of Mg on the release of endothelin and endothelium-derived relaxing factor, two potent vasoactive agents, show that Mg deficiency may influence initiation of coronary vasospasm through these agents (77). Mg deficiency in patients with variant angina, suppression of angina upon intravenous administration of  $MgSO_4$  in patients with coronary artery spasm, association between ischemic heart disease and low myocardial Mg and perturbation in Mg balance in acute myocardial infarction amply illustrate the vascular effects of the element (77, 91).

#### **d. Greater myocardial vulnerability to arrhythmia**

Early after depolarizations are oscillations in membrane potential that cause repetitive depolarizations, referred to as triggered activity, otherwise known as cardiac arrhythmias. Mechanisms underlying early after depolarizations are not clear but are believed to be related to a blockage of the inward

rectifying current (91-93). The inhibitory effect of Mg on early after depolarizations produced by various agents has been described by Davedenko *et al* (94) and Kaseda *et al* (95). The observed effects have been attributed to an increase in inward rectifying K<sup>+</sup> current. It was also suggested that suppression of triggered activity may be related to a stabilizing of surface charge effect that enhances the threshold current requirement for activation of triggered response (94). Ventricular and atrial arrhythmias are related to Mg deficiency in many clinical settings and successful treatment of arrhythmias with parenteral Mg is reported in many cases (91).

#### **e. Pathogenesis of the cardiomyopathy of Magnesium deficiency**

Hypomagnesemia is the hall-mark of experimental Mg depletion in many observed species. Crystalluria, calcification and degenerative changes in various organs, especially in the heart, are prominent in Mg-deficient rat and certain other species maintained on low magnesium diet. Myocardial lesions of Mg deficiency have been described and interpreted in various ways and, it appears that significant species-related differences in the heart's susceptibility to Mg deficiency may exist (77).

Myocardial degeneration with fibrosis and polyblastic infiltrations in rats reared on Mg- deficient diet has been observed by Greenberg *et al* (96). In a comprehensive study of the basic histologic lesions of Mg deficiency in rat, Lowenhaupt *et al* (97) described a wide- spread inflammatory lesion involving loose mesenchymal tissues. In the heart, the lesion began as focal acute perivascular inflammation and progressed through stages of necrosis, granulomatous inflammation and fibroblastic proliferation to scar formation. Heggveit *et al* (10) have reported an array of myocardial changes (as early as two weeks after rats were placed on Mg- deficient diet) which include focal myocardial necrosis, manifesting itself subendocardially, which enlarges and develops areas of calcification. As deficiency advances, fragmentation, vacuolization and eventual myocyte loss are noticed with progressive increase in macrophages, fibroblasts and collagen. Electron microscopic studies revealed mitochondrial swelling and loss of internal fine structure. Mitochondria calcify and coalesce to form mineralized masses within the sarcoplasm of degenerating myocytes. Calcification of large arteries, including the aorta, was also observed.

## Theories on causation of cardiac lesions observed in Magnesium deficiency

### a. Calcium overload

Heggveit (10) stresses the role of calcium overload in the causation of myocardial necrosis in Mg deficiency cardiomyopathy. The earliest observed change in a Mg-deficient animal is a dip in serum Mg and elevation in myocardial Na. Reduction in Mg-dependent  $\text{Na}^+$ - $\text{K}^+$  ATPase activity (77,78) causes  $\text{Na}^+$  accumulation in the myocyte, resulting in the reversal of Na-Ca exchanger and a rise in intracellular  $\text{Ca}^{2+}$  (98). That the observed increase in myocardial Ca level is due to Na-Ca exchange is consistent with the observed lag in the myocardial Ca level behind increase in Na levels (99). Thus, the "Calcium overload" hypothesis stresses the role of elevated levels of myocardial Ca in the development of Mg deficiency cardiomyopathy. This hypothesis is based on the observation that histological lesions parallel elevation of myocardial calcium and do not precede it (100). It is not quite clear whether Ca accumulation in the myocardium in Mg deficiency precedes necrosis or is secondary (dystrophic calcification) to it.

## **b. Defective membrane function**

The possible physiological consequence of alterations in membrane composition and properties caused by dietary depletion of magnesium was studied by Rayssiguier *et al* (101). It was reported that defective membrane function could be the primary lesion in Mg deficiency.

## **c. Ferryl-myoglobin radical formation**

Studies (102) on the oxidation states of intracellular myoglobin and cytochrome oxidase  $aa_3$  in isolated perfused rat heart subjected to acute Mg-deficient environment showed that upon exposure to low extracellular  $[Mg^{2+}]_o$  (ie 0.3 mM) for 30 mins, more than 80% of the oxymyoglobin was converted to its deoxygenated form. The levels of reduced cytochrome oxidase  $aa_3$  also increased by about 80% in low  $[Mg^{2+}]_o$ . Further, it was observed that deoxymyoglobin was converted to a species identified as ferryl-myoglobin. This process, set in motion by acute Mg deficiency, resulted from direct accessibility of the exogenous peroxide to the cytosolic protein. On the basis of the above findings, the authors suggest that a pathway leading to cardiac tissue damage, induced by Mg deficiency, probably involves the generation of ferryl-myoglobin radical, the formation of which could be prevented

by ascorbate. These studies could have wide implications as indicating a new mechanism by which low extracellular magnesium,  $[Mg^{2+}]_o$ , can induce myocardial injury.

#### **d. Alterations in G-protein function**

Considerable attention has been given to the association between heart muscle disease and G-protein function (103). The  $\beta$ -adrenergic-G-protein (guanine nucleotide binding regulatory protein) - cAMP ( $\beta$ -RGC) system is the primary signaling system in the heart that mediates the inotropic effects of  $\beta$ -adrenergic agonists and is altered in heart failure (104). Crucial role of Mg in the functioning of  $\beta$ -RGC system has been demonstrated with the discovery of G-proteins (105, 106). The primary effect of Mg on the  $\beta$ -RGC system is to activate G-proteins by two mechanisms:

- i. stimulating nucleotide hydrolysis through high-affinity binding sites of G-proteins and
- ii. by stimulating hormone - receptor nucleotide exchange through low-affinity binding sites. Studies *in vitro* have shown that  $Mg^{2+}$  ions are absolutely required for activation of G-protein. Changes in levels and functions of G-protein account at least in part for alterations of the  $\beta$ -RGC system and have been observed in patients with endstage heart

failure (107). Experiments in pursuance of the hypothesis, that effects of Mg deficiency on the recovery of heart function after myocardial infarction might relate to its influence on G-protein, have shown that Mg deficiency increases the expression of  $G_i$  (increase in  $G_i$  levels is associated with impaired heart function) in the heart after myocardial infarction while  $G_o$  levels were not altered by either Mg deficiency or by myocardial infarction alone. The function and level of  $G_i$  are not altered by dietary Mg deficiency alone, and, hence, it is reasonable to conclude that dietary Mg deficiency alone does not cause significant changes in the function and levels of myocardial G proteins, but that existing Mg deficiency increases levels of  $G_i$  in the heart after myocardial infarction (107). It has been suggested by the authors that alterations in G-protein levels could be due to elevated levels of plasma catecholamine seen in Mg-deficient rat.

#### **e. Oxidative injury**

The involvement of  $O_2$ -derived free radical production in the pathogenesis of Mg deficiency cardiomyopathy has been proposed (108- 113). They have demonstrated, in a rat model of acute Mg deficiency, elevated circulating levels of cytokines

like IL-1, IL-6, and TNF- $\alpha$  after 3 weeks on Mg-deficient diet. Sequential analysis of changes in cytokine levels in the serum of rats revealed an initial rise on day 12, followed by major elevation in all 3 cytokines by day 21. Further, elevation of the circulating levels of the neuropeptide, Substance P, occurs on day 5. Substance P, a neuronal tachykinin thought to be released from neural tissue, is known to produce several mediators of inflammation and both Substance P and TNF- $\alpha$  trigger free radical production. Increase in plasma thiobarbituric acid-reactive substances (TBARS) and the protective effect provided by vitamin E during Mg deficiency further support the theory that Mg deficiency does promote free radical generation. Further, a recent report from Weglicki *et al* ( 112 ) showed that Substance P blockade by CP96954 reduces accumulation of Substance P in cardiac lesions and TBARS levels. Based on these observations, Weglicki and associates have proposed the oxidative injury hypothesis, envisaging a role for cytokine/neurogenic mediators in the pathobiology of Mg deficiency.

**LANTHANIDES**

## **Chemistry of lanthanides**

Lanthanides or rare- earth metals constitute a series of fifteen metals starting with lanthanum (atomic number 57) and ending with lutetium (atomic number 71). They are sub-grouped as follows:

- a. The light lanthanides or cerium group from lanthanum to samarium;
- b. The medium lanthanides or terbium group from europium to holmium, and
- c. The heavy lanthanides or erbium group from erbium to yttrium.

These lanthanides occur together as oxides of the following minerals : xenotime, fergusonite, gadolinite, cerite, lanthanite, euxenite, polycrase, samarskite and monazite : monazite sand serving as the major source : cerium and lanthanum occur predominantly in rare- earth minerals, in concentrations of about 40 % and 25 % respectively.

## **Uses of lanthanides**

In the past, the use of lanthanides was restricted to 5 main areas; glass polishing, carbon arcs, cracking catalysts, flints for lighters and modulating agents for iron. Cerium compounds were used to treat tuberculosis, and as antinausea

agents during early pregnancy. Therapeutic use of lanthanides has been discontinued in some countries because, although lanthanide poisoning is rare, the potential for exposure to these metals leading to intoxication is growing (12).

### **Absorption and distribution of lanthanides in the biological system**

Normally, lanthanides do not occur in plant or animal tissues, but traces of these metals ( $0.05 \mu\text{g/g}$ ) were detected in the bones of man and animals exposed to them. In mammals, the gastro-intestinal absorption of soluble simple lanthanides is poor, less than 0.05%. However, dietary absorption of water-soluble and stable lanthanide-citrate and lanthanide-EDTA complex is rapid and complete in experimental animals (12). Dietary deficiency of Ca, P or vitamin A increases Ce absorption (12).

Studies on the distribution of lanthanides following parenteral injections show that the transport and removal of lanthanides from the blood to the reticuloendothelial system, soft tissues and bones depend on the route of administration, the concentration and the nature of lanthanide compounds. Intravenously administered Ce salts are rapidly distributed from the blood to the liver, skeleton, spleen, and adrenals

with a gradual uptake and retention in the bone. Lanthanide ions are unable to penetrate phospholipid bilayers and probably cannot enter healthy cells (13). The possibility that lanthanides enter specific types of cells under certain conditions cannot, however, be excluded (114). When present as aggregates, lanthanides are internalized through phagocytosis.

### **Biological effects of lanthanides**

The light lanthanides are slightly more toxic than the heavy lanthanides on the basis of LD<sub>50</sub> values, and intermediate lanthanides are the least toxic (115). Among parenteral administration routes, intravenous injection appears to be the most toxic, and intraperitoneal administration is more toxic than either intramuscular or sub-cutaneous injections.

The lanthanides interfere with normal metabolism in two general ways. The first concerns the damage inflicted by radioactive lanthanide and the second is through specific biochemical interactions producing a variety of toxic pharmacologic sequelae.

Most of the physiological responses of isolated cells, tissues and organs to lanthanides can be explained in terms

of the interaction of lanthanide ions at the outer surface of the plasmalemmae, with resulting disturbances in cellular transport of metal ions (13). Physiological studies suggest that  $\text{Ca}^{2+}$  channels provide high-affinity binding sites for the lanthanide ion (13). Since lanthanides usually block the cellular uptake of  $\text{Ca}^{2+}$ , they generally inhibit those physiologic processes which depend upon  $\text{Ca}^{2+}$  influx. Those physiologic processes which depend upon the release of intracellular  $\text{Ca}^{2+}$  or which are mediated by an alternative second messenger such as cyclic nucleotides are unaffected (13).

Lanthanides have high affinity for phosphate with which they form insoluble complexes. This enables them to function at low concentrations as non-specific "Phosphatases" cleaving phosphate groups from ATP, glycerophosphate, nucleotides and nucleic acids (13). Lanthanides can precipitate DNA from aqueous solutions by combining with the phosphate group of adjacent chains.

Fatty liver degeneration, with increased levels of neutral fats from extra-hepatic sources, liver mitochondrial damage, and decreased liver glycogen levels are symptoms of Ce toxicity in the liver (116). Ce salts appear to have a marked adreno- and sympatho-mimetic effect (117). This has been

attributed to an increase in the catecholamine content of peripheral and central adrenoreactive centers in the brain, adrenals and heart by Ce.

### **Effects of Cerium on cardiac tissue**

Among the first physiologic experiments to be carried out on the effects of lanthanides on cardiac tissue were those of Mines (118) who showed that micromolar concentrations of lanthanides reversibly and quickly produced a diastole arrest in frog's heart. Fawzi and McNeill (119) produced a 50% inhibition in contraction of guinea pig heart at a very low concentration of lanthanide. Reports are also available on the production of cardiac injury and polycythaemia in small animals by cerium tartrate. Cardiopolygraphic studies on rats following parenteral injection of  $^{144}\text{Ce}$  revealed a slight cardiotoxic effect of Ce.

The presence of Ce in conjunction with lower levels of Mg in endomyocardial tissues from EMF patients forms the basis of the geochemical hypothesis that tropical EMF could be the cardiac expression of Ce toxicity in combination with Mg deficiency (6, 7, 8).

Studies in pursuance of the geochemical hypothesis had shown that Ce at  $>1 \mu\text{M}$  concentration has an inhibitory effect

on protein biosynthesis in cultured cardiac myocytes (120). Further, heart cells exposed to a Mg-deficient medium were reported to be more susceptible to the action of Ce (120). However, at nanomolar levels, Ce stimulated collagen and non-collagen protein syntheses in cardiac explants and fibroblasts (121). These findings suggested that Ce may influence the expression of collagen and lead to its accumulation as seen in EMF. However, the effects of Ce *in vivo* remain to be probed.

# **DESIGN OF THE STUDY**

**Section A - MATERIALS**

## **Fine Chemicals**

Medium M-199, Cerium chloride, Calf-thymus DNA, Bovine serum albumin, Nitroblue tetrazolium, Phenazine methosulfate, Nicotinamide adenine dinucleotide, Sodium pyrophosphate, Mg and Ca standards for atomic absorption spectrometry were obtained from Sigma Chemical Company, St. Louis, MO, USA.

## **Routine Chemicals**

Ammonium alum, Ammonium sulfate, Alanine, Boric acid, Calcium chloride, Charcoal, Chloramine - T, Copper sulfate, D- dextrose, DPX, Diphenylamine, Eosin, Fastgreen FLF, Glass slide, Hydroxyproline, Hematoxylin, Lanthanum oxide, Magnesium sulfate, Ninhydrin, Naphthalene, L-phenylalanine, Potassium chloride, Potassium dihydrogen phosphate, p-dimethyl aminobenzaldehyde, Potassium alum, L-proline, Potassium hydroxide, PPO, POPOP, Sodium bicarbonate, Sodium acetate, Sodium chloride, Sucrose, Sodium carbonate, Potassium-Sodium tartrate, Sodium thiosulfate, Trichloroacetic acid, Thiobarbituric acid and Wax were obtained from Sisco Research Laboratories, India.

## **Solvents :**

Benzene, n-butanol, Chloroform, conc . HCl, conc.  $H_2SO_4$ , conc.  $HClO_4$ , 1-4 dioxane, Ethanol, Ether, Ethylacetate, Folin-Ciocalteau reagent, Glacial acetic acid, Isopropanol, Methanol, Orthophosphoric acid, Phosphotungstic acid, Triton X-100 and Toluene were obtained from Sisco Research Laboratories, India.

## **Radiochemicals**

$^3H$ -L-phenylalanine (Specific activity 6400mCi/mmol)  $^3H$ -L-proline (Specific activity 7500mCi/mmol)  $^3H$ -thymidine (Specific activity 17,000 mCi/mmol ) and  $^3H$ -Uridine ( Specific activity, 16,000 mCi/mmol) were from Bhabha Atomic Research Center, India.

## **Animal feed**

Animal feed, based on the nutrient requirement of rats proposed by the American Institute of Nutrition, was purchased from Zeigler Bros. Inc., USA. Mg- sufficient (Control), - deficient and - restricted diets contained 0.0515%, 0.0008% and 0.012% Mg respectively . The composition of the experimental rat diet is given in Table 1.

## **Instruments used**

Liquid scintillation counter (Wallac, 1409), Atomic absorption spectrometer (IL-551, USA), UV-Visible spectrophotometer (Shimadzu, Japan) ICP-MS (Perkin-Elmer SCIEX, ELAN 250, SCIEX, Canada), High speed refrigerated centrifuge (Hitachi, Japan), weighing balance (Sartorius, Germany), Water - bath (LKB, Sweden), Ice-machine (Hoshizaki, Japan), pH meter (Elico, India), Tissue homogeniser (Remi, India), Microtome (Leica, Germany).

## Composition of rat feed

TABLE 1

Cellulose	-	50.0 g
Casein lactate	-	20.0 g
Corn Starch	-	15.0 g
Cellulose	-	5.0 g
Fat, Corn oil	-	5.0 g
American Institute of Nutrition vitamin mix	-	1.0 g
American Institute of Nutrition mineral mix	-	3.5 g
Dimethionine	-	0.3 g
Choline bitartrate	-	0.2 g

## **Section B - EXPERIMENTAL DESIGN**

In keeping with the objectives, this study consisted of a long-term experiment and several short-term experiments. The experimental design was as follows:

### **I. Long - term experiment**

The aim was to look for histological changes in the heart, if any, in response to Ce and dietary deficiency of Mg.

Sprague - Dawley rats of both sexes (approximately equal in number) and weighing  $55 \pm 5$ g, were randomly distributed into four experimental groups following a  $2 \times 2$  factorial design. Animals in Groups A and B were fed a Mg-sufficient diet (containing 0.0515 % Mg) while those in Groups C and D were fed a Mg - restricted diet (containing 0.012 % Mg). Cerium chloride dissolved in double distilled water to a concentration of 35 ppm of Ce was given to Groups B & D, while Groups A and C were given double distilled water. Feed and water were provided *ad libitum*.

Group A + Mg

Group B + Mg + Ce

Group C - Mg

Group D - Mg + Ce

At thirteen months, the animals were killed under ether anaesthesia and cardiac tissue was collected for elemental analysis, determination of collagen concentration and histology. Femur bone was removed, cleared of blood and

muscle and used for elemental analysis. Blood was collected at sacrifice and serum was separated for elemental analysis. Levels of Mg and Ca were determined by atomic absorption spectrometry and Ce concentrations in the cardiac tissue and bone were determined by inductively coupled plasma-mass spectrometry (at IGCAR, Kalpakkam, Madras) as described in Methods. Determination of collagen concentration and processing of tissues for histology were carried out as described in Methods.

## **II. Short-term experiments**

The overall aim of these experiments was to delineate the molecular pathogenesis of cardiac muscle damage and fibrosis produced by Mg deficiency and Ce.

### **i. Effects of acute Magnesium deficiency on lipid peroxidation and cardiac fibrogenesis in rat heart**

Sprague-Dawley rats (M:F, 1:1) weighing approximately  $110 \pm 5$  g were used for the study. They were divided into control and deficient groups after 2 days on control feed. The rats were pair-fed a Mg-sufficient feed (containing 0.0515 % Mg); or a Mg-deficient feed (containing 0.0008 % Mg) for 12, 21, 28, 60 or 80 days as indicated in the Tables. The animals

were provided double distilled water *ad libitum*.

At sacrifice, blood was collected by cardiac puncture and serum separated for Mg and Ca analysis; the cardiac tissue obtained was also processed for elemental analysis as described in Methods.

Parameters of oxidative stress in terms of TBARS levels in serum and cardiac tissue and activity of antioxidant enzymes, SOD and catalase, in cardiac tissue and SOD activity in plasma of Mg- sufficient and Mg- deficient rats were studied as described in Methods.

Studies on collagen metabolism addressing fractional rates of collagen synthesis, % degradation of newly synthesised collagen, collagen deposition rates and total collagen concentration in heart were carried out as described in Methods.

Fibroblast proliferation in response to Mg deficiency was measured *in vivo* as described under Methods.

## **ii. Effect of Cerium administered *in vivo*, on lipid peroxidation and fibrogenesis in rat heart**

Female Sprague - Dawley rats weighing approximately  $200 \pm 5$  g were used for these experiments. All animals were fed a standard rat chow. Rats were administered a

single dose of  $\text{CeCl}_3$  (1.3 mg Ce/Kg body weight, a dose which is well below the  $\text{LD}_{50}$  but sufficient to induce fatty liver in female rats) intravenously through the tail vein. Control rats received an equal volume of physiological saline. Rats were sacrificed 24 hours or 48 hours after administration of  $\text{CeCl}_3$ .

TBARS levels in serum and cardiac tissue, fractional rates of collagen synthesis, % degradation of newly synthesised collagen, deposition rates of newly synthesised collagen, rates of total protein synthesis and transcription and fibroblast proliferation were measured as described in Methods.

## **Section C - METHODS**

## 1. Elemental analysis

### i. Determination of Magnesium and Calcium levels by atomic absorption spectrometry

#### a. Serum

Serum was diluted 1:100 times with 0.05% lanthanum solution for Mg and Ca determinations.

#### b. Heart and bone

Tissue obtained at sacrifice was washed to remove blood and dried to constant weight at 100°C. Approximately, 50 mg of heart tissue and 20 mg of bone were then subjected to acid digestion first with conc.  $\text{HNO}_3$  and then with conc.  $\text{HNO}_3$ :  $\text{HClO}_4$  (1:1) in a fume-hood. The digested material was evaporated to near dryness and made up to 5 ml with 0.5 % lanthanum solution.

For Mg, the acid digested samples were diluted 1:25 times for heart and 1:100 for bone with 0.5 % lanthanum solution.

For Ca, the acid digested samples were diluted 1:5 times for heart and 1:100 for bone with 0.5 % lanthanum solution.

Standard solutions of Mg and Ca were obtained from Sigma Chemical Company. Air : acetylene mixture was used as fuel. Wavelength settings were 285.2nm for Mg and 422.7 nm for Ca. Citrus leaves containing known amounts of Mg and Ca were processed as above for analysis and used as reference. All solutions were prepared in deionised double distilled water and all glassware thoroughly rinsed with deionised double distilled water before use.

**ii. Determination of Cerium levels by inductively-coupled plasma mass spectrometry**

Approximately, 50 mg of heart tissue and 20 mg of bone were subjected to acid digestion as described above. The digested material was evaporated to near dryness and made up to 5 ml with 0.5 N HNO<sub>3</sub>.

Cerium concentration was determined by inductively-coupled plasma mass spectrometry (at IGCAR, Kalpakkam, Madras). The acid-digested samples were diluted 1:5 for heart and bone. Argon was used as fuel. Solutions containing known amounts of Ce were used as standard.

## 2. Histology

### Fixation, processing and staining of tissue sections

The cardiac tissue was fixed in 10 % formalin. Thin sections ( 5  $\mu$  thickness) were cut on microtome. Hematoxylin - eosin staining and Masson's trichrome staining were carried out following standard procedures (122).

## 3. Metabolic Studies

### i. Studies on collagen metabolism *in vivo*

#### a. Measurement of rates of collagen synthesis

Rates of collagen synthesis were measured *in vivo* exactly as described by Mays *et al* (123). The animals were injected  $^3\text{[H]}$ - L- proline at a dose of 0.1  $\mu\text{Ci/g}$  body weight along with a flooding dose of unlabelled proline (1.4 mmol/100g body wt.) in phosphate - buffered saline intraperitoneally. Animals were killed 60 minutes later. The heart was removed and washed with ice- cold saline to remove blood.

The tissue was homogenized in ice- cold phosphate- buffered saline. An equal volume of ice- cold 10 % trichloroacetic acid (TCA) was added to precipitate the proteins. The precipitate was washed

thrice with 5 % TCA, twice with ethanol: ether (1:1) and finally with ether. The pellet was dried overnight. The TCA supernatant was also collected.

The proteinaceous pellet and TCA supernatant were hydrolyzed overnight in 6N HCl and the hydrolysate decolourised with charcoal. The hydroxyproline content, total radioactivity and specific radioactivity of hydroxyproline in proteinaceous material and TCA supernatant as well as the proline - associated specific radioactivity in TCA - supernatant were determined.

Collagen fractional synthesis rates ( $k_s$  %/day) were calculated using the equation (3)

$$K_s = \frac{S_B}{S_A \times t} \times 100$$

where  $S_B$  is the specific radioactivity of total hydroxyproline in both proteinaceous material and in the tissue-free pool,  $S_A$  is the specific radioactivity of proline in the tissue-free pool (precursor pool for protein synthesis) and 't' time (in days) between injection and death.

**b. Determination of %degradationof newly synthesised collagen**

Degradation of newly synthesised collagen (%) was calculated from the relative proportion of hydroxyproline in tissue free pool and proteinaceous fraction (124).

**c. Determination of deposition rate of newly synthesised collagen**

Deposition rate of newly synthesised collagen was determined by the difference between the fractional synthesis rate and the proportion of collagen degraded rapidly after synthesis (125).

**d. Estimation of hydroxyproline content & radioactivity associated with hydroxyproline**

Hydroxyproline content in acid hydrolysate was determined by the procedure of Kivirikko *et al* (126). Briefly, the acid hydrolysate was subjected to chloramine - T oxidation, and the product of hydroxyproline oxidation namely,  $\alpha$  pyrroline-4-hydroxy-2-carboxylic acid and pyrrole-2-carboxylic acid extracted into toluene.

An aliquot of the toluene extract was used for the colour reaction with p-dimethylamino benzaldehyde (Ehrlich's reagent) and optical density measured at 560 nm. The amount of hydroxyproline was calculated from the standard curve obtained by processing known amounts of hydroxyproline through the steps described above.

Radioactivity associated with hydroxyproline was determined using a second aliquot of the toluene extract.

**e. Estimation of proline content and radioactivity associated with proline**

Proline content of TCA supernatant was determined following the procedure of Troll and Lindsley (127). Briefly, the acid hydrolysate was heated with glacial acetic acid and ninhydrin reagent in a boiling water-bath for 1 hour. The chromogen formed was extracted into benzene and optical density was measured at 515 nm. The amount of proline was determined from a standard curve obtained by treating known concentrations of proline as above. Radioactivity associated with

proline was determined using an aliquot of the benzene extract.

#### **f. Determination of collagen concentration**

Collagen concentration in rat heart was calculated from the hydroxyproline content obtained during the analysis of collagen synthesis and was expressed as the amount of collagen/g wet weight of tissue (125).

#### **ii. Measurement of rates of DNA synthesis**

DNA synthesis was measured by the method of Tan *et al* (128)  $^3\text{[H]}$ -thymidine was administered intraperitoneally at  $0.1 \mu\text{Ci/g}$  body weight 1 hour before sacrifice. The heart was excised, weighed, minced and homogenised in 2 ml ice - cold phosphate- buffered saline. 2 ml of ice - cold 10 % TCA was added to the homogenate to precipitate DNA and proteins. The pellet was then washed three times with ice-cold TCA and then with 2% sodium acetate in 90 % alcohol to remove unincorporated thymidine. After centrifugation, the supernatant was discarded and the precipitate was resuspended in 2 ml of 5 % TCA. DNA was extracted by heating the samples at  $90^\circ \text{C}$  for 30 mins. Following

centrifugation, aliquots of the supernatant were used for determination of radioactivity. DNA content was determined by the method of Burton (129). Rates of DNA synthesis in the heart were expressed as cpm/g tissue. Concentration of DNA was expressed as mg DNA/g tissue.

### **iii. Measurement of RNA synthesis**

Rates of RNA synthesis in the heart tissue were measured as described elsewhere (130). Briefly, cardiac tissue slices were incubated in M-199 for 2 hours with  $^3\text{[H]}$ -uridine at  $2\mu\text{ Ci/ml}$ . Tissue slices were washed, homogenised and an equal volume of ice-cold 10 % TCA was added to the homogenate. After centrifugation at 10,000 g for 10 minutes, the pellet was washed twice with ethanol: ether (1:1) and finally with ether and dried overnight. The dried pellet was dissolved in 0.2 N NaOH. Aliquots were used for protein estimation by Lowry's method (131) and determination of radioactivity by liquid scintillation counting.

### **iv. Measurement of rates of protein synthesis in cardiac tissue slices**

Rates of protein synthesis were measured as

described by Robert *et al* (132) with slight modifications. Briefly, rats were killed 24 h after injection of  $\text{CeCl}_3$ . Heart was removed and placed in a solution containing (mM)  $\text{CaCl}_2$  2.5,  $\text{KCl}$  4.7,  $\text{MgSO}_4$  1.2,  $\text{KH}_2\text{PO}_4$  1.1,  $\text{NaHCO}_3$  24.0, Na acetate 10.0,  $\text{NaCl}$  98.0, dextrose 10.0 and L-phenylalanine 0.4. This solution was equilibrated with 95 %  $\text{O}_2$  and 5 %  $\text{CO}_2$  and a resultant pH of 7.4 at 37°C. Thin slices of heart were removed and placed in medium M-199 with Earle's salts containing 20 amino acids and additional vitamins and minerals but no serum or other mitogens. Also added to this muscle superfusate were  $\text{NaHCO}_3$  24 mM, Na acetate 10mM, dextrose 10mM, and unlabelled L-phenylalanine 0.4 mM to ensure rapid equilibration of extracellular phenylalanine with that in the intracellular pools (2). Finally,  $^3\text{H}$ -L-phenylalanine was added at 0.4  $\mu\text{Ci/ml}$  and incubation carried out for 45 mins. For measurement of kinetics of incorporation, tissue slices were incubated with  $^3\text{H}$ -L-phenylalanine at 0.4 $\mu\text{Ci/ml}$  for 0, 30,60,90,120, 150, and 180 minutes.

After incubation, tissue slices were homogenised and an equal volume of ice-cold 10% trichloroacetic

acid (TCA) was added to precipitate proteins. The pellet was processed for determination of radioactivity as described elsewhere. (121). Briefly, the pellet was washed four times with ice-cold 5 % TCA, heated in trichloroacetic acid for 20 mins at 80°C to remove tRNA - bound <sup>3</sup>[H]- phenylalanine, cooled and washed once with ethanol; ether (1:1) and finally with ether. The final pellet was dried overnight and dissolved in 0.2 N NaOH. An aliquot was used for protein estimation by Lowry's method (131). Rates of protein synthesis were expressed as pmoles of phenylalanine incorporated/mg protein.

## **v. Studies on oxidative damage to the myocardium**

### **a. Measurement of lipid peroxidation**

Lipid peroxidation was assessed by the thiobarbituric acid reaction, following the method of Chandra *et al* (133). Ice-cold TCA was added to the tissue (heart) homogenate or serum to a final concentration of 10 %. The samples were kept in ice for 10 mins, spun at 600g and the supernatant was collected. 1 ml of 0.67 % (w/v) thiobarbituric acid (pH 7.5) was added to an equal volume of the TCA

supernatant. The tubes were shaken well, heated on a boiling water-bath for 10 minutes and cooled under tap water. Absorbance was then measured at 535 nm. The amount of malondialdehyde formed was calculated from the molar extinction coefficient of malondialdehyde ( $1.56 \times 10^5$  /M per cm) and expressed as nmol malondialdehyde per gram tissue wet weight or as mmol malondialdehyde/100ml serum.

## **b. Measurement of superoxide dismutase activity**

### **i. in Plasma**

SOD activity was measured according to the method of Kakkar *et al* (134). The reaction mixture contained, in a total volume of 2 ml, 0.02  $\mu$  mol of sodium pyrophosphate (pH 8.2), 0.019  $\mu$  mol of phenazine methosulfate, 0.09  $\mu$  mol of nitroblue tetrazolium, enzyme protein (plasma) and 0.3 $\mu$  mol of NADH. After 90 seconds, 1 ml of glacial acetic acid was added. In the control tubes, glacial acetic acid was added before NADH. The chromogen formed was extracted into isobutanol and optical density read at 560 nm. Protein was estimated by the procedure of

Lowry *et al* (131). SOD activity was expressed as units/mg protein.

## **ii. in the cardiac tissue**

Tissue (heart) was homogenised in phosphate buffer (pH 7.0) and the homogenate was centrifuged at 12,000 g for 10 minutes. To the supernatant, ammonium sulfate was added to a final concentration of 90% and the tubes were kept in ice for 10 minutes. Following centrifugation at 12,000 g for 10 minutes, the pellet was discarded and supernatant again centrifuged at 40,000 g for 10 minutes. This supernatant was then dialysed overnight against 0.02 M Tris HCl (pH 7.3) with 3 changes of buffer. The dialysate was used for SOD assay as described for plasma and specific activity was expressed as units/mg protein. One unit of enzyme activity is defined as enzyme concentration required to inhibit the optical density, at 560 nm, of chromogen produced by 50% in one minute under assay conditions.

## **c. Measurement of catalase activity in the heart**

Catalase activity in the heart was assayed according to the method of Sinha (135). The reaction mixture

contained, in a volume of 1 ml, 5 mmol phosphate buffer (pH 7.0), 0.08 mmol of  $H_2O_2$  and enzyme protein (tissue homogenate). Reaction with catalase was followed by disappearance of  $H_2O_2$  at 240nm. Activity was expressed as units/mg protein. (Unit = Velocity constant/Sec).

#### **4. Statistical analysis**

Statistical analysis of data was done using Student's 't' test.  $p < 0.05$  was taken to be significant.

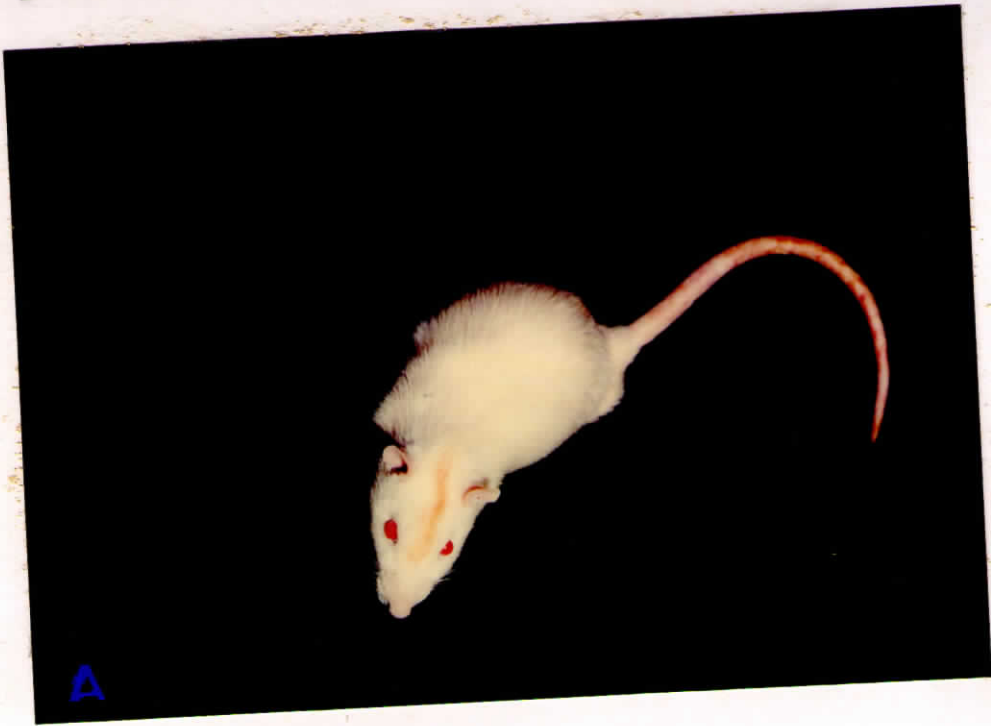
# RESULTS

Although the clinical and pathological features of EMF have received much attention, fundamental to an understanding of the disease is the pursuit of clues to its aetiology which still remains a matter of speculation. This study examined the geochemical hypothesis that envisages a role for Mg deficiency and Ce in the causation of tropical EMF. The focus is on histological changes and changes in collagen metabolism in rat heart.

### **I. Investigations into myocardial injury induced by Magnesium deficiency and Cerium**

Animal experiments were initiated (refer Design of the study Section B-I for experimental design) to induce Mg deficiency in rats through dietary means and to study the effects of Ce supplementation to these animals. This section deals with changes in elemental levels in the serum and heart, histological changes in the heart and changes in cardiac collagen concentration in response to dietary deficiency of Mg and Ce administration.

Animals belonging to Groups C (-Mg) and Group D (-Mg + Ce) exhibited severe skin manifestations (Fig 2). Animals in Group D developed a yellowish colouration of the skin.





**Figure 2 : Photographs of rats**

A - Group A (+Mg)

B - Group B (+Mg +Ce)

**Figure 2: Photographs of rats**

C - Group C (- Mg)

D - Group D (- Mg + Ce)

## **i. Elemental analysis**

### **a. Serum**

Changes in serum levels of Mg and Ca are shown in Table 2.

1. Serum Mg decreased by 21 % ( $p < 0.01$ ) and 40% ( $p < 0.001$ ) in Groups C and D respectively, compared to initial values.
2. Serum Ca was 15 % ( $p < 0.001$ ) and 12 % ( $p < 0.01$ ) higher in Groups C and D respectively, compared to initial values. These observations are in agreement with earlier reports that in rats, hypomagnesemia is associated with hypercalcemia (136).

### **b. Heart**

#### **1. Magnesium**

Though not statistically significant, a small difference in cardiac tissue levels of Mg was observed between Groups A and C. Cardiac Mg levels in Group D were 26% lower ( $p < 0.01$ ) than in Group A (Table 3).

#### **2. Calcium**

Calcium levels in the heart were higher by

TABLE 2  
SERUM MAGNESIUM AND CALCIUM LEVELS

Element	Group A		Group B		Group C		Group D	
	Initial	Final	Initial	Final	Initial	Final	Initial	Final
Magnesium (mg/100 ml)	2.09 ± 0.1 (5)	2.45 ± 0.2* (5)	2.15 ± 0.3 (8)	2.28 ± 0.2 (8)	2.22 ± 0.3 (8)	1.76 ± 0.3* (8)	2.26 ± 0.3 (8)	1.35 ± 0.2 <sup>Δ</sup> (8)
Calcium (mg/100ml)	10.50 ± 0.7 (5)	10.75 ± 0.4 (5)	10.55 ± 0.4 (8)	10.99 ± 1.3 (8)	10.60 ± 0.4 (8)	12.19 ± 0.7 <sup>Δ</sup> (8)	11.18 ± 0.7 (8)	12.43 ± 0.7* (8)

Values are expressed as mean ± SD

Numbers in brackets represent number of animals used for the experiment

Initial value Vs Final value \*p < 0.01, <sup>Δ</sup>p < 0.001

61% ( $p < 0.001$ ) and 70% ( $p < 0.001$ ) in Groups C and D respectively, compared to Group A (Table 3).

### **3. Cerium**

It is known that gastrointestinal absorption of orally administered Ce is poor but is enhanced during dietary deficiency states(13). Ce levels in the cardiac tissue (Table 4) of Ce-treated rats were  $0.86 \pm 0.33$  ng/mg dry tissue and  $2.895 \pm 0.51$  ng/mg dry tissue in Group C (+Mg+Ce) and Group D (-Mg + Ce) respectively. This shows that Mg deficiency enhances Ce levels in the heart. However, even without Mg deficiency, Ce was found to be deposited in the heart.

#### **c. Bone**

##### **1. Magnesium**

Compared to Groups A and B, Mg levels in bone were 14% ( $p < 0.001$ ) and 25% ( $p < 0.001$ ) lower in Groups C and D respectively. (Table 3).

##### **2. Calcium**

Calcium levels in bone were higher in Groups B, C and D compared to Group A (Table 3).

TABLE 3

TISSUE LEVELS OF MAGNESIUM AND CALCIUM ( $\mu\text{g}/\text{mg}$  DRY TISSUE)

Tissue	Element	Group A	Group B	Group C	Group D
Heart	Magnesium	$0.898 \pm 0.15$ (6)	$0.963 \pm 0.07$ (8)	$0.827 \pm 0.04$ (9)	$0.663 \pm 0.04^*$ (8)
	Calcium	$0.574 \pm 0.07$ (6)	$0.509 \pm 0.05$ (9)	$0.924 \pm 0.09^{\Delta}$ (9)	$0.977 \pm 0.05^{\Delta}$ (8)
Bone	Magnesium	$3.530 \pm 0.25$ (5)	$3.690 \pm 0.28$ (8)	$3.030 \pm 0.09$ (9)	$2.660 \pm 0.22^{\Delta}$ (8)
	Calcium	$227.9 \pm 34.0$ (6)	$334.7 \pm 18.5$ (8)	$287.7 \pm 8.8^{\Delta}$ (7)	$305.6 \pm 4.3^{\Delta}$ (8)

Values are expressed as mean  $\pm$  SD

Numbers in brackets represent number of animals used for the experiment

Values compared to Group A \* $p < 0.01$ ,  $\Delta p < 0.001$

TABLE 4

## TISSUE LEVELS OF CERIUM (ng/mg DRY TISSUE)

Tissue	Group A	Group B	Group C	Group D
Heart	0.065 ± 0.07 (6)	0.860 ± 0.33 (9)	0.184 ± 0.15 (8)	2.895 ± 0.51 <sup>△</sup> (8)
Bone	0.458 ± 0.25 (6)	0.864 ± 0.86 (7)	0.350 ± 0.24 (7)	13.780 ± 1.87 <sup>△</sup> (8)

Values are expressed as mean ± SD

Numbers in brackets represent number of animals used for the experiment

Group D Vs Group B <sup>△</sup>p < 0.001

### **3. Cerium**

In the bone, Ce levels were significantly higher in rats on Mg-deficient diet compared to those in Group B ( Table 4 ).

#### **ii. Histology**

##### **a.. Gross changes**

No gross changes were observed in the heart in rats in any of the 4 groups.

##### **b. Histological changes**

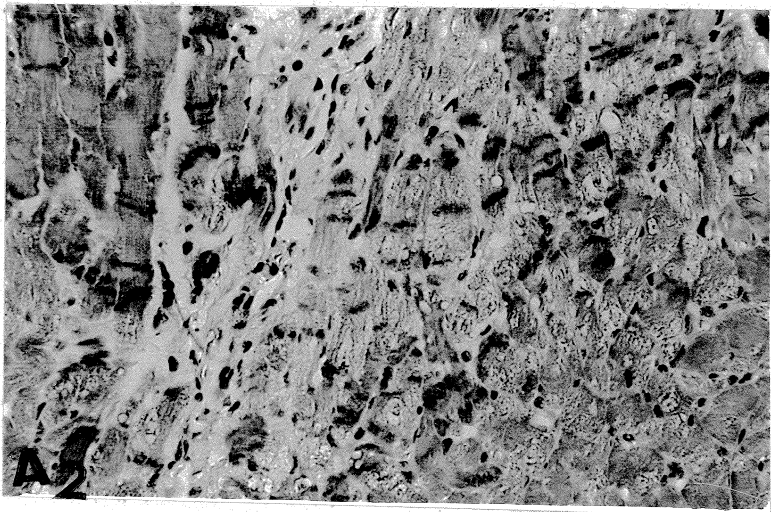
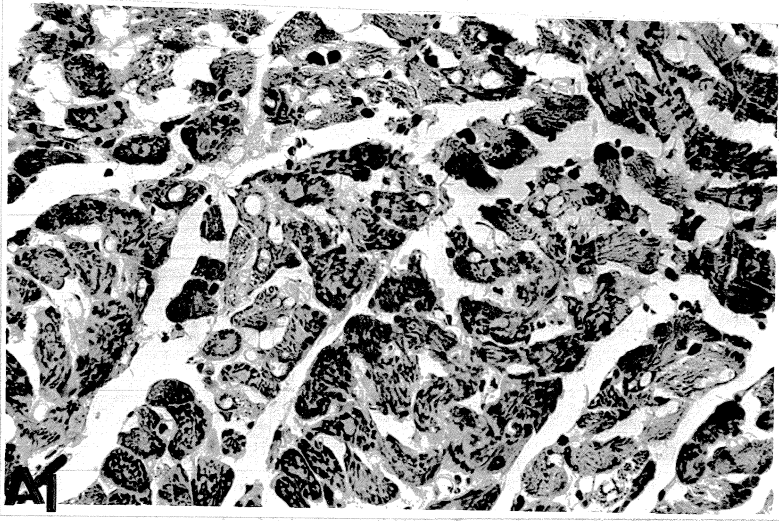
Histological changes in the heart in response to a Mg-deficient diet and Ce administration are shown in Fig 3 (A-D).

The lesions included myocytolysis, contraction bands, myofibrillar lysis, interstitial fibrosis and foci of scarring, particularly in the subendocardial myocardium and one animal in Group D had scarring around an intramyocardial artery. Animals in Group A did not manifest such histological changes.

Qualitatively, the lesions, as described above, were comparable in Groups B (+ Mg + Ce), C (- Mg) and D (- Mg +Ce); but 3 out of 9 animals in Group B, 2 out of 9

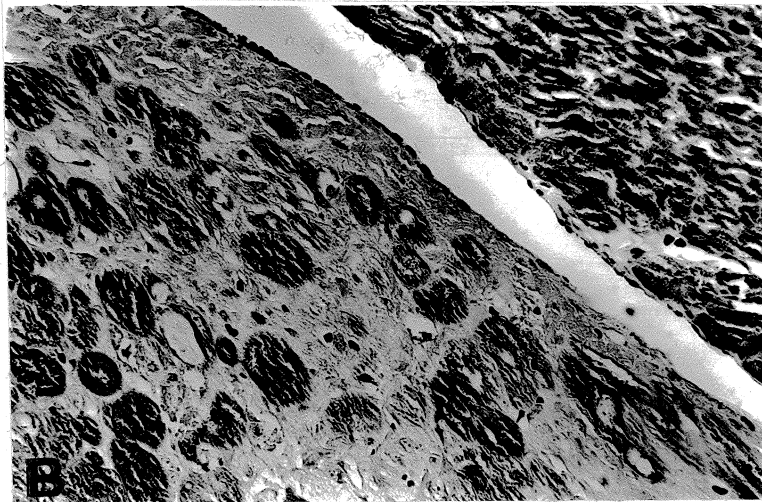
**Figure 3 : Photomicrograph of cardiac lesion in experimental animals**

A. Myocytolysis (A1), contraction bands and myofibrillar lysis (A2) are seen in subendocardial region. Haematoxylin and Eosin x 200.



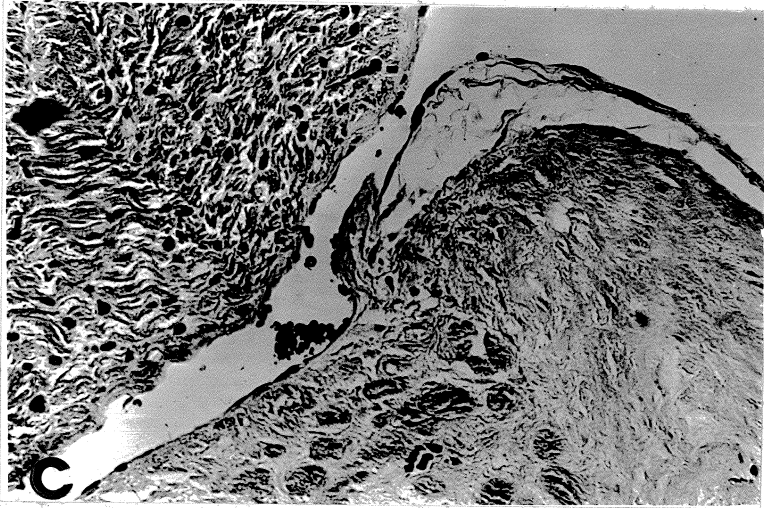
**Figure 3 : Photomicrograph of cardiac lesion in experimental animals**

B. Intermyofibre fibrosis in the myocardium. Some of the myofibres entrapped appear atrophic. Masson's trichrome x 240.



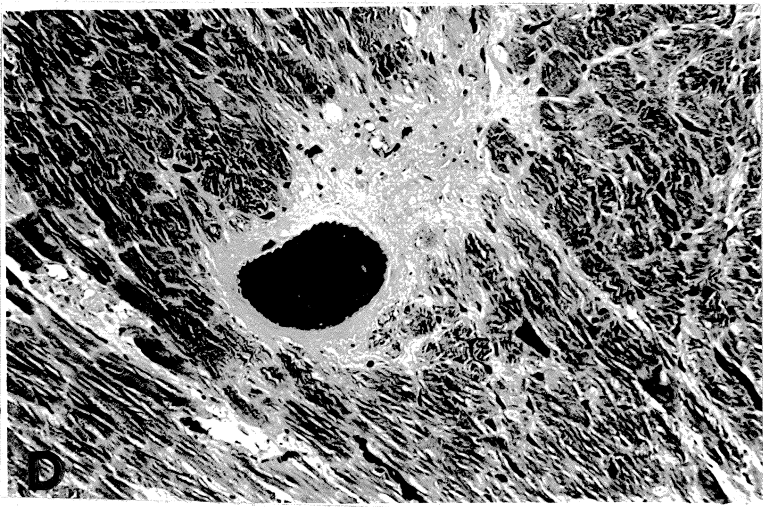
**Figure 3 : Photomicrograph of cardiac lesion in experimental animals**

- C. Endocardial fibrosis with tongues of fibrous tissue creeping in between myofibres into subendocardial myocardium.  
Masson's trichrome x 120.



**Figure 3 : Photomicrograph of cardiac lesion in experimental animals**

D. An area of scarring around an intramyocardial artery.  
Masson's trichrome x 240.



in Group C and 4 out of 8 in Group D had such histological changes. Thus, the number of animals with histological changes were more in Group D than in Group B or C. Although Mg deficiency is known to produce a cardiomyopathy, an important observation made in this study is that Ce *per se* produces cardiac lesions (Group B).

### **iii. Collagen concentration**

With histologic evidence of fibrosis, the collagen concentration in rat heart in the 4 groups was determined. Results presented in Table 5 show a 15 % ( $p < 0.001$ ), 18 % ( $p < 0.001$ ) and 33 % ( $p < 0.001$ ) increase in cardiac collagen concentration in Groups B, C and D respectively, compared to Group A, which is in agreement with the histological findings of cardiac fibrosis in these animals.

Interestingly, changes in many of the parameters studied i.e., Mg and Ca levels in serum, heart and bone, Ce levels in heart and bone, histology and concentration of cardiac collagen were more pronounced in Group D than in Group B or C, implying synergistic effects of Ce and Mg deficiency or potentiation of the effects of Ce in Mg deficiency.

## II. Investigations on the molecular basis of myocardial injury induced by Magnesium deficiency and Cerium

### i. Myocardial injury by Magnesium deficiency

It has been known for a long time that Mg deficiency produces a cardiomyopathy characterised by focal myocardial necrosis and fibrosis. Various theories have been put forward to explain the pathobiology of cardiac muscle damage in Mg deficiency. Using an experimental model of acute Mg deficiency, it has been shown (108-113) that Mg deficiency induces a pro-oxidant, pro-inflammatory state marked by elevated circulating levels of factors like TNF- $\alpha$  and Substance P which are known to trigger free radical generation. A role for these factors in promoting oxidative injury in the heart in Mg deficiency seems plausible and is supported by the observation that inhibition of Substance P receptor resulted in attenuation of the indices of oxidative stress *in vivo* (112). While these observations point to the possibility of oxidative damage to the cardiac muscle in Mg deficiency, the molecular basis of cardiac fibrogenesis associated with Mg deficiency remains unclear in the absence of investigations on changes in collagen metabolism.

Against this backdrop, this study examined the biochemical correlates of cardiac muscle damage and fibrosis associated with Mg deficiency in the rat model of acute Mg deficiency (Refer Design of the study Section B-II (i) for experimental design).

**a. Signs of Magnesium deficiency**

Peripheral vasodilatation and hyperemia of the ears were noted regularly in the Mg-deficient animals. These changes appeared between Days 12 and 20 of the regimen of Mg depletion and persisted for varying periods (1-4 days). A few of the Mg-deficient animals exhibited severe skin manifestations. The fur between the ears and around the eyes were completely denuded and blood stained skin lesions could be seen which were replaced by necrotic hemorrhagic scabs. None of the rats developed convulsions. There was no difference in weight gained during the study period between the control and deficient groups and all rats survived to the day of termination.

## **b. Changes in electrolyte levels**

### **1. Serum levels of Magnesium and Calcium**

Serum levels of Mg and Ca are shown in Table 6. All the deficient rats developed severe hypomagnesemia, defined as serum Mg concentration less than or equal to 1.4 mg/100 ml serum (1.24 mEq/L) (80). Animals on Mg-deficient diet had significantly lower serum levels of Mg by Day 12 i.e., a 65 % decrease ( $p < 0.001$ ) compared to controls and remained so till Day 21. Subsequently, there was a slight increase in Mg levels, although it remained significantly lower than control values till Day 80.

Serum Ca levels were significantly higher by 28% ( $p < 0.001$ ) in the Mg-deficient animals from Day 12 of the study and continued to increase progressively, as the severity of Mg deficiency increased. This observation is in line with earlier reports that in rats hypomagnesemia leads to hypercalcemia (136).

### **2. Tissue levels of Magnesium and Calcium**

Results presented in Table 7 show the levels of

TABLE 7  
 CHANGES IN LEVELS OF MAGNESIUM AND CALCIUM IN HEART  
 IN MAGNESIUM-DEFICIENT RATS

Days on deficient diet	Magnesium ( $\mu\text{g}/\text{mg}$ dry tissue)		Calcium ( $\mu\text{g}/\text{mg}$ dry tissue)	
	Control	Deficient	Control	Deficient
Day 12	1.063 $\pm$ 0.08 (4)	1.101 $\pm$ 0.10 (4)	0.864 $\pm$ 0.12 (4)	0.825 $\pm$ 0.03 (4)
Day 28	1.082 $\pm$ 0.06 (4)	1.088 $\pm$ 0.06 (4)	0.806 $\pm$ 0.06 (4)	0.801 $\pm$ 0.06 (4)
Day 60	1.144 $\pm$ 0.07 (4)	1.168 $\pm$ 0.02 (4)	0.780 $\pm$ 0.01 (4)	0.956 $\pm$ 0.20 <sup>A</sup> (4)
Day 80	1.131 $\pm$ 0.01 (4)	1.055 $\pm$ 0.09 (4)	0.798 $\pm$ 0.02 (4)	1.058 $\pm$ 0.13 <sup>A</sup> (4)

Values are expressed as mean  $\pm$  SD

Numbers in brackets represent number of animals used for the experiment

Control Vs Deficient <sup>A</sup>p < 0.001

Mg and Ca in the cardiac tissue of rats fed Mg-deficient diet for different time intervals. There was no change in the level of Mg in the heart as deficiency progressed. A 23 % increase in myocardial calcium level was observed by Day 60 which became more marked by Day 80 (33 % increase,  $p < 0.001$ ), compared to control values.

### **c. Studies on oxidative stress and anti-oxidant enzyme activity**

#### **1. Lipid peroxidation**

A marked elevation in the amounts of TBARS in the serum and cardiac tissue of Mg-deficient rats was observed on Days 28 and 60, compared to control. In serum, the increase in TBARS was about 97 % ( $p < 0.001$ ) and 66% ( $p < 0.001$ ) on Days 28 and 60 respectively (Table 8). The levels of TBARS in the heart tissue increased by about 7 % ( $p < 0.05$ ) and 39% ( $p < 0.05$ ) on Days 28 and 60 of deficiency respectively (Table 8).

TABLE 8

CHANGES IN LEVELS OF TBARS IN SERUM AND HEART  
OF MAGNESIUM-DEFICIENT RATS

Days on deficient diet	Serum ( $\mu$ mol MDA/100ml serum)		Heart (nmol MDA/g tissue weight)	
	Control	Deficient	Control	Deficient
Day 28	1.55 $\pm$ 0.3 (4)	3.06 $\pm$ 0.4 <sup><math>\Delta</math></sup> (4)	271.99 $\pm$ 9.8 (4)	291.28 $\pm$ 5.2 <sup>+</sup> (4)
Day 60	1.79 $\pm$ 0.1 (4)	2.98 $\pm$ 0.2 <sup><math>\Delta</math></sup> (4)	312.68 $\pm$ 46 (10)	434.66 $\pm$ 58 <sup>+</sup> (10)

Values are expressed as mean  $\pm$  SD

Numbers in brackets represent number of animals used for the experiment

Control Vs Deficient <sup>+</sup>p < 0.05,  <sup>$\Delta$</sup> p < 0.001

## **2. Activity of anti-oxidant enzymes**

Anti-oxidant enzymes SOD and catalase play an important role in protecting tissues from damage induced by free radicals. The observed increase in levels of TBARS in serum and heart prompted further investigations into the activity of these protective enzymes. The activities of SOD in plasma and heart and catalase in heart were measured in rats fed Mg-deficient diet for 21 days. SOD activity decreased by 52% ( $p < 0.001$ ) and 30% ( $p < 0.001$ ) in plasma and heart respectively (Table 9). It was observed that catalase activity (Table 10) in the heart in the test group decreased by 26% ( $p < 0.001$ ). The decreased anti-oxidant enzyme activity is consistent with the observed increase in TBARS levels on Days 28 and 60.

### **d. Changes in collagen metabolism *in vivo* in Magnesium deficiency**

A direct response to cardiac tissue injury is the formation of scar tissue following deposition of collagen. Cardiac collagen synthesis is sensitive to a variety of pathophysiologic stimuli. Although this may

TABLE 9

ACTIVITY OF SOD IN PLASMA AND HEART

Group	Plasma (units/mg protein)	Heart (units/mg protein)
Control	2.32 ± 0.2 (4)	14.29 ± 0.5 (4)
Mg-deficient	1.12 ± 0.1 <sup>o</sup> (4)	9.96 ± 0.7 <sup>o</sup> (4)

Values are expressed as mean ± SD

Numbers in brackets represent number of animals used for the experiment

Control Vs Deficient °p < 0.001

Rats were fed a Magnesium-deficient diet for 21 days

TABLE 10

## ACTIVITY OF CATALASE IN THE HEART

Group	Heart (x 10 <sup>-3</sup> units/mg Protein)
Control	10.65 ± 0.8 (4)
Mg-deficient	7.88 ± 0.9 <sup>Δ</sup> (4)

Values are expressed as mean ± SD  
Numbers in brackets represent number of  
animals used for the experiment  
Control Vs Deficient <sup>Δ</sup>p < 0.001  
Rats were fed a Magnesium-deficient diet for  
21 days

reflect adaptive changes, the alterations may lead to patho-physiologic abnormalities.

Studies on collagen metabolism in whole animals have been hampered by lack of suitable methods to assess rates of synthesis and degradation. The development of the "flooding - dose" method for measuring protein synthesis rates ( 2 ) and its application to collagen by G.J. Laurent in 1982 has provided invaluable information about the dynamic nature of connective tissue synthesis in a variety of species(3).

Applying the flooding dose technique, fractional rates of collagen synthesis, hydroxyproline-associated radioactivity in collagen, % degradation and deposition rates of newly synthesised collagen were determined as described in Methods.

The study demonstrated significant changes in cardiac collagen metabolism *in vivo* in Mg-deficient rats (Tables 11- 15). The changes included.:

1. A 49% increase ( $p < 0.001$ ) in hydroxyproline-associated radioactivity in collagen, compared to control on Day60 and a 12 % increase ( $p < 0.05$ ) in

- fractional rate of collagen synthesis. No changes were observed at earlier time- points (Tables 11, 12).
2. A nearly 22 % increase ( $p < 0.001$ ) in degradation of newly synthesised collagen on Day 28 (Table 13) % degradation remained unaltered on Day 12.
  3. A marked fall in the rate of deposition of newly synthesised collagen (59%,  $p < 0.001$ ) was observed on Day 28. The deposition rate was higher by 24 % ( $p < 0.001$ ) by Day 60, compared to control (Table 14).
  4. Collagen concentration: The observed increase in the fractional rate of synthesis and deposition rate (relative to degradation) pointed to a possible increase in collagen concentration in the heart. Accordingly, analysis of collagen concentration in the heart in Mg- deficient rats (Table 15) showed an increase of 19% ( $p < 0.01$ ) by Day 60. Further, in keeping with the increased degradation of collagen on Day 28, the concentration of cardiac collagen at this time- point was significantly lower (33%  $p < 0.01$ ), compared to control values.

TABLE 11

EFFECT OF MAGNESIUM DEFICIENCY ON HYDROXYPROLINE -  
ASSOCIATED RADIOACTIVITY IN COLLAGEN

Parameter	Day 28		Day 60	
	Control	Deficient	Control	Deficient
Hydroxyproline-associated radioactivity in collagen (cpm/g tissue wet weight)	6251 ± 794 (4)	5975 ± 960 (4)	6474 ± 474 (4)	9649 ± 479 (4)

Values are expressed as mean ± SD

Numbers in brackets represent number of animals used for the experiment

Control Vs Deficient p < 0.001

TABLE 12

EFFECT OF MAGNESIUM DEFICIENCY ON FRACTIONAL SYNTHESIS RATES  
OF COLLAGEN IN RAT HEART

Parameter	Day 12		Day 28		Day 60	
	Control	Deficient	Control	Deficient	Control	Deficient
Fractional synthesis rate (K <sub>S</sub> %/day)	22.99 ± 1.3 (4)	21.84 ± 1.3 (4)	21.01 ± 0.5 (4)	20.88 ± 0.2 (4)	22.64 ± 1.6 (4)	25.30 ± 1.2 <sup>+</sup> (4)

Values are expressed as mean ± SD

Numbers in brackets represent number of animals used for the experiment

Control Vs deficient <sup>+</sup>p < 0.05

TABLE 13

EFFECT OF MAGNESIUM DEFICIENCY ON DEGRADATION RATES  
OF NEWLY SYNTHESISED COLLAGEN

Parameter	Day 12		Day 28		Day 60	
	Control	Deficient	Control	Deficient	Control	Deficient
Degradation (%)	83.30 ± 1.3 (4)	82.70 ± 1.5 (4)	73.15 ± 1.1 (4)	89.30 ± 0.5 <sup>Δ</sup> (4)	74.40 ± 2.0 (4)	71.60 ± 0.7 <sup>+</sup> (4)

Values are expressed as mean ± SD

Numbers in brackets represent number of animals used for the experiment

Control Vs deficient <sup>Δ</sup>p < 0.001, <sup>+</sup>p < 0.05

TABLE 14

EFFECT OF MAGNESIUM DEFICIENCY ON DEPOSITION RATE OF  
NEWLY SYNTHESISED COLLAGEN IN RAT HEART

Parameter	Day 28		Day 60	
	Control	Deficient	Control	Deficient
Deposition rate (% /day)	5.66 ± 0.3 (4)	2.33 ± 0.2 <sup>△</sup> (4)	5.79 ± 0.2 (4)	7.19 ± 0.3 <sup>△</sup> (4)

Values are expressed as mean ± SD

Numbers in brackets represent number of animals used for the experiment

Control Vs deficient <sup>△</sup>p < 0.001

TABLE 15

EFFECT OF MAGNESIUM DEFICIENCY ON COLLAGEN  
CONCENTRATION IN RAT HEART

Group	Collagen concentration ( mg/g tissue wet weight )		
	Day 12	Day 28	Day 60
Control	8.343 ± 0.29 (4)	7.436 ± 0.56 (4)	8.205 ± 0.46 (4)
Deficient	8.325 ± 0.78 (4)	5.013 ± 0.69* (4)	9.752 ± 0.26* (4)

Values are expressed as mean ± SD

Numbers in brackets represent number of animals used for the experiment

Control Vs deficient \*p < 0.01

## e. Fibroproliferative response *in vivo* in Magnesium-deficient rat

Studies on fibroblast proliferation constitute a basic approach to important questions like wound healing. A direct response to cardiac myocyte injury is cardiac fibroblast proliferation which may be detected with great sensitivity by an increase in cardiac DNA synthesis rates (128).

### 1. Rate of DNA synthesis

Studies on proliferative response of cardiac fibroblasts *in vivo*, in Mg deficiency, were undertaken. As shown in Table 16, there was no increase in thymidine incorporation into cardiac fibroblasts on Day 28 or Day 60 of deficiency. However, with increasing severity of the deficiency, stimulation of fibroproliferation in the heart (34% increase,  $p < 0.01$ ) was evident by Day 80 compared to control.

### 2. DNA concentration

A 20 % increase ( $p < 0.01$ ) in the DNA concentration of the heart was also observed on

TABLE 16

EFFECT OF MAGNESIUM DEFICIENCY ON RATES  
OF DNA SYNTHESIS IN RAT HEART

Day	Incorporation of $^3\text{H}$ -thymidine into DNA ( cpm/g tissue wet weight )	
	Control	Deficient
28	626 $\pm$ 50 (4)	617 $\pm$ 25 (4)
60	580 $\pm$ 48 (5)	582 $\pm$ 67 (5)
80	783 $\pm$ 71 (4)	1049 $\pm$ 87* (4)

Values are expressed as mean  $\pm$  SD

Numbers in brackets represent number of animals used for the experiment

Control Vs deficient \*p<0.01

Day 80 (Table 17), in keeping with the increase in cardiac fibroblast proliferation in these animals.

## **ii. Myocardial injury by Cerium**

Histological evidence of cardiac muscle damage and fibrosis in response to Ce led to investigations into the mechanisms underlying cardiac muscle damage and fibrosis induced by the element (Refer Design of the study Section B-II (ii) for experimental design).

### **a. Studies on the effect of Cerium administered *in vivo* on oxidative stress**

To test the possibility that Ce may enhance lipid peroxidation, representing a mechanism by which Ce could induce myocardial injury, TBARS levels in serum and cardiac tissue were measured 24 hours after intravenous injection of the element.

TBARS levels in serum and cardiac tissue were higher by 300 % ( $p < 0.001$ ) and 30 % ( $p < 0.001$ ) respectively, compared to control (Table 18).

### **b. Studies on the influence of Cerium on cardiac collagen metabolism**

It was reported earlier that low levels of Ce (100 nm) stimulate collagen and non-collagen

TABLE 17

EFFECT OF MAGNESIUM DEFICIENCY ON  
DNA CONCENTRATION IN RAT HEART

Day 80	Concentration of DNA (mg DNA/g tissue wet weight )
Control	1.380 ± 0.09 (4)
Deficient	1.663 ± 0.15* (4)

Values are expressed as mean ± SD  
Numbers in brackets represent number of  
animals used for the experiment  
Control Vs deficient \*p<0.01

TABLE 18

EFFECT OF CERIUM ADMINISTERED *IN VIVO*  
ON LEVELS OF TBARS IN SERUM AND HEART

	Control	Ce-treated
Serum ( $\mu\text{mol MDA}/$ 100 ml serum)	$1.268 \pm 0.12$ (4)	$5.034 \pm 0.85^{\Delta}$ (4)
Heart (nmol MDA/g tissue wet weight)	$323.82 \pm 7.2$ (4)	$414.32 \pm 9.1^{\Delta}$ (4)

Values are expressed as mean  $\pm$  SD  
Numbers in brackets represent number of animals  
used for the experiment  
Control Vs deficient  $^{\Delta}p < 0.001$

protein synthesis in cardiac fibroblasts *in vitro* through stimulation of transcription (121). Recognising the possibility that the effects of the lanthanide *in vitro* may not correlate with its effect *in vivo*, experiments were undertaken to examine the influence of Ce on the biosynthetic repertoire of cardiac muscle, particularly cardiac collagen metabolism.

### **1. Changes in collagen metabolism *in vivo* by Cerium**

The influence of the element *in vivo* on rates of collagen synthesis, degradation and deposition in rat heart was studied. No difference was observed in hydroxyproline-associated radioactivity in collagen either 24 hours or 48 hours after administration of Ce (Table 19). The % degradation of newly synthesised collagen was the same at 24 hours but, significantly, there was a 7 % decrease ( $p < 0.001$ ) in degradation of newly synthesised collagen 48 hours after Ce administration (Table 19). Consistent with a decrease in the rate of degradation, a 27 % increase ( $p < 0.001$ ) in the deposition rate of newly

TABLE 19

EFFECT OF CERIUM ADMINISTERED *IN VIVO* ON COLLAGEN METABOLISM IN RAT HEART

Parameter	24 hrs after Ce-injection		48 hrs after Ce-injection	
	Control	Ce-treated	Control	Ce-treated
Hydroxyproline - associated radioactivity in collagen (cpm/g tissue wet weight)	14417 ± 1332 (4)	14178 ± 536 (4)	12479 ± 344 (4)	12645 ± 358 (4)
Fractional rates of collagen synthesis ( $K_s$ %/day)	21.78 ± 2.7 (4)	21.29 ± 2.6 (4)	18.90 ± 1.1 (4)	20.50 ± 1.0 (4)
% degradation of newly synthesised collagen	69.70 ± 3.3 (4)	72.10 ± 2.8 (4)	68.80 ± 1.2 (4)	63.80 ± 0.8 <sup>Δ</sup> (4)
% deposition of newly synthesised collagen	6.53 ± 0.2 (4)	5.89 ± 0.6 (4)	5.90 ± 0.5 (4)	7.50 ± 0.3 <sup>Δ</sup> (4)

Values are expressed as mean ± SD

Numbers in brackets represent number of animals used for the experiment

Control Vs Ce-treated <sup>Δ</sup> $p < 0.001$

synthesised collagen was observed 48 hours after Ce administration (Table 19). Experiments were also carried out to evaluate the effect of the element on total protein synthesis, in terms of incorporation of  $^3\text{[H]}$  - L-phenylalanine into proteins.

## **2. Influence on total protein synthesis and transcription**

Figure 4 shows the kinetics of incorporation of radiolabelled phenylalanine into cardiac muscle proteins. There was a linear increase in the incorporation upto 120 mins, after which there was a small decline. Results presented in Table 20 show that Ce causes a two-fold increase in the incorporation of  $^3\text{[H]}$ -L-phenylalanine into total proteins. To ascertain whether the observed increase in  $^3\text{[H]}$ -L-phenylalanine incorporation into proteins in Ce-treated animals was due to an effect of the cation on transcription, its effect on  $^3\text{[H]}$  - uridine incorporation into RNA was studied. It was found that the element enhances transcriptional rates about two-fold (Table 21), confirming the increase in the rate of protein synthesis. Measurement of rates of total protein

**Figure 4 : Kinetics of incorporation of  $^3\text{H}$  - L-phenylalanine  
into cardiac muscle proteins**

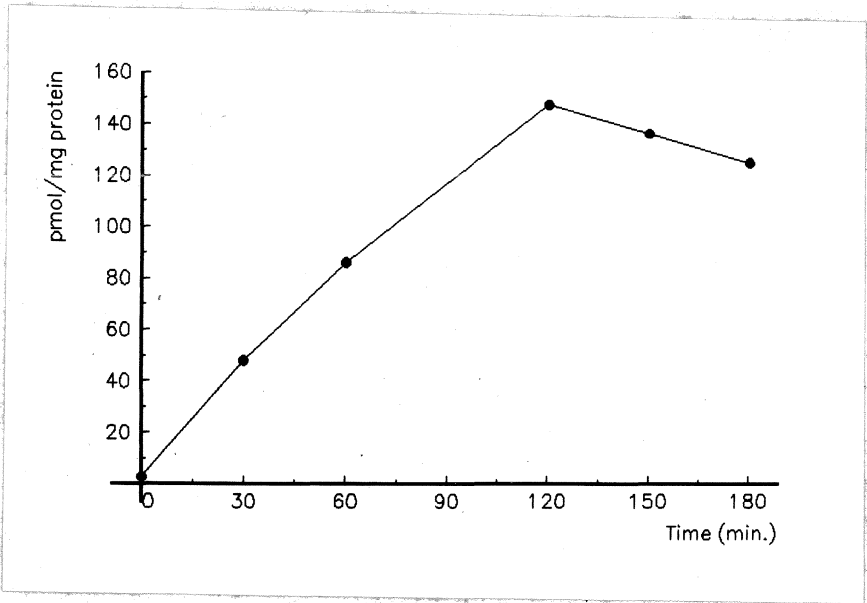


TABLE 20

EFFECT OF CERIUM *IN VIVO* ON RATES OF PROTEIN  
SYNTHESIS IN RAT HEART

Group	Incorporation of $^3\text{[H]-L-Phe}$ into TCA - insoluble material (pmol/mg protein)
Control	$107 \pm 20$ (4)
Ce-treated	$226 \pm 28^{\Delta}$ (4)

Values expressed as Mean  $\pm$  SD

Numbers in brackets represent number of animals used for  
the experiment

Assay was done in duplicate

Control Vs Ce-treated  $\Delta p < 0.001$

TABLE 21

EFFECT OF CERIUM *IN VIVO* ON RATES OF  
TRANSCRIPTION IN RAT HEART

Group	Incorporation of $^3\text{[H]}$ - Uridine into TCA-insoluble material (dpm/mg protein)
Control	13520 $\pm$ 4020 (4)
Ce-treated	28533 $\pm$ 3469.7 <sup><math>\Delta</math></sup> (4)

Values expressed as Mean  $\pm$  SD

Numbers in brackets represent number of animals used for the experiment

Assay was done in duplicate

Control Vs Ce-treated  $\Delta p < 0.001$

synthesis and rates of transcription was done *in vitro* as described in Methods, following intravenous administration of  $\text{CeCl}_3$  to the animal.

c. **Studies on influence of Cerium on fibroblast proliferation *in vivo***

Rates of  $^3\text{H}$ - thymidine incorporation in Ce- treated rats were higher by 23% ( $p < 0.05$ ), compared to control values, 24 hours after administration of a single dose of Ce (Table 22).

The changes in collagen metabolism and fibroblast activity described above suggest that Ce may influence cardiac connective tissue metabolism to trigger fibrogenesis.

TABLE 22

EFFECT OF CERIUM ADMINISTERED *IN VIVO* ON  
RATES OF DNA SYNTHESIS IN RAT HEART

	Control	Ce-treated
cpm/g tissue	849 ± 74 (4)	1041 ± 88 <sup>+</sup> (4)

Experiment was done as described in Methods  
 Values are expressed as mean ± SD  
 Numbers in brackets represent number of animals  
 used for the experiment  
 Control Vs Ce - treated +p < 0.05

# DISCUSSION

Tropical EMF is a restrictive cardiomyopathy prevalent in regions within 15° of the equator. Since its recognition as a clinical entity by Bedford and Konstam in 1946 (36) and description of its clinical features by Davies and Ball (4), the clinical and pathological features of this condition have been studied extensively. Although the gross and histological features of the affected heart have been thoroughly studied, little attention has been paid to the aetiopathogenesis of the fibrotic process observed in EMF.

Nutritional deficiency, serotonin from consumption of bananas, obstruction of cardiac lymphatics, hypersensitivity reaction to malaria and hyper-eosinophilia, etc. have been implicated as causative factors of endomyocardial fibrosis. The predominant tropical distribution and high incidence of EMF in regions rich in monazite elements and the observed high concentration of Ce, a major constituent of monazite soil, in combination with lower concentration of Mg in endomyocardial samples from patients with EMF led to the postulation of the geochemical hypothesis (8) that the disease could be the cardiac expression of Ce toxicity in conjunction with Mg deficiency. Recent epidemiologic studies on geographic distribution of EMF in Kerala point to

co- existence of high density of occurrence of EMF and desposits of monazite elements (72).

Against such a backdrop, the objective of this study was to examine, histologically and biochemically, cardiac changes of relevance to EMF induced by Ce and Mg deficiency. Briefly, this work has generated histological evidence of endomyocardial fibrosis induced by Mg deficiency and Ce. This is further supported by the changes in collagen metabolism triggered in response to Mg deficiency and Ce.

Towards the primary objective of ascertaining whether Mg deficiency and Ce toxicity act synergistically to produce EMF- like lesions in the heart, a long- term experiment was initiated to induce Mg deficiency in rats through dietary means and to study the effect of Ce supplementation to these animals.

Histological changes in the heart (Fig. 3 A - D) in response to Mg-restricted diet and Ce administration included myocytolysis, contraction bands, myofibrillar lysis, interstitial fibrosis and foci of scarring, particularly in the subendocardial myocardium. 3 out of 9 animals in Group B, 2 out of 9 in Group C and 4 out of 8 in Group D had such changes. Thus, the number of animals with histological

changes was more in Group D than in Groups B or C. One animal in the Mg- restricted and Ce- supplemented group had scarring around an intramyocardial artery. It may, however, be noted that at 13 months, morphologic features characteristic of a restrictive cardiomyopathy were not found in any of the test groups nor were there any histological changes in Group D, qualitatively different from Groups B and C, to suggest that a combination of Mg deficiency and Ce may lead to a restrictive cardiomyopathy upon an extended duration of treatment.

Interestingly, changes in all the parameters studied, including Mg and Ca levels in serum, heart and bone, Ce levels in heart and bone, number of animals with histological changes and cardiac collagen concentration (Fig. 3 A - D, Tables 2 - 5) were more pronounced in rats which were fed a Mg- restricted diet and supplemented with Ce (Group D) than in other groups.

Of particular significance was the observation that rats on Mg-restricted diet supplemented with Ce had higher levels of Ce in the heart than rats on Mg-sufficient diet supplemented with Ce, suggesting that Mg deficiency increases Ce levels in the heart. The gastrointestinal absorption of Ce is generally

poor in all the mammalian species studied. However, the absorption is greater in younger animals and dietary deficiency of Ca, P and vitamin A have been reported to increase Ce absorption (12). The mechanism by which Mg deficiency promotes accumulation of Ce in the tissue is not clear. It is possible that enhanced vascular permeability in Mg deficiency could cause an increased uptake from the absorptive surface (101) which could contribute to the accumulation of Ce. It has also been reported that lanthanides could gain entry into vascular smooth muscle cells when membrane-bound  $Mg^{2+}$  is removed (137). Moreover, earlier studies in this laboratory had shown that heart cells exposed to a Mg-deficient medium are more sensitive to the effects of Ce (120). Thus, Mg deficiency may enhance both the accumulation of Ce in the heart and its biochemical action on heart cells.

With histological evidence of cardiac fibrosis in response to Mg deficiency and Ce *per se*, several short-term experiments designed to delineate the molecular pathogenesis of the lesions were carried out. Two important events related to cardiac fibrosis are proliferation of fibroblasts and excessive connective tissue synthesis. The study examined in detail the

effect of Mg deficiency and Ce on both the parameters. The findings that the lanthanide causes:

- a. an increase in TBARS levels in serum and heart, indicating increased lipid peroxidation (Table 18), and
- b. increased deposition of newly synthesised collagen and an increase in fibroblast proliferation. (Table 19, 22)

suggest that lipid peroxidation-mediated damage to cardiac muscle in Ce-treated animals could represent a pathway by which Ce may produce myocardial injury, eventually resulting in reparative fibrosis.

The observation that Ce may interfere with collagen degradation and cause increased deposition of newly synthesised collagen is consistent with the observation of myocardial fibrosis (Fig. 3 A - D) and higher cardiac collagen content in rats (Table 5) upon prolonged administration of Ce. Although there was no alteration in collagen synthesis rates under the experimental conditions employed, an increase in cardiac collagen expression in response to prolonged administration of Ce cannot be ruled out as earlier studies (121) had shown that the element has a direct effect on collagen synthesis in isolated cardiac fibroblasts. Moreover, results presented in Table 20 show that Ce has a stimulatory

effect on rates of total protein synthesis and transcription *in vivo*. The results in this study may represent early events in which cardiac fibroblasts become responsive to a proliferative and fibrogenic stimulus. Taken together, the stimulatory effect of Ce on collagen synthesis in cardiac fibroblasts *in vitro* (121), increase in cardiac collagen content upon continued exposure to the element ( Table 5), accelerated fibroproliferation *in vivo* ( Table 22) and higher rates of collagen deposition in the heart following reduced rates of degradation ( Table 19) indicate the involvement of the cardiac interstitium in what is possibly a wound healing response triggered by Ce in rat heart. This could eventually lead to cardiac fibrosis.

Mg deficiency is known to produce a cardiomyopathy characterised by myocardial necrosis and fibrosis (100). Despite several theories put forward to explain the cardiac lesions observed in Mg deficiency (see literature review), the molecular basis of cardiac muscle damage and cardiac fibrogenesis associated with Mg deficiency remains unclear.

The involvement of O<sub>2</sub>- derived free radical generation in the pathogenesis of Mg deficiency

cardiomyopathy has been proposed (108 - 113). Weglicki *et al* (112) have shown increased TBARS levels in plasma of rats fed a Mg- deficient diet. Further, the observation of Freedman *et al* (108) that Vitamin E intervention limits the size of cardiomyopathic lesions of Mg deficiency supports the oxidative injury hypothesis. This work expands the oxidative injury theory by analysing TBARS levels in cardiac tissue and activities of anti- oxidant enzymes SOD and catalase in the heart and SOD activity in the plasma of Mg- deficient rats. The increase in TBARS levels in the cardiac tissue and decreased activities of SOD in plasma and SOD and catalase in cardiac tissue of Mg- deficient rats observed in the study (Tables 9,10 ) are consistent with the postulation of oxidative damage to the myocardium in Mg deficiency.

The present study also revealed that dietary deficiency of Mg produces marked alterations in collagen metabolism in the heart. While an increase in fractional rates of synthesis and rates of degradation and deposition was observed on Day 60 of deficiency, changes in the Mg-deficient animals on Day 28 were marked by a significant drop in collagen deposition rates following an increase in the rate of degradation of newly synthesised collagen. Fractional synthesis rates remained unchanged on Day 28. Consistent

with these findings, the concentration of collagen in the heart was found to be higher on Day 60 but lower on Day 28 in the test group (Tables 11 - 15 ). Further, although the aforementioned changes in collagen metabolism were not accompanied by proliferation of fibroblasts on Day 28 or even Day 60, a fibroproliferative response to dietary deficiency of Mg was observed on Day 80, a finding corroborated by an increase in the DNA content (Tables 16,17 ). A rise in collagen synthesis, initially mediated by existing fibroblasts and subsequently by fibroblast proliferation, has been reported to occur in response to other stimuli also (138). The changes in collagen turnover may represent a process of remodeling in the heart in this model. Increased collagen deposition would lead to increased myocardial stiffness with its functional sequelae but this aspect has not hitherto been probed in relation to Mg deficiency. Taken together, these observations suggest that cardiac fibrogenesis associated with Mg deficiency may be reparative in nature, upon oxidative damage to the myocardium.

It may be noted that levels of myocardial Mg remain unchanged in response to acute dietary Mg deficiency (Table 7 ) but a marked drop in serum Mg has been observed. It is in fact difficult to achieve a reduction in the

concentration of intracellular free Mg, the biologically active form of the element by changing the extracellular Mg concentration (139, 140 ). It appears therefore that biochemical changes in the heart in Mg deficiency are mediated through a mechanism not involving a reduction in myocardial Mg. Admittedly, the mechanism remains unclear at present but a few possibilities, as discussed below, are noteworthy.

Using an experimental model that is nearly identical to the one employed in this study, it has been shown (108-113) that Mg deficiency produces a pro-oxidant, pro-inflammatory state marked by elevated circulating levels of factors like TNF- $\alpha$  and Substance P, which are known to trigger free radical generation. A role for these factors in promoting oxidative injury to the heart in Mg deficiency seems plausible and is supported by the observation that inhibition of Substance P receptor resulted in attenuation of indices of oxidative stress *in vivo* (112). Speculating on an alternative mechanism of free radical production, it was postulated by Shivakumar (91) that, in the absence of a reduction in myocardial Mg, hypomagnesemia may, through effects on vascular smooth muscle, induce mild episodes of ischemia and reperfusion, which can result in free radical production. Such

changes could exacerbate the oxidative stress imposed by the cytokines. Though speculative, the possibility that pathological changes in the myocardium in response to dietary Mg deficiency are secondary to hypomagnesemia-induced vascular changes warrants further study.

Increased rates of collagen synthesis, as observed in the present study, reflect increased fibroblast activity. Transforming growth factor- $\beta$  is a potent mitogen that has a stimulatory effect on cardiac fibroblasts (141). It is up-regulated in tissue injury and is considered to be the cytokine most implicated in fibrosis (142) but there is no evidence, at present, of its involvement in cardiac fibrogenesis associated with Mg deficiency. On the other hand, the concentration of Substance P is reported to be elevated not only in plasma but also at the sites of lesions in the cardiac muscle in Mg deficiency. Further, up-regulation of Substance P receptors in target cells has been suggested (112). As Substance P is known to stimulate fibroblast proliferation and collagen synthesis (143), it is tempting to speculate that the neuropeptide triggers the changes in collagen metabolism observed in this model.

Thus, it is possible that induction of pro-oxidant and

mitogenic factors in hypomagnesemia may trigger not only oxidative damage but also subsequent fibrogenesis in the myocardium, even without a fall in tissue Mg levels, in a model of acute Mg deficiency.

### **The findings in perspective - an epilogue**

It takes several lines of evidence to establish the validity of a hypothesis on the causation of any disease. The results presented in this thesis are consistent with the geochemical hypothesis on the aetiopathogenesis of tropical EMF to the extent that Mg deficiency and Ce produce cardiac fibrosis and promote changes in cardiac collagen metabolism that would favour fibrogenesis. Gross changes characteristic of a restrictive cardiomyopathy were not found in any of the test groups. This work is significant also for the reason that it has focussed on fibroblast activity and collagen turnover in the heart and, in recent years, there has been a tremendous surge of interest in such changes in the cardiac interstitium which may provide a new perspective on ventricular dysfunction, irrespective of the aetiology.

## Future work

Histological evidence of endomyocardial fibrosis, admittedly, does not by itself constitute an animal model of a restrictive cardiomyopathy like EMF. Future studies should therefore address the functional sequelae of the histological changes described here. Further, the outcome of this work calls for more incisive investigations on the molecular events that lead to cardiac fibrogenesis in response to Mg deficiency and Ce. The interaction between cardiac fibroblasts and growth factors, which is being increasingly viewed as the molecular basis of regulation of collagen expression in the heart, is an area that holds promise for the future in terms of mechanistic explanations and development of therapeutic strategies to check cardiac fibrosis.

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## LIST OF PUBLICATIONS

1. Cerium Stimulates Protein Biosynthesis in Rat Heart *in vivo*.  
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4. Alterations in Collagen Metabolism and increased fibroproliferation  
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# **SYNOPSIS**

## INTRODUCTION

The heart is a muscular pump composed of the parenchyma and the stroma. The parenchyma consists of highly differentiated cells having specialized functions (like cardiac myocytes) while the stroma includes pluripotent, highly undifferentiated cells (like cardiac fibroblasts) and a structural protein network formed largely by fibrillar collagen (1). To date, most of the research carried out to understand the pathogenesis of heart failure has focused on the parenchyma which was presumed responsible for organ failure. This was because structural protein turnover was assumed to be too slow to be of any pathophysiologic consequence. However, it has now become clear that in many pathological states, it is the continued accumulation of the stroma, not faulty parenchyma, that impairs organ function. The abnormal accumulation of fibrous tissue creates a pathological remodeling of the myocardium. The progressive and extensive nature of remodeling negates what is initially an adaptive response and may eventuate into a pathologic state with muscular entrapment, cell loss, and abnormal diastolic stiffness and systolic wall stress

of the myocardium. A classic example of such abnormal accumulation of fibrous tissue leading eventually to cardiac failure is endomyocardial fibrosis (EMF).

Characterised as a distinctive endocardial fibrosis which tends to grow into the myocardium and is distributed largely over the inflow tracts of either or both ventricles, resulting in restriction to ventricular filling and arterioventricular valve incompetence, tropical endomyocardial fibrosis represents an important cause of restrictive cardiomyopathy.

Ever since its recognition by J.N.P. Davies as a distinct clinical and pathologic entity, studies on the causative factors of tropical EMF, such as nutrition, viral, parasitic infection, and immunologic abnormalities have not so far conclusively demonstrated the aetiological link. The spatial coincidence of distribution of EMF with abundant deposits of monazite-rich mineral in the tropical zone, significantly higher levels of the monazite element cerium (Ce) and lower levels of magnesium (Mg) in endomyocardial tissue samples from patients with EMF compared to control samples had led to the geochemical hypothesis that tropical EMF may be the cardiac expression of Ce toxicity in conjunction with Mg deficiency (2).

## OBJECTIVES OF THE STUDY

Admittedly, it takes several lines of evidence to assess the validity of a hypothesis on the causation of any disease.

The objectives of this study were

1. To ascertain whether a combination of Mg deficiency and Ce would produce fibrotic changes in rat heart as in EMF.
2. If so, to establish the biochemical correlates of cardiac fibrogenesis promoted by Mg deficiency and Ce, focussing on changes in collagen metabolism.

## METHODOLOGY

In keeping with the objectives, this study consisted of a long-term experiment and several short-term experiments. The experimental design was as follows:

### I. Long-term experiment

The aim was to look for histological changes, particularly fibrosis, in rat heart, if any, in response to Mg deficiency and Ce.

Sprague-Dawley rats of both sexes (approximately equal in number) weighing  $55 \pm 5$  g were randomly distributed into four experimental groups following a 2 X 2 factorial design. Animals in Group A and B were fed a Mg-sufficient diet

(containing 0.0515% Mg) while those in Groups C and D were fed a Mg-restricted diet (containing 0.012% Mg). Cerium chloride, dissolved in double distilled water to a concentration of 35 ppm of Ce, was given to Groups B and D. While groups A and C were given double distilled water. Feed and water were provided *ad libitum*.

Group A + Mg

Group B + Mg + Ce

Group C - Mg

Group D - Mg + Ce.

At thirteen months, the animals were killed under ether anaesthesia and cardiac tissue was collected for elemental analysis, determination of collagen concentration and histology. Blood was collected at sacrifice and serum was separated for elemental analysis. Levels of Mg and Ca in the serum and cardiac tissue were determined by atomic absorption spectrometry and Ce concentration in cardiac tissue and bone were determined by inductively coupled plasma-mass spectrometry. Determination of cardiac collagen concentration (3) and processing of cardiac tissue for histology were done according to standard procedures.

## II. Short-term experiments

The aim of these experiments was to investigate the

biochemical basis of cardiac muscle damage and fibrosis induced by Mg deficiency and Ce.

**(i) Cardiac muscle damage and fibrosis induced by Magnesium deficiency.**

Sprague-Dawley rats (M:F:1:1) weighing approximately  $110 \pm 5$  g were used for the study. They were divided into control and deficient groups after 2 days on control feed. The rats were pair fed a Mg-sufficient diet (containing 0.0515 % Mg) or Mg-deficient diet (containing 0.0008 % Mg) for 12,21,28,60 or 80 days. The animals were provided double distilled water *ad libitum*.

**a. Elemental analysis**

At sacrifice, blood was collected by cardiac puncture and serum separated for Mg and Ca analysis; cardiac tissue was collected and processed for elemental analysis.

**b. Oxidative damage to the muscle**

Parameters of oxidative stress, namely, levels of thiobarbituric acid reactive substance (TBARS) in serum and cardiac tissue and activity of antioxidant enzymes in plasma and cardiac tissue of Mg-sufficient and Mg-deficient rats, were studied (4, 5).

**c. Changes in collagen metabolism and fibroproliferative response in the heart**

Investigations on collagen metabolism addressed fractional rate of collagen synthesis, % degradation of newly synthesised collagen and collagen deposition rates. These were measured using standard procedures, following intraperitoneal administration of  $^3\text{[H]}$ -proline (6). Cardiac collagen concentration was determined from the hydroxyproline content per gram of the tissue (3).

Fibroblast proliferation, as a response to cardiac myocyte injury, was measured in vivo in terms of incorporation of  $^3\text{[H]}$ -thymidine into cardiac DNA (7).

**(ii) Cardiac muscle damage and fibrosis induced by Ce.**

Female Sprague-Dawley rats weighing approximately  $200 \pm 5$  g were used for these experiments. All animals were fed a standard rat chow. Rats were administered a single dose of  $\text{CeCl}_3$  (1.3 mg Ce/Kg body weight, a dose which is well below the  $\text{LD}_{50}$  but sufficient to induce fatty liver in rats) intravenously through the tail vein (8). Control rats received an equal volume of physiological saline. Rats were sacrificed 24 hours or 48 hours after administration of  $\text{CeCl}_3$ .

- a. TBARS levels in serum and cardiac tissue were determined (4).
- b. Fractional rates of collagen synthesis, % degradation of newly synthesised collagen and collagen deposition rates were measured (6).
- c. Fibroblast proliferation was studied using  $^3\text{[H]}$ - thymidine (7).

## RESULTS

### I. Long - term experiment

Important observations in pursuance of the first objective included:

- a. Reduction in serum levels of Mg (hypomagnesemia) and rise in serum levels of Ca (hypercalcemia) in Group C and D.
- b. A significantly lower concentration of Mg in the heart in rats fed a Mg-restricted diet (Groups C & D) compared to rats fed a Mg-sufficient diet (Groups A and B).
- c. Rats on Mg-restricted diet supplemented with Ce (Group D) had higher levels of Ce in the heart than rats on Mg-sufficient diet supplemented with Ce (Group B).
- d. Histologic changes in the test groups (B, C & D) included myocytolysis, contraction bands and myofibrillar lysis, intermyofibre fibrosis in the myocardium, endocardial fibrosis

with tongues of fibrous tissue creeping in between myofibres into sub-endocardial myocardium. Scarring around an intramyocardial artery was observed in one of the rats belonging to Group D. These histological changes were seen in 3 out of 9 animals in group B, 2 out of 9 animals in Group C and 4 out of 8 animals in Group D.

- e. Collagen concentration was higher in Groups B, C & D compared to Group A, the changes being most marked in Group D.

## **II. Short-term - experiments**

**(i) Studies on acute Magnesium deficiency yielded the following results**

- a. Severe and consistent hypomagnesemia and progressive hypercalcemia.
- b. No change in the concentration of Mg in the heart but Ca concentration increased by Day 60 of deficiency.
- c. TBARS levels in serum and cardiac tissue were higher by Day 28 of deficiency, compared to control values.
- d. Activities of anti-oxidant enzymes, SOD and catalase, in plasma and heart decreased by Day 21 of deficiency, compared to controls.

- e. Changes in collagen metabolism included a significant increase in degradation of newly synthesised cardiac collagen on Day 28 of deficiency and increase in fractional rates of synthesis and deposition of collagen by Day 60 of deficiency.
  - f. An increase in the rate of fibroblast proliferation by Day 80 of deficiency.
- (ii) Investigations into possible mechanisms of myocardial injury by Cerium showed that the element**
- a. Increases levels of TBARS in serum and heart, indicating increased lipid peroxidation.
  - b. Increases the rate of deposition of newly synthesised cardiac collagen (relative to degradation). The effect of Ce was, however, not specific to collagen as there was a two-fold increase in the rates of total protein synthesis as well. Preliminary experiments showed an increase in the overall rates of transcription in response to Ce.
  - c. Increases the rate of fibroblast proliferation in the heart.

## **DISCUSSION**

While Mg deficiency has for long been known to produce a cardiomyopathy, the cardiac effects of Ce remain unexplored. The objective of the study was to examine, histologically and biochemically, cardiac changes of possible

relevance to EMF, induced by Ce in a context of Mg deficiency. This work has generated histological evidence of endomyocardial fibrosis induced by Ce *per se* and Mg deficiency *per se*. A combination of the two seems to have a synergistic effect with respect to most of the parameters examined. The histological finding of cardiac fibrosis is consistent with the observed increase in cardiac collagen concentration in the test groups (B, C, D). Further, the changes in collagen metabolism and increased fibroproliferative activity in the heart observed in this study indicate that Mg deficiency and Ce may promote cardiac fibrogenesis. Another observation of importance was the elevation in Ce levels in the heart in Mg deficiency. It was demonstrated earlier (9) that heart cells exposed to a Mg-deficient medium are more sensitive to the effects of Ce. It appears therefore that Mg deficiency may enhance both the accumulation of Ce in the heart and its biochemical action on heart cells. The present study also suggested that cardiac fibrogenesis associated with Mg deficiency and Ce toxicity may be reparative in nature, upon oxidative damage to the myocardium.

Stated briefly, the histological and biochemical changes observed are consistent with a role for Mg deficiency and Ce in

the causation of tropical EMF. Further, the study has established the biochemical basis of myocardial injury induced by Mg deficiency and Ce.

Future studies should address the functional sequelae of these histological changes. Further, the outcome of this study calls for more incisive investigations on the molecular mechanisms underlying cardiac fibrogenesis in response to Ce toxicity and Mg deficiency. The interaction between cardiac fibroblasts and growth factors, which is being increasingly viewed as the molecular basis of regulation of collagen expression in the heart, is an area that holds promise for the future in terms of mechanistic explanations and development of therapeutic strategies to check cardiac fibrosis.

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