STIMULATION OF CARDIAC FIBROBLAST PROLIFERATION BY LANTHANIDES : A SUPEROXIDE ANION MEDIATED RESPONSE

A THESIS PRESENTED

BY

PREETA R

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FOR

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Dedicated to my parents

CERTIFICATE

I, PREETA R, hereby certify that I had personally carried out the work depicted in the thesis entitled "STIMULATION OF CARDIAC FIBROBLAST PROLIFERATION BY LANTHANIDES: A SUPEROXIDE ANION MEDIATED RESPONSE", except where external help was sought and is acknowledged.

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DECLARATION

This is to certify that Ms. PREETA R 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 her thesis entitled "STIMULATION OF CARDIAC FIBROBLAST PROLIFERATION BY LANTHANIDES: A SUPEROXIDE ANION MEDIATED RESPONSE" was carried out under my direct supervision.

4/11/99

(R. RENUKA NAIR)

Guide

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SYNOPSIS

INTRODUCTION :

Excessive proliferation of connective tissue and its hyperfunction are characteristic features of several common human diseases involving fibrosis. A fibroproliferative reaction could be triggered by a number of factors such as cytokines, growth factors, low oxygen potential, exposure to metals, etc.

Endomyocardial fibrosis is a condition prevalent in the tropics, in regions with latasolic soil and is characterised by excessive endocardial thickening that grows into the inflow tracts of either or both the ventricles. The lanthanide cerium has been implicated in the etiopathogenesis of the disease. Investigations carried out in pursuance of the postulation furnish histological evidence of a fibroproliferative reaction and increased cardiac collagen content in rats and rabbits on administration of cerium. This investigation was carried out to study the effect of cerium on proliferation of isolated cardiac fibroblasts and delineate the mechanism that mediates the proliferative response. Cerium, a trivalent ion with a large ionic radius, does not penetrate the cells, but it raises the possibilty of induction of lipid peroxidation of the cell membrane thus leading to the generation of an array of free radicals. With evidences citing the emerging role of free radicals as a stimulant for cell growth, **it was hypothesized that the lanthanide cerium at low levels may induce a fibroproliferative response with the superoxide anions as the biologic mediators.**

OBJECTIVES OF THE STUDY:

This study was therefore carried out with the following objectives

1. To ascertain whether low levels of cerium could induce a stimulatory response on cardiac fibroblast proliferation
2. To investigate whether superoxide anions could mediate the cerium induced stimulatory response on cardiac fibroblast proliferation
3. To assess the tissue specific reaction of the lanthanide

EXPERIMENTAL DESIGN :

Cardiac fibroblasts isolated from newborn rat was used as the experimental model. In keeping with the objectives, the experimental protocol was designed to examine ;

1) The effect of different concentrations of the lanthanide cerium on cardiac fibroblast proliferation by determination of :
   a) the proportion of cells immunoreactive for proliferating cell nuclear antigen
   b) cell density
2) Stimulation of superoxide anion generation by cerium by measurement of :
   a) nitroblue tetrazolium reduction for assessment of intracellular generation of superoxide anions
   b) superoxide dismutase inhibitable reduction of cytochrome c for extracellular generation of superoxide anions

c) levels of thiobarbituric acid reactive substances to assess lipid peroxidation

3) Role of superoxide anions in the mediation of fibroblast proliferation as assessed by:

a) the effect of enzymatic and non-enzymatic free radical scavengers - superoxide dismutase, catalase, N-acetyl-L-cysteine (NAC)

b) the response on exposure to exogenously generated superoxide anions

4) Cardiospecific response to cerium by comparison with the behaviour of skeletal muscle fibroblasts

METHODOLOGY :

The experiments designed to study the effect of cerium toxicity were carried out on cardiac fibroblasts isolated and cultured from the heart of newborn rats of the Wistar strain.

The cells were isolated from 2-3 day old rats by enzymatic dispersion method. Fibroblast rich cultures were obtained following selective adhesion. Pure fibroblast cultures were obtained by repeated passage. In addition to its typical morphology fibroblasts were identified immunohistochemically. Vimentin positive, desmin and factor VIII negative cells were characterised as fibroblasts. Before carrying out the experiments the cultures were synchronised by serum deprivation for 24 hours. Serum concentration in

the medium used for experiments was nominal (0.4%).

Cardiac fibroblasts were treated with different concentrations of cerium (μM) so as to determine the concentration that stimulates growth and generation of superoxide anions.

To ascertain the role of superoxide anions on cell proliferation, cerium treated cultures were exposed to free radical scavengers - superoxide dismutase(100 U/ml), catalase (120 U/ml) and NAC (20 mM). The proportion of PCNA reactive cells and cell count was determined. The proliferative response to superoxide anions was also verified by using known generators of superoxide anions.

To examine tissue specific response to cerium or to oxygen radical stress, fibroblasts from skeletal muscle were isolated and the results were compared.

STATISTICAL ANALYSIS :

The data are presented as mean ± SEM values for each set. Each experimental observation was based on a minimum of 4 replicates. A level of $p < 0.05$ was selected to indicate statistical significance. Group means were compared by a one-way ANOVA where necessary and the difference between selected means were evaluated using unpaired Student's t-test.

RESULTS:

Effect of cerium on cardiac fibroblast proliferation :

a) Adopting PCNA as a marker for cell proliferation, a significant increase in the proportion of immunoreactive cells was observed at 0.5 μM concentration of cerium (38%±4.18 *vs* 16%±2.7, $p < 0.005$). The proportion of immunoreactive cells were lower at higher concentration of cerium.

b) The total cell number was determined after an exposure period of 96 hours to different concentrations of cerium and a statistically significant difference ($p<0.005$) compared to control at 0.5 μM concentration of cerium was observed.

Effect of cerium on superoxide anion generation in cardiac fibroblasts :

a) Intracellular generation of superoxide anions : Cardiac fibroblasts exposed to different concentrations of cerium for 1 hr showed a significant increase in the generation of superoxide anions as assessed by the reduction of NBT to formazan. The formazan production was lowered on inclusion of SOD (100 U/ml) and NAC (20 mM) in the culture medium.

b) Extracellular generation of superoxide anions : Release of superoxide anions to the extracellular medium was found to be higher in the cerium treated cultures compared to cerium free controls.

c) Lipid peroxidation : Increased levels of Thiobarbituric acid reactive substances was observed on exposure to 0.5 μM cerium concentration indicating increased peroxidation.

Effect of free radical scavengers on cerium induced cardiac fibroblast proliferation :

To ascertain whether free radicals mediated the cerium stimulated fibroblast proliferation, extracellular free radical scavengers SOD (100 U/ml) and catalase (120 U/ml) and cell permeant antioxidant NAC (20 mM) were included in the cultures exposed to 0.5 μM concentration of cerium. It was observed that SOD neutralized the cerium induced stimulatory response, thus fortifying the role of superoxide anions in fibroblast proliferation. With NAC the decrease in cerium induced stimulatory response was higher.

Effect of known generators of superoxide anions on cardiac fibroblast proliferation :

Exposure of cardiac fibroblasts to extracellular source of superoxide anions (Hyp + XO) was shown to increase the total cell count, the proportion of cells immunoreactive for PCNA and the intracellular superoxide anion content ($p<0.0005$) in the cardiac fibroblasts. Inclusion of SOD and NAC lowered the Hyp+XO induced stimulatory response suggesting that extra -

-cellular anion mediated rise in intracellular superoxide anion level is responsible for the stimulatory effect on cardiac fibroblast proliferation.

Assessment of tissue dependent variation in response to cerium:

Exposure of skeletal muscle fibroblasts to 0.5 μM cerium, showed an increase in total cell count, PCNA reactive cells and intracellular superoxide anion content. A pattern similar to that seen in cardiac fibroblasts was observed. The response of skeletal muscle fibroblast to cerium was found to be similar to that of cardiac fibroblasts.

CONCLUSION :

The increase in the proportion of PCNA reactive cells and the increase in total cell number supports the hypothesis that low levels of cerium stimulates cardiac fibroblast proliferation. The increase in superoxide anion content further ensures the fact that low levels of cerium is capable of generating free radicals. Neutralization of the cerium induced stimulatory response in cardiac fibroblasts by superoxide dismutase fortifies the role of superoxide anion in the mediation of cardiac fibroblast proliferation by cerium. From this experimental data, it is inferred that cerium at low levels can stimulate cardiac fibroblast proliferation and the superoxide anions function as biologic mediators of cerium induced fibroproliferative response. This study signifies the prominence of superoxide anions in the transduction of biochemical

responses. A remarkable outcome of this investigation is that superoxide anions, the molecules till recently associated with cell death is attributed to evoke growth factor like responses when produced in low levels. The study also stresses upon the role of superoxide anion in the initiation of fibrotic reaction in general.

INTRODUCTION

CHAPTER - 1

INTRODUCTION

Inappropriate proliferation of fibroblasts is characteristic of fibrotic disorders which can occur either as a reactive or reparative pheomenon. The fibroproliferative reaction in the normal course limits the spread or extension of potentially injurious agents. However, in some circumstances an abnormal accumulation of fibrous material interferes with the normal functioning of the affected tissue. Many common debilitating diseases involve the excessive proliferation of the connective tissue cells - the fibroblasts; and it is the mechanism leading to this aberration that requires further elucidation.

Current understanding of the factors initiating such a proliferative response in the fibroblasts include hormones, growth factors and cytokines (Booz and Baker, 1995), oxidants (Burdon, 1995), action and interaction of inflammatory cells (Nicoletti and Michel, 1999), metals and minerals (Nemery, 1990). Lanthanides, a group of rare earth metals, have also been associated with this pathological phenomenon. The lanthanide cerium has been implicated in the etiology of endomyocardial fibrosis; based on the analysis of the cardiac tissue of the patients (Valiathan and Kartha, 1990). This postulation is supported by the observation in rats and rabbits where increased collagen content (Kumar et al, 1996; Kartha et al, 1998), elevated lipid peroxidation and heart cell proliferation was seen (Kumar and Shivakumar, 1998) on administration of cerium either orally or intravenously

in conjunction with a magnesium deficient diet. Cardiac fibroblasts have a multifarious role in the maintenance of the functional and structural integrity, and the excessive proliferation of these cells culminating in fibrosis leads to dysfunction of the organ.

In fibrotic disorders, the cause of the connective tissue proliferation is not clear, other than the fact that increase in the number of fibroblasts or a hyperfunction of individual cells is involved. The lack of understanding of the regulatory processes of the fibrotic reaction thus restricts the development of rational therapeutic measures. This study was taken up with the aim of elucidating the action of cerium on cardiac fibroblasts and to obtain a deeper understanding of the mechanism that induces a fibroproliferative response.

A number of extracellular signals and exogenic factors such as growth factors and other cytokines released from inflammatory cells have been shown to induce a proliferative response. It has recently been observed that reactive oxygen species, traditionally viewed only as toxins can also play a role in the cell's signaling pathways. Superoxide anion has been found to help a protein called Ras that transmits growth stimulating messages to the cell interior; and this Ras pathway is one of the cell's most important growth stimulating pathways (Irani et al, 1997). A fibrotic reaction due to silicon has been reported in pneumoconiosis and a superoxide anion mediated fibrosis has been suggested (Gusev et al, 1990). Studies have also shown that the active oxygen species stimulate growth factor like responses such as

intracellular alkalinization (Shibanuma et al, 1988a) and increase in c-myc and c- fos proto - oncogene mRNA levels (Crawford et al, 1988).

In view of the reports on the induction of fibroproliferative response by cerium and also the stimulatory effect of free radicals on cell systems, it was hypothesized that: **low levels of cerium can stimulate cardiac fibroblast proliferation; and that superoxide anions mediate the fibroproliferative response.**

This hypothesis is based on the **assumption** that cerium being a trivalent ion cannot penetrate the cell (Altura and Altura, 1985) but may bind to the cell membrane (Langer and Franks, 1972); and as metals are known to induce lipid peroxidation, similar induction of the phenomenon may lead to generation of oxygen radicals. The superoxide anions so released could mediate the cerium induced fibroproliferative response.

The experimental studies have been carried out on cultured cells. It has been assumed that the characteristics of cardiac fibroblasts are retained in culture with all the specialized functions.

The study has been designed with the following **objectives** :

1) To ascertain whether low levels of cerium can induce a stimulatory response on cardiac fibroblast proliferation
2) To examine whether cerium stimulates superoxide anion generation
3) To investigate whether superoxide anions mediate the cerium induced trophic response on cardiac fibroblasts

4) To assess whether there exists a tissue dependent variation in response to cerium.

Advances in mammalian cell culture technology have provided numerous systems and approaches for both routine toxicological screening and for studies on the mechanisms at the cellular and molecular level. The cell culture system has certain **advantages** and **limitations.** Cultured cells serve as an effective system for the study of various physiological and biochemical aspects as they are free from influences of dynamic hormonal and circadian regulatory variables.

In biological sciences a significant factor in the development of an area of research is often the choice of the most suitable experimental system. This system should permit ready manipulation of its components, rigid control of environmental factors and reproducibility. Tissue culture as a research tool has much to commend it. One can study tissue and cell types and observe cells of one type modulating into other forms. Nevertheless, one strong criticism is that the cells growing in vitro are far removed from their normal environment and spatial relationships. Hence, what may happen in vitro need not necessarily occur in vivo. It is therefore misconstrued that the in vitro system is not a suitable experimental model on the grounds that in vivo situation, such as the influence of the other organs cannot be simulated in culture. This is true only if an experimental finding is extrapolated to describe the end result or an expected pathological condition. But, for delineating the mechanism of action leading to a pathological state it is essential to have a

system devoid of a number of influencing factors. Such a controlled system helps in understanding whether the changes observed in vivo are primary or secondary to the factors under consideration and this is essential for delineating the etiopathogenesis of a condition. The system also finds application in pharmacology. In a study designed to obtain a deeper understanding of cellular mechanisms underlying a fibrogenic reaction, tissue culture is an ideal model for delineating the response and behaviour of fibroblasts.

For assessment of trophic responses, the proliferative activity of cells can be measured by many techniques which include mitotic index, thymidine labelling index, s phase fraction measured by flow cytometry, PCNA and Ki 67 index. Immunocytochemical staining method for proliferating cell nuclear antigen (PCNA) as a method for assessment of cell proliferation was preferred over the traditional ^{3}H - thymidine (^{3}H - TdR) or ^{3}H - deoxycytidine incorporation because it is an easier and reliable method. Proliferating cell nuclear antigen expression and synthesis is correlated with the proliferative phase of the cell and is used as a molecular marker of replicating cells. Its presence subsequent to immunocytochemical processing can be observed microscopically thereby providing direct evidence of cell proliferation under various experimental conditions.

An important theme in the study is that superoxide anions can mediate a mitogenic response. For the detection of intracellular content of superoxide anions in cell culture system, nitroblue tetrazolium reduction assay is a simple

and non invasive method. Tests of the specificity of NBT reduction assay have shown that nitroblue tetrazolium reduction is more than 600 - fold more sensitive to superoxide anion than to hydrogen peroxide. Optical density at 550 nm, 15 sec after adding 1 mMol/l and 100 mMol/l hydrogen peroxide was 0.001 and 0.030 respectively whereas that for 1 mMol/l potassium superoxide was 0.620. The small reduction of NBT by 100 mMol/l hydrogen peroxide was unaffected by catalase, whereas that caused by potassium hydroxide was reduced by superoxide dismutase (150 U/ml) to 0.024. This substantiates that NBT reduction assay is specific for superoxide anion (Wang, 1998).

This study on assessment of the proliferative response to cerium and elucidation of the mechanism inducing the mitogenic response was therefore carried out on cardiac fibroblasts isolated from rat heart. The proliferative response was assessed both by determination of cell density and the proportion of cells immunoreactive for PCNA. As superoxide anions were postulated to be the biologic mediators of the proliferative response, the superoxide anion content of cells on exposure to cerium was measured by the NBT reduction assay. The role of superoxide anions in cell proliferation was also confirmed by determination of cell response in the presence of superoxide anion scavengers and on exposure to known generators of superoxide anions. The response of cardiac fibroblasts was compared to the behaviour of skeletal muscle fibroblasts to examine whether the fibroproliferative reaction is tissue specific.

DEFINITION OF TERMS

Antioxidants - Any substance that when present at low concentrations compared to those of an oxidizable substrate, significantly delays or prevents oxidation of that substrate.

Apoptosis - An innate cellular program of cell death which can be regarded as the opposite of proliferation.

Cirrhosis - Condition where some cells of liver die and are replaced by hard fibrous tissue.

Dupuytren's contracture - Condition where the palmar fascia becomes thicker causing the fingers to bend forward.

Endomyocardial fibrosis - Extensive thickening of the endocardium that grows into the inflow tracts of either or both the ventricles.

Fibrosis - Formation of fibrous tissue as a reparative or reactive process, as opposed to formation of fibrous tissue as a normal constituent of an organ or tissue.

Free radicals - Any atom or molecule that contains one or more unpaired electrons.

Free radical scavengers - Substances that do not eliminate a free radical but replaces or substitutes the highly reactive radical with a less reactive one.

Heamochromatosis - Hereditary disorder in which the body absorbs and stores too much iron, causing cirrhosis of liver and giving the skin a dark color.

Ischemia - Local anemia due to mechanical obstruction of the blood supply.

Mitogen - A substance that stimulates mitosis and lymphocyte transformation.

Necrosis - A pathological form of cell death caused by physical , chemical or osmotic damage with consecutive disruption of internal and external membrane leading to cell swelling and lysis and release of cytoplasmic material.

Oncogenes - Genes whose products are associated with neoplastic transformation.

Oxidative stress - State in which exposure to free radicals or other oxidants represents a challenge to normal function or even to survival.

Proto - oncogenes - Normal cellular genes that affect growth and differentiation.

Phagocytosis - Destruction of bacteria, cells and foreign bodies by phagocytes.

Pneumoconiosis - Lung disorders caused by the inhalation of any aerosols, including mineral dusts, organic dusts, fumes, and vapors.

Reactive oxygen species - A collective term that includes not only the oxygen radicals ($O_2^{\cdot-}$, $RO_2^{\cdot-}$, $RO^{\cdot}$ and $OH^{\cdot}$) but also H_2O_2, $ONOO^-$, HOCl and even the non radical ozone (O_3).

Receptor - Molecular structure within the cell surface characterised by selective binding of a specific substance and accompanied by specific physiological changes in the cell.

Signal transduction - Involves an intracellular cascade of biochemical events that follow the interaction between extracellular factor and their membrane receptors, ending in a switch of nuclear mechanisms controlling the proper biological responses.

Silicosis - Form of pneumoconiosis caused by inhaling silica dusts from mining or stone crushing operations.

ABBREVIATIONS

ACE - Angiotensin converting enzyme

Al - Aluminium

AngII or AII - Angiotensin

ANP - Atrial natriuretic peptide

As - Arsenic

ATP - Adenosine triphosphate

ATR - Angiotensin receptor

Be - Beryllium

BNP - Brain natriuretic peptide

BSA - Bovine serum albumin

Ca - Calcium

CAT - Catalase

Cd - Cadmium

Ce - Cerium

$Ce(NO_3)_3$ - Cerium nitrate

Co - Cobalt

DAB - 3, 3' Diaminobenzidine tetrahydrochloride

DMH - 1, 2 Dimethyl hydrazine

DNA - Deoxyribonucleic acid

EDTA - Ethylenediaminetetracetic acid

EMF - Endomyocardial fibrosis

ET - 1 - Endothelin -1

FCS - Fetal calf serum

FGF - Fibroblast growth factor

GSH - Glutathione

HBSS - Hank's balanced salt solution

H_2O_2 - Hydrogen peroxide

Hyp - Hypoxanthine

IGF - Insulin like growth factor

IL - Interleukin

K - Potassium

KRB - Kreb's Ringer buffer

Ln - Lanthanum

Lu - Luteutium

MAPK - Mitogen activated protein kinases

Mg - Magnesium

Mn - Manganese

Na - Sodium

NAC - N- acetyl -L-cysteine

NADPH - Nicotianmide dinucleotide phosphate (reduced)

NBT - Nitroblue tetrazolium

$O_2^{\cdot}$ - Superoxide anion

PCNA - Proliferating cell nuclear antigen

PDGF - Platelet derived growth factor

PKC - Protein kinase C

SEM - Standard error of mean

Si - Silica

SOD - Superoxide dismutase

TBARS - Thiobarbituric acid reactive substance

TGF -β - Transforming growth factor - β

Th - Thorium

Ti - Titanium

TNF - Tumor necrosis factor

TPA - 12 - O - tetradecanoyl phorbol - 13 - acetate

XO - Xanthine oxidase

$ZnCl_2$ - Zinc chloride

REVIEW OF RELATED LITERATURE

CHAPTER - 2

REVIEW OF RELATED LITERATURE

Fibrosis is an essential element in physiological repair. It can also occur as a pathological process which leads to a diversity of serious and chronic diseases. The eliciting factor may be similar or dissimilar in different tissues. Cardiac fibrosis can be classified as reactive and reparative processes based on the mode of initiation of the proliferation of cells. Though the active role of low levels of free radical species in evoking growth responses in fibroblasts and other cells have been documented, metals provoking such a reaction in the cardiac tissue has not been reported. Recent literature pertinent to the study have been reviewed under the following headings :

Biological effects of lanthanides

Free radicals in health and disease

Cardiac fibrosis

BIOLOGICAL EFFECTS OF LANTHANIDES

Lanthanides or the rare earth metals constitute a series of fifteen metals beginning from Lanthanum (atomic number : 57) and ending with Lutetium (atomic number : 71) and are subgrouped as -

1) Light lanthanides or Cerium group - Lanthanum to Samarium
2) Medium lanthanides or Terbium group - Europium to Holmium
3) Heavy lanthanides or Erbium group - Erbium to Lutetium

Rare earths, as the name implies, are not abundant in the earth's crust. Among the rare earths, cerium is the most plentiful element and is about 100 times more abundant than cadmium, one of the most well known heavy metals in toxicology. These lanthanides occur together as oxides of the following minerals : xenotine firgusonite, gadolinite , cerite, lanthanite, euxenite, polyerase samarikite and monazite. Monazite sand serves as the major source. Cerium and lanthanum occur predominantly in rare earth minerals at concentration of about 40% and 25% respectively.

One of the most prominent features of lanthanoids is what is called as lanthanoid contraction (Hirano and Suzuki, 1996). From La to Lu, the radius of lanthanoid ions (+3) decreases as the atomic number increases. This phenomenon is due to the attraction of electrons in the 4f orbitals by increasing positive charge of the nucleus with the

increase in atomic number. Because the radius of calcium (0.99 A°) is very close to those of lanthanoids, these metals have been used as calcium probes in biochemical and physiological studies.

In the past, the use of lanthanoids was restricted to five main areas, glass polishing , carbon arcs, cracking catalysts, flints for lighters and modulating agents for iron. Cerium in particular was used in the treatment of tuberculosis and as anti-nausea agent during early pregnancy. It is also reported that cerium is a potent antiseptic drug for gram - negative bacteria and fungi (Monafo et al, 1976). Swabbing of lanthanum is effective in protecting teeth from caries (Sakurai,1982; Ozeki et al, 1979). Cerium in specific has the following industrial uses - lighters, catalysts, glass additives, ceramics, magnets, abrasives (Ito, 1985; Ohmachi, 1988). Lanthanides have marked anticoagulant properties which may result from antagonism of certain clotting factors which require calcium (Johansson et al, 1968). Intracranially injected lanthanides share the analgesic properties of opiates (Harris et al, 1975). They are thought to modify calcium fluxes across cellular membranes in specific regions of brain. At higher doses, lanthanides cause epileptoid fits and loss of motor function (Weinmann et al, 1984). Long term exposure to rare earth plays a role in the pathogenesis of the observed pulmonary fibrosis of industrial workers (Vocaturo et al, 1983). Haley (1991) suggests that the toxicity of lanthanides is related to the type and physicochemical form of the material inhaled and to the dose and

duration of exposure. Chronic exposure of industrial workers to dusts which contain lanthanides lead to a condition called "rare earth pneumoconiosis" (Heuck and Hoschek, 1968; Napee et al, 1972; Husain et al, 1980).

The chemical forms of rare earth compounds primarily determine deposition and retention of the element following intravenous, per oral, subcutaneous, intra tracheal and inhalation exposure. The clearance of chelated rare earth metals from the body depends on the stability of the complexes. The chelated rare earths are excreted rapidly via urine, while unchelated ionic rare earth easily form colloid in blood, and the colloidal material is taken up by phagocytic cells of the liver and spleen (Oksendal, 1993).

Although bone is one of the target organs of the rare earth, it is not clear what cells in the bone has maximum affinity for rare earth. Both macrophages (Berry et al, 1989) and erythroid cells or light reticular cells (Shaklai and Tavassoli, 1982; Tavassoli et al, 1980) have been reported to assimilate rare earth metals. In the bone marrow of rats, only macrophages were found to take up intra peritoneally injected cerium chloride (Berry et al, 1989). However, lanthanum was found in the bone marrow cells and the cell sap of light stromal cells when rat bone marrow cells were exposed to lanthanum nitrate in vitro under fixing conditions (Shaklai and Tavassoli, 1982; Tavassoli et al, 1980).

Whole body retention and tissue distribution of intravenously injected rare earth primarily depend on the stability of rare earth in blood. Chelated rare earth is excreted mainly via urine after transient accumulation in the kidney. Rare earth chlorides are taken up by the liver and spleen and are not easily excreted. Rosoff et al (1963) have suggested that in mice rare earth chlorides are changed into colloidal forms of hydroxide, phosphate, and carbonates in the blood.

Intravenously injected rare earth chlorides in mice increase vascular permeability for low molecular weight substances (Marciniak et al, 1988) and cause necrosis in the liver (Salonpaa et al, 1992). Intra venous injection of lanthanum chloride and cerium chloride increased vascular permeability of the spleen in mice (Marciniak et al, 1988), and both subcutaneous and per oral administration of cerium citrate caused hypertrophy, reticuloendothelial hyperplasia and hyperactive lymphoid follicles (Stineman et al, 1978).

Inhaled or intratracheally instilled rare earth chlorides have been shown to accumulate in alveolar and tissue macrophages and alveolar walls in humans and rats (Hirano et al, 1990; Suzuki et al, 1992; Galle et al, 1992; Berry et al, 1989). In macrophages, rare earth compounds have been shown to localize in lysosomes, according to Gomori's (phosphatase) reaction (Galle et al, 1992). The dose of the compounds influences the pulmonary retention and translocation of rare earths in the lung, because it has been shown in the rats that translocation of

yttrium from the lung to the bone decreased as the deposition in the lung increased (Wenzel et al, 1969).

It is reported that intra - peritoneally injected cerium chloride or cerium citrate was deposited mainly in the liver and skeleton in hamsters (Sturbaum et al, 1970) and rats (Kargacin et al, 1986). On oral intake through drinking water or peroral administration, ionic rare earth was absorbed mainly from the ileum of rats and swines (Sullivan et al, 1984; Kostial et al, 1987 and 1989) and deposited in the skeleton, teeth, soft tissues such as the lungs, liver and kidney (Sakurai, 1982; Rabinowitz et al, 1988; Eisele et al, 1980; Menczel et al, 1982). It has also been reported that intra peritoneal injection of cerium chloride causes lipid peroxidation and a decrease in glutathione reductase activity in the chick liver (Basu et al, 1984).

It has been shown that only 13.3% of perorally administered cerium chloride was excreted via bile during the first four hours in rats (Kitani et al, 1977), suggesting that a significant amount of cerium was absorbed from the intestine. However, the intestinal absorption of rare earths seems to depend on the diet. Fasting significantly increased the absorption of rare earth from the gastrointestinal tract in adult rats (Sagan and Lengemann, 1973; Sullivan et al, 1986). Oral administration of cerium citrate has been shown to cause focal hemorrhage, necrosis of mucosa, and neutrophil infiltration in the stomach and duodenum (Stineman et al, 1978).

Absorption of the rare earth by the skin is known to be negligible as evident from studies in guinea pigs (Inaba and Yasumoto, 1979). However, when the skin was lacerated or wounded, rare earth was seen to be absorbed into the body to some extent (Inaba and Yasumoto, 1979; Takada, 1978). Inaba and Yashumoto (1979) reported that 4% of applied cerium chloride was absorbed from the stripped guinea pig's skin while 89% of cesium chloride and 79% of cobalt chloride were absorbed from the skin under the same experimental conditions. Intra muscularly injected cerium chloride has been reported to accumulate in the lysosomes of the liver in rats and hamsters (Seidel et al, 1986).

The pathological consequences of cerium intake are dependent on a number of variables including species and sex and also the mode of entry. Subcutaneous administration of $Ce(NO_3)_3$ has also been found to cause hepatic necrosis (Stineman et al, 1978). Hepatic endoplasmic reticulum has been shown to be the primary target of intra -venously injected cerium chloride in the rat liver. Dilation, disorganization and degranulation of rough endoplasmic reticulum and proliferation of smooth endoplasmic reticulum occured (Salas et al, 1976). It has been shown that intra venous injection of cerium chloride caused fatty liver in female rats but not in male rats (Salas et al, 1976; Mugnusson , 1963). The reason that fatty liver was limited to female rats that received cerium chloride remains unknown. It is observed that intra venous

injection of cerium chloride produces lipid droplets in the liver of male mice (Salonpaa et al, 1992).

It has also been shown that subcutaneous or intra dermal injection of rare earth chlorides caused local calcification, called calcergy. (Selye, 1962; Haley and Upham, 1963). Histological examination of the calcification site by Garrett and McClure (1981) revealed close association of the mineral with collagen fibers of the dorsal fascia. Deposits were surrounded by mild fibrosis and accumulation of multinucleated giant cells; and the calcification area was proportional to the dose (upto 2 mg of rare earth chlorides) in mice (Garrett and McClure, 1981). It has been shown that cerium was deposited in the liver, spleen and bone of mice following subcutaneous injection of cerium citrate (Morganti et al, 1978; Stineman et al, 1978).

Cerium exposed mice exhibited significantly reduced open - field behavior. Ambulations were depressed after 10 subcutaneous injections (at 3 day intervals) of cerium nitrate at 20 mg Ce/ kg body weight (Morganti et al, 1978), and ambulation and rearing were depressed following subcutaneous injection of cerium citrate at doses of 136 to 173 mg Ce/ kg body weight (Stineman et al, 1978).

A number of experimental studies have been carried out on the effect of lanthanides on growth and cell proliferation. Evans (1913) reported that the carbonates of cerium and lanthanum increased the rate of cell division in hyacinth roots while yttrium carbonate had the opposite

effect. Chinese scientists have investigated the effects of cerium chloride upon the growth of corn seedlings. At low concentrations (0.5 ppm), growth was slightly stimulated, while a tenfold higher concentration inhibited growth of the shoots and particularly the roots (Tang and Li, 1983).

Following their findings that lanthanides can enhance the growth of plants under certain conditions, the Chinese have developed a fertilizer known as "Nong-le" which contains lanthanides (Guo, 1985). When applied to crops at appropriate stage of growth, at a concentration of 450-750 g Ln/ ha, the yield apparently increased. Excess amounts of the fertilizer are toxic to crops, and application after the primary growth stage depresses the yield. "Nong-le" is reported to promote the growth and quality of sugarcane, apples, wheat, rice and various other crops. The mode of action is unknown, but it has been suggested that lanthanides increase the uptake and transport of phosphatases.

The effects of lanthanum ions on cell division are interesting. Hepler in 1985 reported that 0.1 mM lanthanum extended the metaphase of plant stamen hair cells. In some cases, metaphase was completely arrested. There was no change in the rate of cell plate initiation or in the rate of chromosome motion. However, micromolar concentrations of various lanthanum ions accelerated another stage in the cell cycle, namely the G_o / G_1 $\rightarrow$ S transition. As little as 1 μM lanthanum had a stimulatory effect. In 3T3 fibroblasts, 5 μM lanthanum

acted synergistically with insulin or colchicine in stimulating DNA synthesis. In 3T6 fibroblasts, it had a direct effect of its own, which was nevertheless stimulated by insulin. Although lanthanum ions slowly precipitated in the culture medium, stimulation of DNA synthesis was independent of this effect, as a 2 hour exposure of the cells to lanthanum ions in buffer was sufficient for its induction. The mode of action is unclear (Smith and Smith, 1984).

These findings may be related to the ability of lanthanum and terbium (0.01-1 mM) to induce the anchorage independent growth of a preneoplastic, murine epidermal cell line. Lanthanum proved as effective as TPA (12 - O - Tetradecanoyl ` phorbol - 13 - acetate) in this regard, and was even able to induce such growth in a variant line which resisted induction by TPA. Unlike TPA, lanthanum failed to activate protein kinase C in intact cells (Smith et al, 1986). Anchorage independent growth is usually associated with malignant transformation. Although subcutaneously injected pellets of metallic lanthanides have been reported to cause neoplasms (Talbot et al, 1965; Ball et al, 1970) and $Y(NO_3)_3$ in the drinking water produces malignant tumors in the rats (Schroeder and Mitchener, 1971), the bulk of the evidence from animal studies suggests that lanthanides are not strong carcinogens (Ball and Gelder, 1966; MacDonald et al, 1952). Lanthanides promote DNA synthesis in cultured fibroblasts, but not lymphocytes (Yamage and

Evans, 1989). The early stages of embryogenesis is also found to be stimulated by the metal ion (Abramczuk, 1985; Whittingham, 1980).

A number of toxicological studies have been reported, following investigation of the effect of lanthanides on microorganisms. Lanthanides have been found to inhibit the growth of bacteria, fungi and yeast (Muroma, 1958), but none have addressed the mode of inhibition. The concentrations required to induce this effect are in the range of 10^{-4} - 10^{-2} M. Even higher concentrations are required for cidal effects. The toxic mechanisms involved are unknown, although microbial respiration is strongly suppressed by lanthanides. It has also been reported that low concentrations of around 10^{-5} M may stimulate bacterial growth (Muroma, 1958).

Among the first physiologic experiments to be carried out on the effects of lanthanides on cardiac tissue were those of Mines in 1910, who showed that micromolar concentrations of lanthanum reversibly and quickly produced a diastole arrest in frog's heart. This was seen when the heart was perfused with Ringer solution containing lanthanum, despite the presence of bicarbonate. Diastolic arrest did not occur when lanthanum ions were injected into the bloodstream, which could be because of the non availability of free lanthanum ions to the cardiac tissue under in vivo conditions.

Fawzi and McNeill (1985) produced 50% inhibition in the contraction of adult guinea pig hearts at 0.19 ± 0.01 μM lanthanum, although higher

concentrations were needed to inhibit the positive inotropic effect of isoproterenol.

Certain cardiac responses to perfused lanthanum depend upon age. Although lanthanum quickly inhibited the developed tension in both adult and newborn rabbit ventricular preparations, it increased the resting tension in the newborn only (George and Jarmakani, 1983). The explanation is unknown, but these results may reflect maturational changes in membrane structure.

Sanborn and Langer (1970) found 5 - 20 μM lanthanum to be a potent uncoupler of excitation and contraction which did not alter the action potential in arterially perfused lapine interventricular septa.

Addition of lanthanum ions (0.1 - 4 mM) to cultures of neonatal rat ventricular myocardiocytes diminished contraction frequency and strength along with reduction of membrane potential and overshoot, and the action potential tended to be prolonged. Complete inhibition of spontaneous contraction was always accompanied by membrane depolarization and absence of action potentials. These effects were reversible (Kitzes and Berns, 1979).

Lanthanides not only suppresses the basal contraction and isoproterenol inotropy of perfused rat hearts, but they also inhibit isoproterenol - induced glycogenolysis (Bockman et al, 1973). Glycogen breakdown involves a calcium dependent activation of phosphorylase b to phosphorylase a. Isoproterenol increases the percentage of

phosphorylase present as the active (a) form. Although, lanthanum ions do not alter the basal level of active phosphorylase, they inhibit the isoproterenol induced increase in phosphorylase a. At a concentration of 1 mM, lanthanum ions had no effect on basal or stimulated transport, and intracellular sodium and potassium levels were unaffected (Bihler et al, 1980).

The presence of cerium in conjunction with lower levels of magnesium in the cardiac tissue of patients suffering from endomyocardial fibrosis, led to the postulation of a geochemical basis for the disease (Valiathan et al, 1990). The role of magnesium deficiency may be synergistic in so far as it enhances the absorption of cerium. The presence of cerium in conjunction with magnesium deficiency suggested the possibility that endomyocardial fibrosis could be the cardiac expression of an elemental interaction.

In pursuance of the geochemical hypothesis several studies were conducted both in vitro and in vivo. Enhanced levels of cerium was found with associated myocardial lesions in hypomagnesaemic rats fed on cerium adulterated diet (Kartha et al, 1992). Though the gastrointestinal absorption of cerium is poor in all mammalian species , absorption has been found to be greater in younger animals; and also dietary deficiencies of calcium, phosphorous, and vitamin A have been reported to increase the accumulation of cerium (Venugopal and Luckey, 1978). The mechanism by which magnesium deficiency promotes cerium accumulation in the tissues is not clear. Magnesium

deficiency may enhance vascular permeability (Gunther, 1990) and increase uptake from absorptive surfaces which could contribute to the accumulation of cerium. A second probability could be the irreversible bonding of cerium in place of magnesium in tissues. Renal lesions which characterize magnesium deficiency may also play a role in cerium accumulation due to impaired excretion.

Cerium is relatively non toxic. However, when compared with other metals within the lanthanide group, cerium is more toxic (Venugopal and Luckey, 1978). The continued administration of cerium *per se* did not cause morphological lesions in rats. But the number of animals with lesions and the severity of the myocardial lesions were greater in the group which was administered a magnesium deficient and cerium adulterated diet (Kartha et al, 1992). At concentrations as low as 100 nM, cerium was found to stimulate collagen and non- collagen protein synthesis in cardiac explants and fibroblasts and the stimulation of collagen synthesis by low levels of cerium may contribute to the accumulation of collagen as observed in endomyocardial fibrosis (Shivakumar et al, 1992). Perivascular and subendocardial fibrosis was observed in rats and rabbits on magnesium deficient diet rich in cerium (Kumar et al, 1996; Kartha et al, 1998). In another study increased lipid peroxidation was associated with elevated rates of cell proliferation and collagen deposition in the heart of cerium treated rats, implicating the element to trigger a wound healing response in the cardiac tissue leading to

cardiac fibrosis (Kumar & Shivakumar, 1998). However, the mechanism of induction of a fibroproliferative response remains unexplained.

FREE RADICALS IN HEALTH AND DISEASE

The propagation and maintenance of life requires substantial energy. The evolution of life on earth led to utilization of oxygen as the major mediator of energy release from organic molecules. The participation of oxygen in energy release is not absolutely essential for maintenance of life, as demonstrated in anaerobic and sulfur bacteria that function without it. Oxygen mediated energy release is favored by most organisms for the following reasons

1) its ready availability
2) high energy yield from oxidation
3) reversibility of the process (oxygen is consumed by energy - releasing process such as cellular respiration and evolved in energy trapping processes such as photosynthesis)
4) the easy distribution of oxygen due to its gaseous state and its solubility in bio - components under normal conditions.

The outstanding benefits from oxygen mediated oxidative energetics, as everything else in life , has a darker side. The dark side of oxygen is manifested either directly or in less obvious ways. It has long been known that breathing pure oxygen (100% O_2 1 atm) instead of air (20% O_2 1 atm) is detrimental to living organisms because uncontrolled and undesirable oxidative processes are enhanced by higher oxygen concentrations in

tissues. A less obvious and only recently known , detrimental facet of oxygen is the formation of oxy-radicals. The reasons for the substantial lag between the discovery of free radicals in model systems and of free radical processes in vivo were the generally short life span of radicals and the difficulties associated with the direct detection and monitoring of free radicals in vivo. It is ironic that recognition of free radicals in vivo often resulted from the discovery of biological defence systems and agents capable of neutralizing free radicals and their products in cells and intercellular regions.

An historic overview as presented by Simic and Taylor in 1988, reveals that the modern concept of free radicals is a product of this century , although the rudimentary idea was first conceived by French and German organic chemists in the 1800s. According to the authors, during the 1930s, Haber and Weiss dealt with the interrelationship between H_2O_2 , $^{\cdot}OH$ radicals and $^{\cdot}O_2^-$ and Michealis discussed free radicals in biochemistry. The generation of superoxide anions by Xanthine - Xanthine Oxidase was demonstrated by Fridovich and McCord much later, in the 1960s and the participation of free radicals in the arachidonic acid cascade was recognised by Samuelson in the 1970s. The authors further state that most of the major advances in the area have been due to the development of pulse radiolysis and highly sensitive analytical instrumentation . The implication of free radical processes are beginning to touch every biologically and medically oriented discipline, such as

replicative inactivation of DNA, mutation, carcinogenesis, atherosclerosis, arthritis, and aging. What began as an obscure idea outside the mainstream of chemistry, has now become essential for understanding the basic processes of life, conception, growth, aging, disease and death. In 1900, radicals (i.e. groups of atoms such as $-CH_3$ in free form) were recognised by Gomberg, who discovered the relatively stable triphenylmethyl radical. The current broader definition of a free radical is a molecular or atomic species with an unpaired electron.

Free Radicals

Oxy radicals are a class of free radicals with the unpaired electron residing predominantly on an oxygen atom. The best known examples are superoxide radical, hydroxyl radical and various other peroxy radicals. Free radicals are generated via numerous processes in the atmosphere, environment, foods, chemical systems and living organisms. One of the most convenient modes of free radical generation, especially for studying their properties is ionizing radiation such as X and γ rays and high - energy electrons. In aqueous systems radiation splits water

$$H_2O \rightarrow OH^{\cdot} + e^{-}aq + H^{\cdot +}$$

where as in organic media it breaks chemical bonds for example

$$RH \rightarrow R^{\cdot} + H^{\cdot}$$

Atmospheric pollutants such as ozone or the nitrogen oxides (NO & NO_2) can react with biologic molecules to form reactive free radicals

(Pryor, 1982) . Free radicals are also found in burning organic matter such as bonfire smoke (Pryor et al, 1983) .

A more important source of free radicals normally is those that are constantly produced within the body from oxidation- reduction reactions (Del Maestro et al, 1981; Halliwell & Gutteridge, 1984). A variety of enzyme systems catalyse the univalent reduction of molecular oxygen to superoxide anion radical eg - xanthine oxidase, aldehyde oxidase, flavin dehydrogenases and peroxidases for example, Urate oxidase, D-amino acid oxidase, glycolate oxidase (McCord & Fridovich, 1968).

Such univalent reduction of molecular oxygen also occurs in vivo in non-enzymatic electron-transfer oxidation-reduction reactions. For example

$$\text{hydroquinone} + O_2 \rightarrow \text{semiquinone} + O_2^{\cdot -} + H^+$$

and also during auto-oxidation reactions, including those that involve catecholamines, flavins, thiols, reduced ferridoxins and tetrahydropterins. However, an important source of superoxide anion radical remains the univalent leak of superoxide anion radical from mitochondrial electron transport system (Boveris, 1977). Superoxide anion radical is also produced by the reduced nicotinamide- adenine dinucleotide phosphate (NADPH) oxidase system present on the surface of inflammatory cells including neutrophils, eosinophils, monocytes and macrophages (Babior, 1978 a,b; Babior and Peters, 1981).

A major source of H_2O_2 in the body is the dismutation of superoxide anion radical, a reaction catalysed by the superoxide dismutase enzyme (Fridovich, 1983). This dismutation reaction occurs in two stages with superoxide radical first combining with a proton to form the hydroperoxyl radical $HO_2^{\cdot}$.

$$O_2^{\cdot} + H^+ \rightarrow HO_2^{\cdot}$$

$$HO_2^{\cdot} + O_2^{\cdot -} + H^+ \rightarrow H_2O_2 + O_2$$

Hydroxyl radicals are formed in the Fenton reaction whenever hydrogen peroxide comes into contact with ferrous or cupric ions (Halliwell and Gutteridge, 1984) . This radical may also be formed by an iron-catalysed Haber-Weiss type of reaction, the net effect of which is an interaction between hydrogen peroxide and superoxide anion radical in the presence of traces of transition metal ions to form the hydroxyl radical, hydroxyl ion, and oxygen (Halliwell and Gutteridge, 1984). This radical is also a product of ionizing radiation.

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{\cdot} + OH^-$$

$$O_2^{\cdot} + H_2O_2 \rightarrow O_2 + OH^{\cdot} + OH^-$$

Free radicals vary in their activity. Some are relatively stable, but most free radicals of biologic interest tend to be extremely reactive and

unstable ; as a result , they have a very short life span. Because of their reactivity, most free radicals exist only at low concentrations (from 10^{-9} to 10^{-4} M) and do not travel far from their site of formation. Thus, a hydroxyl radical formed in the mitochondria is unlikely by itself to have a direct effect in other parts of the cell - for eg. DNA in the nucleus.

Free radicals can act both as oxidants and as reducing agents. When a free radical reacts with a non radical compound, other free radicals are formed. This enables free radicals to induce chain reactions that may be thousands of events long for example lipid peroxidation involving polyunsaturated fatty acids. When two free radicals react with each other, a stable molecule may be formed. This fact helps explain the eventual termination of free radical induced chain reactions.

The superoxide anion radical is not a particularly reactive species but is potentially toxic. It may directly influence local homeostasis by, for example, oxidizing catecholamines (Wolin and Belloni, 1985). More importantly , it can be transformed into the highly dangerous hydroxyl radical which may react with any biologic molecule present in its vicinity. The hydroxyl radical may be considered as the ultimate damaging species, whenever superoxide is formed. Cellular components damaged by free radicals are:

Lipids : Peroxidation of polyunsaturated fatty acids in organelles and plasma membranes.

Proteins : Oxidation of sulfhydryl containing enzymes - Inactivation of enzymes.

Carbohydrates : Polysaccharide depolymerization.

Nucleic acids : Base hydroxylation, cross linkage, scission of DNA strands.

Protection against free radical injury

Free radicals are extremely reactive in general and can inflict considerable damage to biomolecules such as DNA, enzymes, membranes and proteins, which may lead to serious biological consequences such as cancer, damage to organs and numerous other pathologic disorders. Each cell has its inherent protection system to combat the free radical stress. However, when free radical generation exceeds the buffering capacity of the inherent defensive mechanism, pathological consequences set in, and then the supplementation with essential antioxidants becomes necessary to overcome the oxidative stress and the pathological conditions associated with it. The endogenous defence system includes both enzymatic and non enzymatic free radical scavengers.

Protection from free radicals may occur by intervention at different stages of free radical processes. One way to intervene is at the pre-radical stage by eliminating H_2O_2 and ROOH with catalase or glutathione peroxidase or by inactivating metal ions with complexing agents . Once free radicals are generated, they can be scavenged by

different agents. Protection agents could be either scavengers, repair agents, antioxidants or antioxyenzymes.

Free radical scavengers - The most popular free radical scavenger in biosystems has been DMSO (dimethyl sulfoxide) due to its high solubility in biomaterials. DMSO does not eliminate free radicals but replaces it with a methyl radical. Another example of commonly used scavenger is mannitol.

Repair Agents - Sulfhydryl compounds with a weak S-H bond strength (~ 85 Kcal / mol) are capable of repairing C- centered radicals which usually have bond strengths > 90 Kcal / mol.

Antioxidants - These can be divided into two broad classes- those with enzymatic and those with non-enzymatic activities. In the first group are enzymes that remove reactive oxygen species (superoxide dismutase, catalase, glutathione peroxidase), molecules blocking enzymatic activity (eg. Allopurinol - a xanthine oxidase inhibitor) and molecules capable of trapping metal ions, which are potent catalysts of free radical reactions (eg. desferrioxamine or lazaroid compounds).

In the second group are molecules, which interact mole by mole with the free radical and are therefore consumed during the reaction for eg. Vitamin A (a quencher of singlet oxygen), vitamin C, glutathione, mannitol, albumin, probucol, N-acetyl-L-cysteine. Vitamin E and butylhydroxyl toulene are the chain breaking antioxidants included in this group.

Free radicals and disease

Free radicals are the primary means by which neutrophils kill bacteria , by which radiation injures both malignant and normal tissues and by which many drugs, carcinogens and other toxic agents exert their effects.

In normal phagocytosis, cytotoxic oxygen species are used to destroy invading microorganisms (Babior, 1978a,b; Klebanoff, 1980). When activated, polymorphoneutrophils and macrophages immediately consume large quantities of oxygen, which is transformed almost quantitatively into superoxide anion radicals. This process is called the respiratory burst and is due to the enzyme reduced NADPH oxidase, which is located on the exterior surface membranes of the cell and the lining of the phagocytic vacuole. The superoxide anion is subsequently converted into H_2O_2, $OH^{\cdot}$ and singlet oxygen. The cytotoxicity of hydrogen peroxide itself is considerably enhanced in the presence of myeloperoxidase which is simultaneously released from the azurophil granules into the phagocytic vacuoles. The enzyme-H_2O_2 complex that is formed can oxidize various halides to produce hypochlorous acid which has a potent bactericidal action. Although this oxidase system is not solely responsible for the cytotoxic effects of phagocytes, its importance in human host defense is exemplified by the genetic disorder chronic granulomatous disease - where polymorphoneurtrophils from patient can ingest microorganism normally, but cannot generate

sufficient reactive oxygen species to kill catalase containing microorganism. Patients with this disease have recurrent infections, abscesses and tissue granulomas.

It has also been found that oxygen radicals released during ischemic preconditioning contribute to cardioprotection in the rabbit myocardium (Baines et al, 1997). Studies have documented that low levels of free radicals can activate protein kinase C directly (Gopalakrishna and Anderson, 1989). In addition, reactive oxygen species may stimulate phospholipase D (Natarajan et al, 1993). The resulting production of diacylglycerol could then lead to the activation of PKC. Hence, a mechanistic rationale that oxygen radicals could be involved in the induction of cardioprotection exists. Studies by Baines et al (1997) indicate that oxygen free radicals produced at the end of the preconditioning ischemia represent a third trigger for protection which appear to act in parallel with adenosine and bradykinin.

Evidences have shown that controlled generation of these highly reactive molecules has important roles in the blastocyst implantation (Laloraya et al, 1989), disintegration of the structural elements of the sperm cells (Chatterjee et al, 1994), iodination of tyrosine in the thyroxine biosynthesis (Prakash et al, 1993; Verma et al, 1990), and secretion of mucous in goblet cells (Parihar & Dubey, 1995; Prakash et al, 1998)

Uncontrolled generation of free radicals are known to cause an array of diseases. Conditions are many where free radicals may be the sole

cause of disease or in some may just be a predisposition factor. Diseases may be classified into two groups depending on the target - those that affect the organism as a whole and those in which only one organ is affected. Inflammatory-immune injury (Lunec, 1991), ischemia reflow states (Powell and Tortolani, 1992), drug toxicity (Hecht, 1986), iron overload (Young et al., 1994), alcohol toxicity (Peters et al, 1986), radiation injury (Korkina et al, 1993), aging (Mecocci et al, 1993), cancer (Frey et al, 1987), and amyloid diseases (Harman, 1984) belong to the first group. In the primary single organ group are found erythrocytes (Clark & Hunt, 1983), blood vessels (Janero, 1991; Illingworth, 1993), lung (Pincemail et al, 1989; Malvy et al, 1993; Leuenberger 1994), the heart and cardiovascular system (Coghlan et al, 1991a), kidney (Pincemail et al, 1993), the gastrointestinal tract (Scott et al, 1993), joint abnormalities (Humad et al, 1988; Meier et al, 1990), brain (Hall & Braughler, 1989; Adams & Odunze, 1991; Girotti et al, 1991), eye (Spector, 1991) and skin (Fuchs & Packer, 1991).

Cellular redox state is an important functional parameter that modulates gene expression (Marui, 1993), activity of signalling pathways (Rao, 1996) and paracrine factors (Gryglewski et al, 1986), apoptosis (Stoian et al, 1996) and cell growth (Rao and Berk, 1992). Cellular redox state reflects a balance between processes that promote oxidative or reductive pathways in the cell.

Free radicals in cell proliferation

From various studies it is clear that certain free radical derived species can have a significant modulatory influence on components of major growth signal transduction mechanisms. Free radicals themselves appear to have a down regulatory effect on cell proliferation in as much as protection from oxidative stress enhances cell proliferation. On the other hand, in certain cases low non toxic levels of free radicals can exert a stimulatory effect on cell proliferation rather than promoting apoptosis or cell necrosis (Burdon, 1995; Suzuki et al, 1997).

Growing experimental evidence suggests that the generation of reactive oxygen species participates in cellular activation and intracellular signal transduction. For example superoxide anion and hydrogen peroxide have been implicated in the activation of phospholipase D, p42/p44 MAPK, p38 MAPK (Huot et al, 1997; Baas and Berk, 1995; Natarajan et al, 1993)

Recent reports on the role of free radicals in cell proliferation suggests that hydrogen peroxide formed during γ- glutamyl transpeptidase enzymatic activity appears to stimulate or maintain U937 cell proliferation (Bello et al, 1999). Lee and others (1998), in their study found that superoxide anion acted as an intermediate signal for serotonin stimulated mitogenesis in bovine pulmonary artery smooth muscle cells. Extensive proliferation of connective tissue around vitallium implants can be observed in young patients who had limb salvage for primary malignant bone tumors. Windhager et al (1998) based on their finding suggest

that hydroxyl radical attack lead to free radical mediated cross linking of collagen with subsequent collagen accumulation, as collagen cross linked to a higher degree is less susceptible to proteolytic degradation. The hydroxyl radical attack seems to be generated by the many transitional metals of vitallium - alloy. Gamberini and Leite in 1997 reported that activation of 1,2 - dimethylhydrazine (DMH) by prolonged auto-oxidation (24 hour) induced proliferation of mouse fibroblasts at low hydrazine concentrations (0.1-1.0 mM) and attributed that hydroxyl radicals mediate the cell proliferation following auto-oxidation. The antioxidant N - acetyl - L - cysteine (NAC) has been increasingly used as an experimental tool to assess the involvement of reactive oxygen species in cell signaling and is currently being evaluated as a preventive and therapeutic agent for cancer and pulmonary diseases related to inflammation and oxidative stress. Sekharam et al (1998) studied the modulation of cell cycle progression by NAC in mouse fibroblast NIH3T3 cells and found that NAC blocked the cell cycle in the G_1 phase.

Yabe and Matsui (1997), infer that proliferation of dermal fibroblasts isolated from rats may be stimulated through the active oxygen generation mediated by a redox cycling between Fe^{3+} and Fe^{2+}, which are dissolved in the medium at a high concentration, rather than through delivery of iron into the cells. H_2O_2 at a low concentration (1 μM) has been found to stimulate proliferation of BHK - 21 cells (Burdon et

al, 1996); and Yang et al (1996) have shown that subtoxic concentrations of adriamycin induces cell proliferation via an H_2O_2 mediated mechanism in human lymphoblastic leukaemic cells. The much endangered species, the hydroxyl radical too has been implicated in cell proliferation. Burch et al (1997) in their study on Grave's opthalmopathy suggested that oxygen free radicals may contribute to the retro - ocular fibroblast proliferation.

The search for the causative factors in Dupuytren's contracture has historically progressed from gross anatomical dissection, through microscopical tissue studies, to the biochemistry of the collagen produced. Studies (Murrell et al, 1990) show that the fibroblasts in Dupuytren's contracture is identical to palmar fascia fibroblasts in people unaffected by the disease and also to all other fibroblasts. The only difference is that there are more of them in Dupuytren's contracture and are clustered around narrow microvessels. Hueston and Murrell (1990) suggested that a number of conditions including localized microvascular ischemia and high alcohol concentrations transform the benign xanthine dehydrogenase of endothelial cells to the oxygen free radical releasing xanthine oxidase which can stimulate fibroblast proliferation.

Increased generation of active oxygen species such as H_2O_2 and superoxide anions stimulate vascular smooth muscle cell growth and DNA synthesis (Rao and Berk, 1992). This proliferation was found to be

associated with the induction of several growth related proto - oncogenes including c - myc and c - fos and the induction of proto-oncogene mRNA expression was found to be in a protein kinase C dependent manner (Rao and Berk, 1992; Rao et al, 1993 a; Rao et al, 1993 b). Other investigators have demonstrated that H_2O_2 and superoxide anion stimulate growth related events such as cell alkalinization and proto-oncogene induction (Shibanuma, 1988 a; Shibanuma, 1988 b). Active oxygen species may act as growth factors by direct oxidation of sulfhydryl groups, leading to activation of growth regulatory factors, or by formation of transition metal complexes, which may inhibit protein phosphatases. Examples of active oxygen species acting in this fashion include dimerization of Fos - Jun proteins (Abate et al, 1990), activation of NF - kB (Schreck et al, 1991), activation of endoplasmic reticulum tyrosine kinases (Bauskin et al, 1991), stimulation of kinases involved in growth related signal transduction (Devary et al, 1992), activation of mitogen activated protein kinases by H_2O_2 and superoxide anion in vascular smooth muscle cell growth and DNA synthesis (Baas and Berk, 1995). These studies indicate that active oxygen species share properties with growth factors. These species at submicromolar levels appear to act as novel intra and intercellular messengers capable of promoting growth response in culture. The mechanisms may involve direct interaction with molecules such as protein kinases, protein phosphatases, transcription factors or

transcription inhibitors. However, critical balances may exist in relation to cell proliferation on one hand and cell death on the other.

Free radicals are now considered as true signal molecules under subtoxic conditions, molecules that are able to transfer information from outside of the cell to inside thus modulating diverse biological activities.

CARDIAC FIBROSIS

During the early nineteenth century, medical research focused on understanding the structural basis of disease (as opposed to its functional basis, in which persistent structural abnormalities were not thought to be involved). In a review by Weber et al (1992), it is stated that the workers in the nineteenth and twentieth centuries, laid the ground work for the study of anatomic pathology in which the importance of structurally remodelled tissue, mediated by cell behaviour was recognized.

The uniformity of cellular and structural elements of the heart and circulation is altered by disease. This remodelling of tissue structure, mediated by transcriptional, translational or posttranslational events within resident cells and extracellular space, may be adaptive or pathologic. Cardiac myocyte growth accompanying increased myocyte work load, for example is an adaptive alteration in myocardial structure seen with excercise training. However, left ventricular hypertrophy in hypertension, an adaptive phenomenon, is associated with the subsequent appearance of symptomatic heart failure (Kannel, 1989). The growth or altered metabolism of non myocyte cells, such as cardiac fibroblasts, vascular smooth muscle cells and endothelial cell, alters myocardial structure and ultimately its function. Therefore factors that regulate the growth and metabolism of non-myocyte cells require elucidation.

Fibrosis is any excess of fibrous tissue. It is primarily a defense process that can be helpful by contributing to the walling off of infected areas and is also the end point of wound healing; the scar that restores the continuity of severed tissue. Fibrosis can even contribute to disease - an excess or inappropriate stimulation resulting in fibrosis of an organ, can impair its function. It is the mechanism of this aberration that remains to be deciphered.

The myocardium comprises many different cells. Cardiac myocytes, the largest of these cells occupy 75% of its structural space; but constitute only one-third of the cell population (Zak, 1973; Frank and Langer, 1974). The other cells found in the cardiac tissue include :

(1) Endothelial cells, forming the ubiquitous lining of the coronary and lymphatic vasculature and endocardium and which are known to influence the vasomotor reactivity of blood containing vessels (Vanhoutte, 1989)

(2) Vascular smooth muscles, which are found in epicardial and intramyocardial coronary arteries and arterioles and like endothelial cells, influence the reactivity and vasodilatory capacities of these vessels (Owens, 1989)

(3) Macrophages and mast cells, found in the circulation, which are defenders against invasion by foreign proteins

(4) Cardiac fibroblasts, the stromal cells that constitute greater than 90% of the non myocyte cells (Eghbali et al, 1988). These cells are the multipotent cells that reside within the interstitial space of the myocardium

and the walls of arteries and veins. They contain the mRNA for types I and III collagens, the major fibrillar collagens of the heart and circulation (Eghbali et al, 1988 and 1989) that constitute its normal structural protein network (Medugorac and Jacob 1983; Weber at al, 1988). These collagens are involved in the interstitial and perivascular fibrosis of the myocardium (Weber et al, 1988) and the replacement scarring that follows cell death (Clore et al, 1979; Whittaker et al, 1989). Like endothelial and vascular smooth muscle cells, fibroblasts are capable of reentering the cell cycle and can therefore undergo mitosis or hyperplastic growth.

Fibroblasts consist of subpopulations with unique phenotypes and functions (Fries et al, 1994) which may be due to the wide variations of gene expression. Strikingly different responses to extracellular signals is possible among different fibroblast populations. The significance of this heterogeneity is important in understanding the proliferative potential, and also the synthetic and degradative behaviour of these cells. Differences among fibroblasts in structure and physiology are many. They may be heterogenous in their morphology, expression of surface markers, antigen presentation to T-lymphocytes, ability to synthesize collagens and cytokine production. As a result of this heterogenous behaviour, one subset of resident fibroblasts may control the inflammatory responses whereas another subset may be important for the fibroblast hyperplasia and extensive extracellular matrix production which are the hallmarks for fibrosis. Striking differences in the

proliferative potential of human skin and lung fibroblasts have been observed (Martin et al, 1974). Dawes and his group (1996) suggest that different populations of fibroblasts exhibit heterogenous responses to endothelin-1. The heterogeneity of the collagenolytic response of gingivial fibroblast strains and their subpopulations to cyclosporine treatment has been reported (Tipton et al, 1991). Several different fibroblast forms are identifiable by morphologic criteria and phenotypic markers. Fibroblasts found in adult tissue retain plueripotentiality and accordingly exhibit diversity in their functions, a trait presumptively related to distinct phenotypic subtypes (McCulloch and Bordin, 1991; Sappino et al, 1990; Schor and Schor, 1987). Between tissues and within a given tissue, fibroblasts demonstrate extensive clonal heterogeneity as shown in various experimental studies where fibroblast like cells appear at the site of cardiac myocyte necrosis and repair several days after myocardial infarction due to coronary artery ligation or freeze-thaw injury (Vracko and Throning, 1991; Sun et al, 1994). These cells are larger than the usual fibroblast, have a prominent nucleus and endoplasmic reticulum, and in addition have acquired α- smooth muscle actin microfilaments during phenotypic transformation from presuambly quiescent fibroblasts of the interstitium and pericytes that normally reside in the adventitia of the intramural coronary vasculature. These actin filaments contribute to the ability of these cells to contract (Gabbiani et al, 1972). Contractile fibroblasts involved in tissue repair are termed myofibroblasts (Gabbiani, 1981).

Other fibroblast like cells that also contain α- smooth muscle actin and ACE are found within the connective tissue of the adventitia surrounding intramyocardial coronary arteries. They are referred to as pericytes. Because of their α- smooth muscle actin microfilaments, pericytes have contractile properties (Sims, 1986; Tilton et al, 1979; Crocker et al, 1970; Arora and McCulloch, 1994; Miller and Sims, 1986).

Heart valve leaflets are composed largely of connective tissue; and collagen turnover is normally very high. Within the interior of the leaflet are valvular interstitial cells. These fibroblast like cells are normal residents of leaflet tissue. They contain α- smooth muscle actin filaments and demonstrate marked ACE binding.

Fibroblasts respond rapidly to physiological and pathological stimuli resulting in the production of new matrix which ultimately may lead to a compromise in heart function. In addition to the stimuli for the compensatory increase in collagen deposition, there are other factors that induce co-ordinate increases in collagen and non-collagen proteins that eventually leads to a disproportionate increase in collagen content. Such factors may include locally produced autocrine or paracrine agents, or factors that originate from the circulation and which are localized in the adventitia or interstitium.

Fibroblasts are subject to regulation by diverse stimuli that includes hypoxia (Agocha & Eghbali-Webb 1997; Tamamori et al, 1997), mechanical stretch (Sadoshima et al, 1992), TGF -β (Siegel et al, 1996) and other local

and systemic factors such as angiotensin II (Schorb et al, 1993), PDGF (Simm et al, 1997) and cytokines (Long et al, 1993).

The view that biochemical signals alone regulate cell function has been challenged by evidence that mechanical load per se exerts important influences on cellular activity. Mechanical load is not only critical for organ or tissue development but also for maintenance and functional adaptation in the adult. Abnormal mechanical forces may directly modulate gene expression leading to compensatory hypertrophic growth and ultimately to pathological conditions. Cardiac growth due to pressure overload is associated with hypertrophy of cardiac myocytes and hyperplasia of fibroblasts and endothelial cells (Grove et al, 1969) indicating that all cardiac cells may be influenced by mechanical load. Cells respond to mechanical forces by getting the signal transduction pathways activated, such as opening of ion channels and activation of membrane bound complexes, by which cells recognize changes in the mechanical environment and convert the mechanical event to chemical or electrical signals which is followed by changes in gene transcription. This inturn may modulate the morphology such as cell orientation and cell shape subsequent to the changes in protein synthesis, the rate of cell division and cell differentiation.

Pressure overload leads to fibroblast proliferation in the heart in vivo, suggesting that mechanical load influences cell replication (Leslie et al, 1991; Skosey et al, 1972). Cell replication also occurs in response to mechanical load in vitro. Enhanced replication of fibroblasts have been found and it has

been observed that fibroblasts need only a trigger of 24 hour mechanical load in order for replication to occur to the same degree as that of 5 days of stretch (Bishop et al, 1993). Mechanical load also stimulates the synthesis of actin and myosin (Pender and McCulloch, 1991) and collagens (Carver et al, 1991; Butt et al, 1995a) in cardiac fibroblasts.

Polypeptide growth factors such as platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and transforming growth factor-β (TGF-β) have been identified in the heart and are known to stimulate fibroblast proliferation in vitro. Platelet -derived growth factor is mitogenic to fibroblast and is produced by a number of cell types including fibroblasts (Fabisiak et al, 1992). It has been implicated in development (Seifert et al, 1984), atherosclerosis (Barret and Benditt, 1988) and wound healing (Lynch et al, 1987). Butt et al (1995 b) demonstrated that it also stimulates cardiac fibroblast collagen synthesis and replication. However Sarzani et al (1991) found no change in PDGF - α or β receptor mRNA or PDGF B chain mRNA in hypertension induced cardiac hypertrophy. Thus a role for PDGF in cardiac hypertrophy has not yet been elucidated.

Fibroblast growth factor -2 found in heart tissues (Casscells et al, 1990) also stimulates cardiac fibroblast collagen synthesis and replication (Butt et al, 1995b).

Insulin - like growth factor - I stimulates myocyte protein synthesis (Fuller et al, 1992) and stimulates cardiac fibroblast synthesis, but not replication (Butt et al, 1995b).

Transforming growth factor -β is the most potent stimulator of fibroblast collagen synthesis in culture (Butt et al, 1995b; Varga and Jiminez, 1986) and decreases the proportion of collagen degraded rapidly in lung (McAnulty et al, 1991) but not cardiac fibroblasts (Butt et al, 1995b).

Vasoactive substances angiotensin II and endothelin -1 have been shown to stimulate cell activity. Angiotensin II has been identified as a growth factor for cardiac fibroblasts from studies in vivo and on cells in culture, and has been implicated as an important factor contributing to fibrosis of the myocardium that is associated with hypertension and ischemia - reperfusion injury. The growth response in cardiac fibroblasts involves increased expression of extracellular matrix proteins as well as cellular hypertrophy and hyperplasia (Weber and Brilla, 1991; Brilla et al, 1992; Lindpaintner et al, 1992). Evidence that Ang II contributes to myocardial fibrosis are many. In a rat model of myocardial infarction, an ACE inhibitor prevented collagen accumulation and DNA synthesis (van Krimpin et al, 1991). In the same model, early treatment with the AT_1 - R antagonist, losartan completely inhibited collagen deposition (Smits et al, 1992). Cardiac myocyte necrosis induced by exogenous or endogenous Ang II was accompanied by enhanced DNA synthesis and microscopic scarring (Tan et al, 1991). Further proof that cardiac fibroblasts are a target cell for the actions of Ang II has been obtained from tissue culture studies, showing that these cells possess functional receptors that couple to second messenger generation and growth responses (Booz and Baker, 1996).

Recent work (Schelling et al, 1991) has demonstrated the intriguing possibility that angiotensin may exert a major influence by acting as a growth factor. The mechanism for the trophic effects of angiotensin suggested by many studies (Taubman et al, 1989; Naftilan et al, 1989) states that the peptide via activation of protein kinase C and modulation of cytosolic calcium, activates the nuclear proto oncogene c-fos. The c- fos protein in turn, has been shown to play an important role in the regulation of cell proliferation and protein synthesis in cardiac muscle (Izumo et al, 1988). Further evidence that peptide hormones influence collagen turnover has been obtained in cultured rat heart fibroblasts. In serum deprived cells, incubation with Ang II increases net collagen production (Villareal et al, 1993) and collagen synthesis (Brilla et al, 1994). Moreover, Ang II reduces collagenolytic activity of culture medium (Brilla et al, 1994).

Endothelin has differential effects on fibroblast function which are highly dependent on the tissue source of the cells. Endothelin-1 increases pulmonary artery fibroblast replication (Peacock et al., 1992). It stimulates collagen synthesis and decreases degradation of newly synthesized collagen in human fetal lung fibroblasts, however it decreases synthesis and increases degradation in whole fetal rat fibroblasts (Dawes, 1996). Endothelin has no effect on cardiac fibroblast replication or on collagen synthesis (Butt et al, 1995a).

Interleukin -1α and IL -1β have not been found to induce fibroblast proliferation as they are either not involved in the induction of proliferation or

they act indirectly. Thorton and co-workers (1990) have defined platelet derived growth factor as a true growth factor and IL-1 as a growth enhancer acting in concert with other growth factors, such as PDGF. Studies suggest that IL-1 apparently induces fibroblasts to secrete PDGF -A chain homodimer, which stimulate cells to enter the cell cycle (Raines et al, 1989). Further amplification of the system during longer culture periods may be the result of PDGF upregulation of IL-1 receptors on fibroblasts (Chiou et al, 1989). Tumor necrosis factor - α has been reported to act as both a growth inducer and inhibitor. At low concentrations TNF- α stimulates fibroblast proliferation and at high concentrations it blocks growth triggered by serum and cytokines (Thorton et al, 1990).

Tamamori et al (1997) demonstrated the stimulation of collagen synthesis in rat cardiac fibroblasts by exposure to hypoxic culture conditions and the stimulatory effect was attenuated by the presence of atrial natriuretic peptides (ANP - 10^{-6} M) or brain natriuretic peptides (BNP -10^{-6} M) in the culture medium. This suggests an interaction between intracellular signals of a physical stimulus (hypoxic stress) and those of a chemical one (ANP or BNP) and demonstrates that both the signals regulate collagen synthesis by cardiac fibroblasts at the levels of mRNA.

The role of female hormones in the prevalence of cardiac diseases are recognized but not fully explored. Study by Lee and Eghbali (1998), showed the effect of estrogen (1/beta - estradiol) on proliferative capacity of cardiac fibroblasts obtained from adult female rat heart. The study demonstrated that

cardiac fibroblasts are cellular targets for different effects of estrogen, and that this hormone enhances proliferative capacity of cardiac fibroblasts via estrogen receptor and MAP kinase - dependent mechanisms.

No reports on cardiac fibrosis induced by metals are available, but the possibility is envisaged by the reports on fibrosis induced by metals in other tissues. Exposure to mineral dusts is associated with the development of chronic airflow obstruction mediated probably in part by dust induced fibrosis of the small airways. In a study by Dai et al, (1998) it is suggested that mineral dusts can induce air way wall fibrosis by directly upregulating proliferative and fibrogenic mediators as well as matrix components in the airway epithelium and interstitium, and that neither airspace nor circulating inflammatory cells are required for these effects. But Robledo and Mossman (1999) in an attempt to study the cellular and molecular mechanisms of asbestos induced fibrosis insists on the participation of a number of cell types and suggests that the fibrosis is characterised by an early and persistent inflammatory response that involves the generation of oxidants, growth factors, chemokines and cytokines which may contribute to cell injury, proliferation and fibrogenesis.

Many reports of respiratory disease attributable to aluminium exposure have appeared in the European medical literature during the last 50 years. Great Britain and Germany are two major industrialized nations that acknowledge a causal relationship between occupational exposure to aluminium and respiratory impairment (Al - Masalkhi & Walton, 1994).

Nemery (1990) reports that the fumes or gaseous forms of several metals for example Cd, Mn, Hg, $NI(CO_4)$, $ZnCl_2$, V_2O_5 may lead to acute chemical pneumonitis and pulmonary oedema or to acute tracheobronchitis; and that inhalation of iron compounds cause siderosis, a pneumoconiosis with little or no fibrosis. The author has also observed that hard metal lung disease is a fibrosis characterised by desquamative and giant cell interstitial pneumonitis and is probably caused by cobalt, since a similar disease has been observed in workers exposed to cobalt in the absence of tungsten carbide. Chronic beryllium disease is a fibrosis with sarcoid like epitheloid granulomas and is presuambly due to a cell - mediated immune response to beryllium. Such a mechanism may be responsible for the pulmonary fibrosis occasionally found in subjects exposed to other metals for example Al, Ti, rare earths.

Iron overload induced heamochromatosis is yet another example of metal induced fibrosis. Iron deposition occurs in the parenchymal cells of the liver in two major defects in human subjects i) primary iron overload (genetic heamochromatosis) and ii) secondary to anemia. Transfusional iron overload results in excessive storage primarily in cells of the reticulate endothelial system. Excessive storage particularly in parenchymal cells eventually results in fibrosis and cirrhosis (Halliday and Searle, 1996). The pathogenesis of liver fibrosis in genetic heamochromatosis and other iron overloaded status remain enigmatic. Recent advances in the cellular and molecular pathogenesis of liver fibrosis have however determined a central role for

hepatic stellate cells which gets activated to a myofibroblastic phenotype with increasing hepatic iron concentration (Arthur, 1996).

Non myocyte cell growth is expressed as a structural remodeling of the interstitium. The accumulation of fibrillar collagen is indicative of fibroblast growth and increased myocardial collagen synthesis, relative to its degradation. Recent in vivo studies have confirmed that myocyte and non myocyte cells grow independently of each other (Lund et al, 1979; Ruskoaho and Savolainen, 1985; Weber et al, 1987).

The hypertrophic remodeling of the myocardium is either a homogenous or a heterogeneous process, based on whether there is a proportionate or disproportionate growth of non-myocyte cells (Weber et al, 1987). When tissue homogeneity is preserved, the proportionality of muscular, vascular and interstitial compartment is maintained and hypertrophy is adaptive. This is the type of hypertrophy that occurs in response to isotonic or isometric exercise training, chronic anemia and arteriovenous fistulas. The adpative nature of the hypertrophy, with preserved myocardial structure is further evidenced by the uneventful regression in hypertrophy and restoration in ventricular chamber size that occur when the overload terminates or is corrected (Sanghvi et al, 1960). In contrast, a heterogeneity in myocardial structure, based on disproportionate non myocyte cell growth and loss of intercompartmental proportionality, will cause pathological hypertrophy. In case of adaptive remodeling, myocardial collagen content is increased, but

disproportionate increase in collagen content leads to pathological hypertrophy.

Morphologically distinct patterns of myocardial collagen accumulation or fibrosis have been identified based on the alignment of thick and thin collagen fibers to one another and to cardiac muscle. According to convention (Sen and Bumpus, 1979), the collagen matrix of cardiac muscle can be distinguished into constituent elements, including an epimysium that surrounds muscle, a perimysium that is an extension of the epimysium serving to separate muscle fibre bundles, and an endomysium or final arborization of the perimysium. The endomysium includes collagen fibrils that join individual muscle cells together and to their capillaries and a collagenous weave that surrounds muscle cells (Abraham et al, 1987; Borg et al, 1981; Robinson et al, 1987). Based on the alignment of collagen fibres, fibrosis is classified into

1) Reactive interstitial fibrosis in which fibrillar collagen is thicker than normal and appears in intermuscular spaces previously devoid of collagen.
2) Reactive perivascular fibrosis or accumulation of collagen within the adventitia of intramyocardial coronary arteries and arterioles.
3) Replacement (reparative) fibrosis which represents microscopic scarring that follows myocyte necrosis.
4) Plexiform fibrosis or swirling arrangement of collagen fibres that is frequently seen in association with muscle fibre disarray.

Less frequent expressions of reactive fibrosis include endomyocardial fibrosis and endocardial fibrosis. Endomyocardial fibrosis, first described by Arthur Williams (Foundation Professor of Medicine at Makeree University) in 1938, has characteristic clinical and pathological features. The large patches of fibrosis without inflammation involving specific regions of either or both ventricles of the heart was initially discovered in Africans (Bedford and Konstam, 1946; Davies, 1948). Soon it became known that the disease is also prevalent in other countries in the tropical belt (Hutt, 1983).

Endomyocardial fibrosis 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. In India, prevalence of EMF is highest in the coastal areas of Kerala with very few cases being reported from Northern India.

It is suggested that EMF is an interstitial disease and that the cardinal feature is abnormal stimulation of cardiac fibroblasts leading to enhanced collagen synthesis. Entry of trophic factors into the interstitium could be mediated by injury to the microcirculation and alterations in permeability. Myocytolysis as seen in EMF is a minor component and could be caused by combination of entrapment by fibrosis and toxicity by the same factors which produce the interstitial injury.

Many theories regarding the causation of EMF have been put forward in the past but none of them has yet found confirmatory evidence (Falase, 1983). Malnutrition, vitamin E deficiency, viral infection, parasitic myocarditis,

cardiac lymphatic obstruction and serotonin toxicity from consumption of bananas were some of the theories vigorously pursued by earlier investigators. A tuberous diet rich in vitamin D is also proposed to lead to EMF (Davies, 1990). Another view holds that endomyocardial fibrosis is an immunological variant of hypersensitivity to Streptococcal infection (Shaper, 1966). Proponents of this view argue that manifestation as EMF is determined by what constitutes a tropical immunological syndrome characterized by presence of high levels of IgM autoantibodies to the heart and bound IgG to myofibres and endocardium (Vander et al, 1966).

The hypothesis which gained wide attention in the 1980's claims that endomyocardial fibrosis in the tropics and Loeffler's endomyocardial fibrosis are different stages of a spectrum of cardiac responses to injury by eosinophils (Olsen and Spry, 1979). Except for a solitary report (Andy et al, 1981), observations by tropical investigators suggest that there is a need for reappraisal of the unitarian concept (Patel et al, 1977; Valiathan and Kartha, 1990). A comparison of tropical EMF and Loeffler's EMF reveals that the two entities are dissimilar not only in their eosinophilic profiles but also in the epidemiologic and clinical features (Valiathan and Kartha, 1990; Davies et al, 1983).

The fact that 730 out of 799 cases of endomyocardial fibrosis reported during the last two decades belong to countries within 15° of the equator is a strong pointer to geographical factors in the causation of the disease (Kartha, 1995). Emphasizing the prevalence of endomyocardial fibrosis in regions with

latasolic soil with abundant monazite elements and striking preference for malnourished children, a geochemical basis for the disease has been suggested (Valiathan et al, 1986; Valiathan et al, 1989). The hypothesis that EMF could be the cardiac expression of cerium toxicity in conjunction with magnesium deficiency (Valiathan and Kartha, 1990) is based on the 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 significantly lower levels of magnesium and higher levels of Na, Ca, Th and Ce (Valiathan et al, 1986 and 1989). It was argued that alteration in Na and Ca levels could be due to salt retention and reciprocal response of Ca to magnesium deficiency as occurs in a variety of myocardial diseases.

It has been postulated that low levels of magnesium promote the absorption and accumulation of cerium (Valiathan and Kartha, 1990). Studies carried out in pursuance of this postulation have shown that the levels of cerium in the cardiac tissue of rats fed on Mg deficient and cerium adulterated diet was higher compared to that of animals on a Mg sufficient diet (Kartha et al, 1992) suggesting that magnesium deficiency promotes absorption or accumulation of cerium. Perivascular and subendocardial fibrosis was observed in rats and rabbits on Mg deficient diet rich in cerium (Kumar et al, 1996; Kartha et al, 1998). The level of cerium in sera of patients was found to be higher than that of unaffected control (Eapen et al, 1997). In vitro studies show that the lanthanide cerium stimulates collagen synthesis (Shivakumar

et al, 1992). Another study provides evidence of increased lipid peroxidation and elevated rates of cell proliferation and collagen deposition in the heart in cerium treated rats and it has been suggested that the element may trigger a wound healing response in the cardiac tissue leading to cardiac fibrosis (Kumar and Shivakumar, 1998).

DESIGN OF THE STUDY

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CHAPTER - 3

DESIGN OF THE STUDY

The investigation is aimed at a better understanding of the factors that could mediate a cerium induced fibrotic reaction in the cardiac tissue. The experiments were carried out on fibroblasts isolated and cultured from the heart of newborn rats. The use of tissue culture techniques in all fields of biomedical research has rapidly expanded during the past decade. The main reason for this intensified interest has been that this technique affords the investigator the advantage of studying viable and functional cells over a long period of time. The purpose of animal cell technology has been to use cultivated cells for experimental studies, whereby manipulating the environment helps to assess the cellular response to hypothetical situations.

The technique of enzymatic dissociation has been successfully adapted to study the morphology , biochemistry and physiology of embryonic , fetal and neonatal heart muscle cells in suspension or as mono and multi layered preparations. It has also been possible to culture enzymatically dispersed cells in tissue - like orientation.

The fibroblast is considered one of the most ubiquitous cells of the mammalian species. It is involved in the construction of tissues such as skin, in tissue repair and in granulation tissue formation during wound healing. The

fibroblast plays an important role in such pathological states as hypertrophic scarring, sclerosis and fibrosis. Due to their physiological importance and because of their mesenchymal origin, fibroblasts are frequently used as a model for studying the characteristic properties and functions of connective tissues. Tissue culture has been found to be an ideal experimental model to study many of the problems of fibroblast structure and function confronting us at the present time.

In keeping with the objectives, the experimental protocol was designed to examine:

The effect of different concentrations of the lanthanide cerium on cardiac fibroblast proliferation by determination of :

a) the proportion of cells immunoreactive for proliferating cell nuclear antigen

b) cell density

Stimulation of superoxide anion generation by cerium by measurement of :

a) nitroblue tetrazolium reduction for assessment of intracellular generation of superoxide anion

b) superoxide dismutase inhibitable reduction of cytochrome c for extracellular generation of superoxide anion

c) levels of thiobarbituric acid reactive substances to assess lipid peroxidation

Role of superoxide anions in the mediation of fibroblast proliferation as assessed by :

a) the effect of enzymatic and non-enzymatic free radical scavengers - superoxide dismutase, catalase, N-acetyl-L-cysteine (NAC)

b) the response on exposure to exogenously generated free radicals

Cardiospecific response to cerium by comparison with the behaviour of skeletal muscle fibroblasts

EXPERIMENTAL DESIGN

Effect of different concentrations of cerium on cell proliferation

The aim was to study the response to low levels of the lanthanide cerium on cardiac fibroblast proliferation and determine the concentration that induces a stimulatory response. The number of proliferating cells and cell density after exposure to cerium was compared with untreated control and the difference was taken as the response to cerium.

Immunocytochemical staining of PCNA was adopted for quantification of the proportion of replicating cells. Using antibody to proliferating cell nuclear antigen (Casasco et al, 1994), the number of quiescent cells that enters the mitotic phase after exposure to cerium was determined. Proliferating cell nuclear antigen synthesis occurs in late G phase, after the restriction point and throughout the S phase. Since PCNA concentration within the nucleus is correlated with the proliferative state of the cell, PCNA is used as a molecular marker of replicating cells. The number of cells carrying PCNA in a synchronised culture will therefore indicate the number of quiescent cells that have entered mitosis.

Measurement of superoxide anion content following exposure of cardiac fibroblasts to cerium

The intracellular superoxide anion content, the level of anions released and lipid peroxidation on exposure to cerium was measured and compared with untreated control.

To assess the intracellular free radical generation by cardiac fibroblasts on exposure to different concentrations of cerium, nitroblue tetrazolium reduction assay (Das et al, 1987) was adopted. In the unreduced state, nitroblue tetrazolium is an artificial electron acceptor, that is soluble and yellow in colour. Upon reduction by superoxide anion, nitroblue tetrazolium becomes a relatively insoluble blue - black coloured substance called formazan, which may be directly visualized microscopically and may also be extracted with an appropriate solvent and quantitated spectrophotometrically. The reduction of nitroblue tetrazolium was used as an indicator of superoxide anion generation in the present study as it is very sensitive to low levels of superoxide anions. It is therefore an integrative measure of the low levels of superoxide anion produced. The specificity of the nitroblue tetrazolium assay was further indicated by showing that the superoxide scavengers, superoxide dismutase and N-acetyl - L - cysteine decreased the reduction of nitroblue tetrazolium when compared with cultures devoid of scavengers.

The measurement of superoxide anions generated extracellularly was based on the SOD inhibitable reduction of cytochrome c (Johnston, 1981).

Superoxide anion mediated reduction of cytochrome c was measured spectrophotomerically by the increase in absorbancy at 550 nm. To normalise for the variation arising due to non specific reduction of cytochrome c, parallel cultures with the superoxide anion scavenger SOD was set up and the difference was taken as the level of superoxide anion released.

Lipid peroxidation is a chain reaction initiated by the hydrogen abstraction or addition by an oxygen radical, resulting in the oxidative deterioration of polyunsaturated fatty acids (Kirshenbaum et al, 1995). Since polyunsaturated fatty acids are more sensitive than saturated ones, it is obvious that the activated methyl bridge represents a critical target site. The resulting fatty acid radical is stabilized by rearrangement into a conjugated diene that forms the more stable products such as hydroperoxides, alcohols, aldehydes and alkanes. The measurement of thiobarbituric acetates is a widely used test of lipid peroxidation. Its main advantage is its capacity to detect many kinds of peroxidation products and intermediates, but its specificity is rather low since various substances not related to the lipid peroxidation process could also react with the thiobarbituric acid during the test procedure. With the use of suitable control the method is applicable for assessment of the presence of lipid peroxidation.

Determination of cell proliferation in the presence of different free radical scavengers

To confirm the role of cerium in the generation of superoxide anions and also the role of free radicals in fibroblast proliferation, free radicals scavenging enzymes superoxide dismutase (100 U/ml) and catalase (120 U/ml) were included and the proportion of PCNA reactive cells and the cell count was determined.

N-acetyl-L-cysteine is a thiol containing compound having diverse pharmacological application due to the multifaceted chemical properties of cysteinyl thiol of the molecule. These include its nucleophilicity and redox activity, role as scavengers, its antioxidant properties and also the ability to undergo transhydrogenation or thiol-sulfide exchange reaction with other thiol redox couples. The interaction with free radical species results in the intermediate formation of NAC thiyl radicals, with NAC disulfide as the major end product. N-acetyl-L-cysteine (20 mM) being a cell permeant antioxidant, it was used in this study to affirm that extracellular cerium can generate superoxide anions intracellularly, and that the superoxide anions stimulate cell proliferation. The cells were treated with NAC (20 mM) and the intracellular superoxide anion content and the stimulatory response on cardiac fibroblasts was assessed.

Influence of exogenously generated superoxide anions on proliferation and intracellular generation of oxygen radicals in cardiac fibroblasts

To substantiate the role of superoxide anions in the induction of proliferative response to cerium, cardiac fibroblasts were exposed to free radicals generated using hypoxanthine - xanthine oxidase. This procedure will also confirm whether the superoxide anions released during inflammatory process, which are commonly encountered, can mediate a fibroproliferative response.

Xanthine oxidase is an enzyme present in large amounts in liver and intestinal mucosa and in traces in other tissues. The enzyme oxidises hypoxanthine to xanthine and superoxide anions and further oxidation results in the production of more superoxide anions and uric acid.

Assessment of tissue specific response to cerium using fibroblasts isolated from skeletal muscle

Fibroblasts consist of subpopulations with extensive site to site and intra site variation (Lekic et al, 1997), with unique phenotype and functions which may be due to the variations of gene expression and differential response to extracellular signals (Fries et al, 1994).

To ascertain whether the response to cerium and to free radical stress is cardiospecific, fibroblasts from skeletal muscle was isolated and treated similarly and the results compared.

MATERIALS

Fine chemicals :

Medium 199, Cerium chloride, Bovine serum albumin (Fraction V, Fatty acid free) Nitroblue tetrazolium, Phenazine methosulfate , Cytochrome c (From horse heart), Collagenase Type I, Trypsin, Superoxide dismutase, Catalase, Deoxyribonuclease, Xanthine oxidase, Hypoxanthine, Diaminobenezidene, EDTA, Anti-vimentin, Anti -desmin, Anti- factor VIII, Anti - mouse IgM (alkaline phosphatase conjugated), Fast red TR/ Napthol AS -MS, N - acetyl - L - cysteine, Ethidium bromide , Acridine orange were obtained from Sigma Chemical company, St. Louis, MO, USA. Penicillin and Gentamycin were supplied by Alembic and Fulford, India.

Fetal calf serum was purchased from PAA Laboratories, Austria. Monoclonal antibody to PCNA, biotinylated goat anti - mouse IgG and Avidin Biotin complex was supplied from DAKO, USA.

Routine chemicals :

Sodium chloride, Potassium chloride, Sodium bicarbonate, di-sodium hydrogen phosphate, Sodium dihydrogen phosphate, Magnesium chloride, Magnesium sulfate, Glucose, Calcium chloride, Phenol red, Potassium dihydrogen phosphate, Thiobarbituric acid, Tris buffer,

Trichloroacetic acid, were purchased from Sisco Research Laboratories, INDIA.

Solvents :

Propanol, Ether, Ethanol, were obtained from Sisco Research Laboratories, INDIA.

Instruments used :

Laminar flow (Clas, India), Auto flow CO_2 water jacketed incubator (Nuaire, USA), UV - visible Spectrophotometer (Shimadzu, Japan), High speed refrigerated centrifuge (Hitachi, Japan), Weighing balance (Sartorius, USA; Anamed, India), Water bath (LKB, Sweden), Ice - machine (Hoshizaki, Japan), pH meter (Elico, India), Phase contrast microscope (Nikon, Japan), Low speed magnetic stirrer (Remi, India), Incubator (Kemi, India), Hot air oven (Tempo, India), Osmometer (Fiske One-Ten, USA).

Experimental animal used :

2-4 day old rat pups (Wistar strain) were supplied from the Toxicology department, Biomedical Technology Wing (SCTIMST) Trivandrum.

METHODOLOGY

Isolation and culture of cardiac fibroblasts

Cardiac fibroblasts were isolated from newborn Wistar rats (2-4 days old) by enzymatic dispersion and maintained in culture (Nair and Gupta, 1989). The heart was excised out through a thoracic incision following mild ether anesthesia. The ventricular tissue from 5-6 rats was passed through 4 or 5 petri dishes containing penicillin (500 U/ml) and gentamycin (100 μg/ml) dissolved in calcium -magnesium containing Hank's balanced salt solution of pH 7.4 (HBSS). The ventricular tissue was placed in a petri dish containing HBSS and minced into bits of 1 mm^3 using sterile scalpel blades. The minced tissue was transferred into a trypsinization flask containing a Teflon magnetic bar and 8 ml of dissociation medium made up of trypsin (0.3 mg/ml), collagenase (0.3 mg/ml), deoxyribonuclease (5.5 μg/ml) and bovine serum albumin (1 mg/ml) in HBSS containing 0.63 mM calcium chloride, 0.25 mM magnesium chloride and 0.2 mM magnesium sulphate; and antibiotics (Penicillin 100 U/ml, Gentamycin 50 μg/ml). The solution was prepared immediately before use and sterilized by membrane filtration (pore size 0.22 μm).

The digestion of the tissue was carried out using 2-3 successive 30 minute incubations in the dissociation medium at 37° C on a magnetic stirrer

at a very low speed. After each incubation, the tissue was allowed to settle by gravity and the supernatant fluid containing the isolated cells were pipetted out. The supernatant from the first dissociation period lasting 10 min, was discarded as it contained mostly erythrocytes, non viable cells and debris. The supernatant from the second through the last dissociation was collected. Inactivation of the proteolytic enzymes was done by adding 4 ml of medium containing 10% fetal calf serum and cells were pelleted by centrifugation at 1200 rpm for 10 min. The pellet was dispersed in culture medium and the cell count was adjusted to approximately 1X 10^6 cells / ml and incubated at 37°C with 5 % CO_2 in a humidified incubator.

Fibroblast rich cultures were obtained by employing the selective adhesion method. The culture supernatant containing the unattached cells, mostly myocytes and dead cells were withdrawn after 90 min from the tissue culture flask, leaving the non myocardial cells attached to the culture surface. Fibroblasts are known to adhere to the culture surface within 60- 90 min. Culture medium was added to the tissue culture flask containing the adhered non myocardial cells from the initial plating and incubated at 37°C with 5% CO_2.

Cultures were examined regularly for cell growth and any signs of possible contamination. The cells were replenished with fresh pre-warmed Medium 199 with 10% FCS every second day till confluency was attained.

The first subculture was done at 7-8 days. During this time growth of cells was monitored regularly. Subcultures were done at near confluence. The usual method of trypsinization was followed for detaching the cells.

Calcium - magnesium free Hank's balanced salt solution (pH 7.4) was added along the side of the flask so as to avoid dislodging of cells, and the cells were washed twice to remove any traces of serum. This was followed by rinse of dissociation medium containing trypsin (0.25% w/v) and EDTA (0.2% w/v) for 1 minute at 37°C. When cells round up and sufficiently loosen from the surface of the tissue culture plate, as observed under the phase contrast microscope, 1 ml of Medium 199 containing 10% FCS was added and the flask was gently agitated. Cell suspension was obtained by squirting the medium gently with the pipette tip to dislodge the attached cells if any from the surface of the flask and care was taken to avoid foaming. Cell count was taken of the dispersed cells on a haemocytometer and the cell count adjusted, and seeded at a density of 5×10^5 cells/ ml into tissue culture flasks.

The cells obtained at the third subculture were used for the experiments. Before carrying out the experiments the cultures were synchronised by serum deprivation for 24 hours (Ashihara and Baserga, 1979) and all the experiments were done using Medium 199 containing 0.4% FCS. For immunostaining studies, the cells were plated on coverslips

at a density of 4.5 x 10^4 cells /ml and for the determination of total cell count, the plating density was 3 x 10^4 cells /well of 96 well plates.

IDENTIFICATION OF CARDIAC FIBROBLASTS The fibroblastic nature of confluent cells as well as the purity of the cultured cell population was determined by morphological identification under a phase contrast microscope and immunocytochemistry.

Cardiac tissue being rich in myocytes and endothelial cells, negative staining for desmin (myocytes) and factor VIII (endothelial cells) and positive staining for vimentin establishes the purity of the cultures. This pattern of staining is widely accepted as a method for characterizing cardiac fibroblasts in culture (Eghbali et al, 1991; Bashey et al, 1992).

The immunostaining of cultured cells were done as follows : The cells were washed twice with phosphate buffered saline (pH 7.4) containing 0.15 M sodium dihydrogen phosphate and 0.15 M disodium hydrogen phosphate. The cells were fixed for 10 min at room temperature with 70% alcohol. After two PBS washings, the fixed cells were incubated for 5 min at room temperature with 0.3% H_2O_2 to prevent the peroxidase activity of the cells and after a PBS wash the cells were incubated with diluted normal rabbit serum 1:5 in PBS buffer to eliminate non - specific staining. Cells were then incubated at room temperature for 30 min with monoclonal anti body against vimentin (1:50) or desmin (1: 20) or factor VIII (1:200). After two washes with PBS, cells were incubated for 30 min at room temperature with the second antibody anti -mouse

IgM, conjugated to alkaline phosphatase (1: 50). Cells were washed again with PBS twice for 5 min and were incubated with fast red TR/ napthol AS - MS for 10-15 min at room temperature. The cells were washed with PBS and then with distilled water after which they were mounted on a slide with 90% glycerol and viewed under brightfield optics.

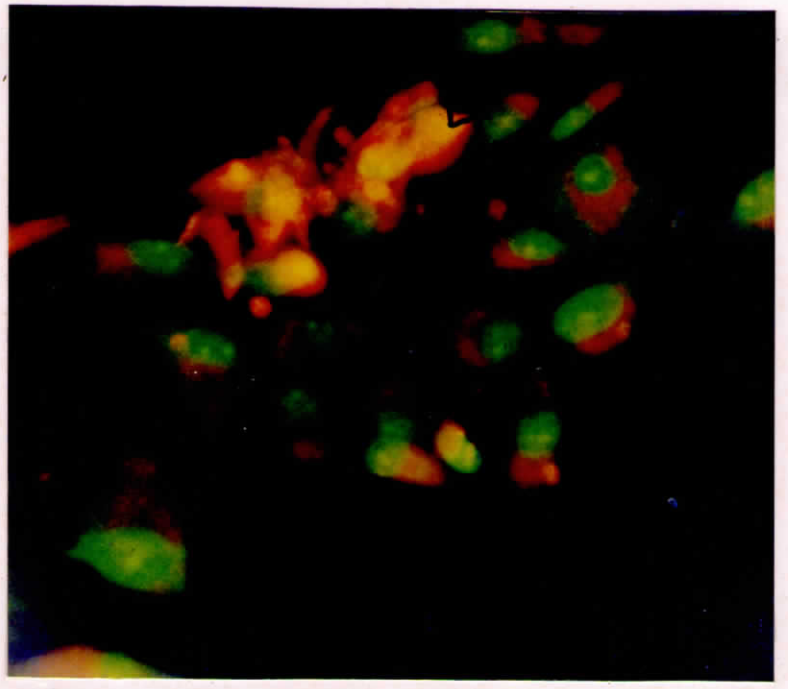
ASSESSMENT OF CELL VIABILITY Cell viability was assessed by using fluorescent DNA binding dyes - acridine orange and ethidium bromide (Zhi - Jun et al, 1997). Both the dyes (100 μg/ ml) were mixed in PBS (pH 7.4). The cardiac fibroblast cultures plated on cover slips were exposed to cerium and for determining the viability of the culture 10 μl of the dye mixture was placed on a slide and the coverslip with the fibroblast culture was placed over it and examined under the fluorescent microscope. Nuclei of viable cells appear green and that of dead cells stain red (Fig. 3-1). A minimum of 200 cells were counted per culture.

Assessment of growth of cardiac fibroblasts

Cell growth was assessed by determining the proportion of proliferating cells and the cell number. The effect of cerium on cell growth was studied by exposing the cells to cerium and comparison with

FIGURE 3-1

MICROGRAPH OF FIBROBLASTS STAINED WITH ACRIDINE ORANGE / ETHIDIUM BROMIDE FOR ASSESSMENT OF CELL VIABILITY



Magnification (230 X)

→ viable cells

> non viable cells

cerium free cultures. A stock solution of 0.1 mM cerium was prepared by dissolving cerium chloride in distilled water. The required concentration of cerium was obtained by dilution in culture medium. The presence of cerium did not affect the osmolality of the culture medium at the concentration used. The pH of the medium was adjusted (pH 7.4) following addition of cerium for the experiments.

DETERMINATION OF THE PROPORTION OF PROLIFERATING CELLS Proliferating cell nuclear antigen (PCNA) is a stable cell cycle regulated nuclear protein which is expressed differentially during the cell cycle. To determine the extent of mitosis, immunocytochemical labelling for PCNA was done, following the method of Casasco et al (1994). Synchronised cultures were exposed to different concentrations of cerium (0.1 μM, 0.5 μM, 1 μM, 10 μM, and 100 μM) for 3 hours . The cells were washed in Tris buffered saline (TBS pH 7.6) twice for 5 minutes and then were fixed in 70 % alcohol for 10 minutes at room temperature. The fixed cells were then processed for immunocytochemical detection of PCNA by PC10 antibody. The fixed cells were sequentially incubated with the following solutions

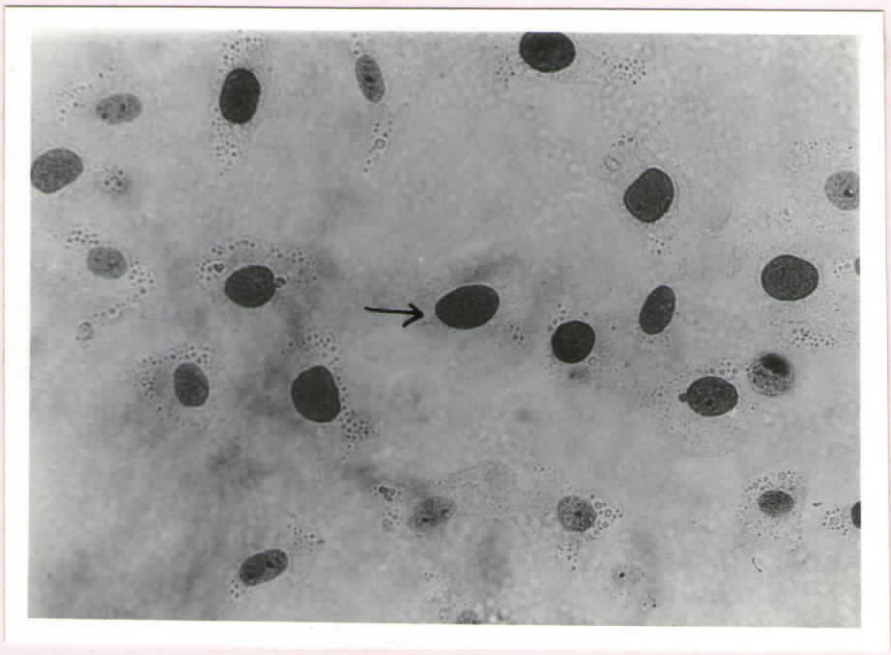
1) 0.3% hydrogen peroxide for 30 min to remove endogenous peroxidase activity 2) normal rabbit serum diluted 1:5 in TBS for 20 min to reduce background staining 3) monoclonal antibody to PCNA/ cyclin, clone PC10, a mouse IgG at a dilution of 1:50 in TBS for 30 min 4) biotinylated goat anti

mouse IgG diluted 1:200 in TBS for 30 min 5) avidin biotin complex for 30 min (5 ml of 0.05M Tris HCl, pH 7.6 was taken in ABC complex mixing bottle and 1 drop of solution A and 1 drop of solution B from the manufacture's Kit were added and mixed well. This was prepared fresh before the experiment). 6) 0.03% 3,3'diaminobenzidine tetrahydrochloride solution, to which a drop of hydrogen peroxide (0.03%) was added just before use, for 10-15 minutes. Each solution was prepared in 0.05 M Tris buffer pH 7.6 containing 0.1 mol/l NaCl (0.15 M Tris buffered saline) and between each step of the staining procedure the fixed cells were washed in the same buffer for 5 min. Immunostained cells were mounted in 90% glycerol and observed under bright field illumination. Cells demonstrating intense brown nuclear staining were considered positive for PCNA expression (Fig. 3-2) and the percentage of positively stained cells was calculated. A minimum of 400 cells were counted. For examining the response to cerium, synchronised cultures were exposed to cerium for 3 hours and immunostained for PCNA. Cell viability was assessed in parallel cultures by acridine orange/ ethidium bromide staining.

DETERMINATION OF CELL COUNT Cardiac fibroblasts, after synchronisation, were exposed to different concentrations of cerium (0.1 μM, 0.5 μM, 1 μM, 10 μM, and 100 μM). Cells were harvested using trypsin EDTA mixture (trypsin - 0.25% w/v - EDTA 0.2% w/v) after 96 hours and the cell count was determined using a haemocytometer. Cell viability was assessed by trypan blue dye exclusion test. Approximately

FIGURE 3-2

PHOTOMICROGRAPH OF CARDIAC FIBROBLASTS STAINED FOR PCNA REACTIVE CELLS



Magnification (320 X)

→ PCNA positive cells

0.9 ml of the cell suspension was mixed with 0.1 ml trypan blue solution (4 mg/ml) in PBS. After 5 min at room temperature the viable (unstained) and non-viable (stained) cells were counted.

Measurement of superoxide anion content

MEASUREMENT OF INTRACELLULAR SUPEROXIDE ANION CONTENT Determination of the superoxide anion generation was done both by the visual method and spectrophotometric assay of reduced nitroblue tetrazolium salt (Das et al, 1987). The assay depends on the principle of substrate free reduction of the tetrazolium salt by superoxide anions to blue insoluble product formazan. Cells were plated at a density of 1.5 x 10^5 cells/well in 4 well plates. Confluent cultures of fibroblasts were incubated for 1 hour at 37°C in a humidified incubator (with 5% CO_2) with or without cerium, with 1 ml of 0.2% NBT dissolved in Kreb's Ringer buffer (pH 7.4). The formazan produced was extracted with propanol and the optical density was read spectrophotometrically at 550 nm. To test for the specificity of the assay, superoxide dismutase (100 U/ml), catalase (120 U/ml) and N- acetyl -L - cysteine (20 mM) was added to the culture and the assay was repeated.

Intracellular superoxide anion generation was also examined cytologically by observation of NBT treated cultures under bright field optics.

MEASUREMENT OF EXTRACELLULAR SUPEROXIDE ANION CONTENT

(Johnston, 1991) Ferricytochrome c (horse heart) was dissolved in HBSS (without phenol red) pH 7.35 to a stock concentration of 1.2 mM. It was filtered through millipore membrane (0.22 μM) and stored at 20°C in airtight containers in aliquots sufficient for a single experiment.

Release of superoxide anion was quantitated using confluent cardiac fibroblast cultures. The cultures were washed twice with KRB, pH 7.4, (kept at room temperature) to remove non adherent cells and traces of medium left in the culture wells. Washing was accompanied by vigorous swirling. After the second wash, the reaction was begun by placing the culture plates in an incubator at 37°C with 95% air and 5% CO_2 . The reaction mixture prepared contained cytochrome c to a final concentration of 80 μM in HBSS. Cerium at 0.5 μM concentration was added simultaneously. Cultures devoid of cerium served as control. For each test well a parallel culture was set up containing SOD at a final concentration of 40 μg/ml. Blanks were prepared by incubating each type of reaction mixture in culture wells without cells.

The reaction was stopped after 1 hour by transfer of the incubation mixture by Pasteur pipette to centrifuge tubes placed in an ice bath, followed promptly by centrifugation at 1200g for 10 minutes. The supernatant was then transferred to separate tubes, and optical density of the absorbance determined in a spectrophotometer at 550 nm. Reaction mixtures from culture wells that did not contain cells were used as blanks.

The cells remaining in the culture wells were washed three times with calcium magnesium free HBSS and the cells were harvested using trypsin (0.25% w/v) and EDTA (0.2% w/v). The cells were counted on a haemocytometer.

The OD_{550} of the reaction mixture was converted to nanomoles of cytochrome c reduced using the extinction coefficient $\Delta E_{550} = 21.0 \times 10^3\ M^{-1}\ cm^{-1}$ (Massey, 1959). As the reaction mixture was 1 ml, the observed OD_{550} was multiplied by conversion factor 47.6 and was expressed as nmoles of $O_2^{\cdot-}$ equivalents per 10^6 cells.

This conversion depends upon the assumption that the cytochrome c in the blank is fully oxidised and therefore the observed OD represents the absorbance of only the reduced product (Δ OD=reduced - oxidised). This assumption was tested by fully oxidising the reagent cytochrome c in solution with a few milligrams of potassium ferricyanide; by fully reducing the cytochrome c with a few milligrams of sodium dithionite and by comparing the OD_{550} of the untreated, oxidised and reduced solutions against that of water. In fresh reagent solution, 98-99% of the ferricytochrome c was found to be oxidised.

MEASUREMENT OF LIPID PEROXIDATION Lipid peroxidation was analysed following the method described by Kirshenbaum et al in 1995. Malondialdehyde formed by the breakdown of polyunsaturated fatty acids, served as an index of the extent of lipid peroxidation. Malondialdehyde

reacted with thiobarbituric acid to a give a red color species and the absorbance was read at 535 nm.

Confluent cardiac fibroblast cultures were treated with 0.5 μM cerium for 1 hour and incubated at 37°C. After the exposure period, the cells were rinsed with HBSS (pH 7.4),were scraped from the dish and then lysed with 6.5% TCA. A mixture containing 15% tricholoroacetic acid, 0.375% thiobarbituric acid and 0.25 N hydrochloric acid was prepared. After mixing this solution thoroughly with the lysed cells, it was heated for 15 min in a boiling water bath. After cooling, the flocculent precipitate was removed by centrifugation at 1000g for 10 min. The absorbance of the supernatant was determined at 535 nm against a blank that contained all the reagents except the cells. The amount of malondialdehyde formed was calculated from the molar extinction coefficient of malondialdehyde (1.56×10^5 /M per cm^3) and expressed as nmoles of MDA equivalents per 10^6 cells. The cell count was determined from parallel cultures.

Use of free radical scavengers to confirm the role of free radicals in cardiac fibroblast proliferation

DETERMINATION OF PROLIFERATING CELLS Synchronised cultures were exposed to 0.5 μM cerium and one of the free radical scavengers : superoxide dismutase (100 U/ml), catalase (120 U/ml) (De Keulenaur et al, 1995) or NAC (20 mM) (Irani et al, 1997) was included in the medium at the initiation of the experiment. After 3 hours,

immunocytochemical labelling for PCNA was done and the number of immunoreactive cells were counted and compared with that in cultures without the scavenger. Cell viability was determined from parallel cultures.

DETERMINATION OF CELL DENSITY Synchronised cultures were exposed to 0.5 μM cerium and the free radical scavenger SOD (100 U/ml), CAT (120 U/ml) or NAC (20 mM) was added simultaneously in the medium along with cerium and incubated for 96 hours at 37°C in a humidified atmosphere with 5% CO_2 . The cells were then harvested using trypsin (0.25% w/v) and EDTA (0.2% w/v) in calcium - magnesium free Hank's balanced salt solution (pH 7.4) as described before and the cell count was taken on a heamocytometer. Cultures without the scavenger served as control. Cell viability was assessed by trypan blue dye exclusion test.

DETERMINATION OF SUPEROXIDE ANION CONTENT To determine the effect of free radical scavengers on cerium induced intracellular generation of free radicals in cardiac fibroblasts, confluent cardiac fibroblast cultures were exposed to 0.5 μM cerium and also to SOD (100 U/ml), CAT (120 U/ml) or NAC (20 mM) for 1 hour and NBT reduction assay was done as described earlier. The extent of NBT reduction was determined on a spectrophotometer at 550 nm. Cultures without the scavenger served as control.

Effect of hypxanthine and xanthine oxidase on generation of superoxide anions and cell proliferation

Confluent cultures of cardiac fibroblasts were exposed to superoxide anion generating system, Hyp+XO (Murrell et al, 1990), in culture medium. Extracellular content of superoxide anions was obtained by measurement of SOD inhibitable reduction of cytochrome c (Johnston, 1991).

The intracellular generation of superoxide anions was measured by NBT reduction assay following exposure to hypoxanthine (1 mM) and xanthine oxidase (10^{-6} U/ml). Cardiac fibroblasts were exposed to the free radical generators with or without the scavenging enzymes SOD, catalase and cell permeant antioxidant NAC in the culture medium for 1 hour at 37°C in a CO_2 incubator. After the exposure time, the formazan produced was extracted using propanol and the extent of NBT reduction was read in a spectrophotometer at 550 nm.

For immunocytochemical study of proliferating cells, synchronised cultures of cardiac fibroblasts were exposed to Hyp (1 mM) and XO (10^{-6} U/ml) for 3 hours in a CO_2 incubator at 37°C. After the exposure time, the cells were washed with Tris buffer (pH 7.6) and were fixed and stained for the PCNA reactive cells. Tests were also carried out with the inclusion of the scavenging enzymes SOD (100 U/ml), catalase (120 U/ml) and NAC (20 mM) in the culture medium.

For studying the effect of superoxide anions on cell density, cardiac fibroblasts were exposed to superoxide anions using hypoxanthine (1 mM) and xanthine oxidase (10^{-6} U/ml). The synchronised cultures were treated with the free radical generators for 96 hours. The culture plate was incubated at 37°C with 5 % CO_2 and 99% humidity. The cells were harvested after 96 hours using trypsin -EDTA mixture and the cell count was taken on a haemocytometer. Cell density was also determined in the presence of the scavenging enzymes superoxide dismutase (100 U/ ml), catalase (120 U/ml) and cell permeant antioxidant N- acetyl - L - cysteine (20 mM).

Effect of cerium on skeletal muscle fibroblasts

ISOLATION AND CULTURE OF SKELETAL MUSCLE FIBROBLASTS Skeletal muscle fibroblasts were isolated following the same procedure as described earlier for cardiac fibroblasts. Identification was based on the morphology and immunocytochemical staining using anti- vimentin, anti - desmin and anti- factor VIII.

ASSESSMENT OF CELL PROLIFERATION IN RESPONSE TO CERIUM Skeletal muscle fibroblasts following synchronisation for 24 hours, were exposed to 0.5 μM concentration of cerium in the culture medium containing 0.4% FCS for 3 hours at 37°C in a CO_2 incubator. The response to cerium was also assessed in the presence of enzymatic and non

- enzymatic scavengers. The cells were washed and stained subsequently for the determination of proliferating cells as carried out for cardiac fibroblasts.

ASSESSMENT OF CELL DENSITY To study the effect of cerium on cell density skeletal muscle fibroblasts were exposed to 0.5 μM cerium in the culture medium containing 0.4% FCS with or without enzymatic and non enzymatic scavengers. The culture plate was incubated at 37°C for 96 hours in a CO_2 incubator. The cells were trypsinised after 96 hours and the cell count was taken on a haemocytometer.

MEASUREMENT OF SUPEROXIDE ANION CONTENT The intracellular generation of superoxide anion was assessed in confluent cultures of skeletal muscle fibroblasts following the method of Das et al (1987) using NBT reduction assay. The extent of NBT reduction by the confluent skeletal muscle fibroblasts after an exposure period of 1 hour to 0.5 μM cerium, was read in a spectrophotometer at 550 nm. Nitroblue tetrazolium reduction assay was also carried out with the inclusion of SOD, catalase and NAC both in presence and absence of cerium.

Effect of exogenous superoxide anion generating system on skeletal muscle fibroblasts

The intracellular generation of superoxide anions was measured using NBT reduction assay. Confluent cultures of skeletal muscle fibroblasts

were exposed to Hyp (1 mM) and XO (10^{-6}/ml) for 1 hour at 37° C in a CO_2 incubator. The formazan produced was extracted using propanol and the absorbance was read on a spectrophotometer at 550 nm. The NBT reduction assay was also carried out following the inclusion of free radical scavengers.

Skeletal muscle fibroblasts following synchronisation, were exposed to Hyp (1 mM) and XO (10^{-6} U/ml). Cultures were also set up with the superoxide anion scavengers included in the culture medium containing 0.4 % FCS. After the exposure period of 3 hours, the cells were washed in Tris buffer (pH 7.6). The cultures were subsequently stained for identification of the PCNA reactive cells following the procedure as described earlier.

For determination of total cell count, the synchronisation of cultures was followed by exposure to the free radicals generated extracellularly using Hyp and XO in the culture medium for 96 hours at 37°C in a CO_2 incubator. The cells were washed in HBSS and were trypsinised using trypsin (0.25% w/v) - EDTA (0.2% w/v) mixture and the cell count was taken on a haemocytometer. Cell density was determined in the presence of SOD, catalase and NAC.

Statistical analysis

The data are presented as mean ± SEM values for each set. Each experimental observation was based on a minimum of 4 replicates. A level of $p < 0.05$ was selected to indicate statistical significance. The difference between selected means were evaluated using unpaired Student's t-test. ANOVA was carried out where necessary, applying Modified LSD (Bonferroni) test for comparison of group means.

ANALYSIS OF DATA

CHAPTER - 4

ANALYSIS OF DATA

RESULTS

The experimental results are presented under the following headings :

Culture and identification of cardiac fibroblasts

Effect of different concentrations of cerium on cell proliferation as assessed by

a) the proportion of PCNA reactive cells

b) total cell count

Effect of cerium on superoxide anion generation

a) intracellular superoxide anion generation

b) extracellular release of superoxide anions

c) lipid peroxidation

Effect of antioxidants and free radical scavengers

a) on cell proliferation

b) on cell density

c) on superoxide anion generation

Response of cardiac fibroblasts to extracellular superoxide anion generating system

Response of skeletal muscle fibroblasts to cerium and extracellular superoxide anion generating system

Culture and identification of cardiac fibroblasts

Cardiac fibroblasts isolated from neonatal Wistar rats were cultured and pure fibroblast cultures were obtained by repeated passage. These cells were identified on the basis of their morphology and also by immunocytochemical studies.

Fibroblasts were identified by their irregular shape with multiple filopodia. The cytoplasm was phaselucent and unstructured. Cytoplasmic granules were found to be arranged around a single oval shaped nucleus often containing two nucleoli (Fig. 4-1).

Immunohistochemical staining was done to characterise the cardiac fibroblasts using antibody to vimentin, desmin and factor VIII. Cultures were immunoreactive to vimentin (Fig. 4-2), but stained negatively for desmin and factor VIII.

Cell viability as assessed by acridine orange/ ethidium bromide staining was more than 90%.

Effect of different concentrations of cerium on cell proliferation

DETERMINATION OF PCNA REACTIVE CELLS Synchronised cultures of cardiac fibroblasts were exposed to different concentrations of the lanthanide cerium (0.1 μM, 0.5 μM, 1μM, 10 μM and 100 μM) for 3 hours and immunocytochemical labelling for PCNA was done. More than 400 cells were scored per preparation. Significant variation in the proportion of

immunoreactive cells was observed (ANOVA $p < 0.05$; Appendix A-2). Compared to untreated control, a significant increase in the proportion of immunoreactive cells was observed on exposure to cerium with a peak at 0.5 μM (Fig. 4-3). Cell viability as determined by acridine orange/ethidium bromide staining was more than 90% in all concentrations of cerium.

DETERMINATION OF CELL DENSITY Cell count was determined in synchronised cultures of cardiac fibroblasts after exposure to different concentrations of cerium for 96 hours. A concentration dependent effect of cerium on cell number was seen (ANOVA $p < 0.005$; Appendix A- 4). Cell density showed an increase at low levels of cerium (from 0.1 - 1μM) and a decrease at higher concentrations (10 and 100 μM) (Fig. 4-4). A statistically significant difference compared to cerium free control was observed at 0.5 μM concentration of cerium. Cell morphology and cell viability were not affected even at higher concentrations of cerium. More than 90% of cells were viable in all cultures.

Inference : Increase in the percentage of immunoreactive cells and the increase in cell number suggests that low concentration of cerium (0.5 μM) induces stimulatory response in cardiac fibroblasts.

Effect of cerium on superoxide anion generation in cardiac fibroblasts

EFFECT OF DIFFERENT CONCENTRATIONS OF CERIUM ON INTRACELLULAR GENERATION OF SUPEROXIDE ANIONS IN CARDIAC FIBROBLASTS Exposure of cardiac fibroblasts to different concentrations of cerium resulted in an increase in free radical production as measured by the reduction of tetrazolium salt to blue formazan. A dose dependent variation in formazan production was observed (ANOVA $p < 0.005$; Appendix A -6). Intracellular superoxide anion content increased with the concentration of cerium, with a peak at 0.5 μM concentration of the element followed by a decrease (Fig. 4-5). However at 100 μM concentration, the free radical content exceeded the value at 10 μM cerium. At 0.5 μM cerium, a distinct difference in NBT reduction to formazan was visible in cells as compared to control (Fig. 4-6).

As the response to cerium both in terms of cell proliferation and intracellular superoxide anion generation was significant at 0.5 μM, this concentration was selected for carrying out all further studies. This concentration falls within the range of the level of the cerium observed in the serum of patients with endomyocardial fibrosis (Eapen et al., 1997).

EFFECT OF CERIUM (0.5 μM) ON EXTRACELLULAR GENERATION OF SUPEROXIDE ANION Extracellularly generated superoxide anions were measured in cerium exposed cultures by assay of SOD inhibitable reduction of cytochrome c. One hour exposure to cerium led to a significant increase ($p < 0.001$) in the release of superoxide anions into the medium (Fig. 4-7).

EFFECT OF CERIUM (0.5 μM) ON LIPID PEROXIDATION When cardiac fibroblasts were exposed to cerium, lipid peroxidation as reflected by the formation of TBARS, was increased ($p < 0.005$) compared to untreated cells (Fig. 4-8).

Inference : Cerium stimulates intracellular and extracellular generation of superoxide anions and the cerium induced stimulation of superoxide anion formation is associated with lipid peroxidation.

Effect of enzymatic and non enzymatic scavengers on cerium induced cell proliferation in cardiac fibroblasts

IMMUNOSTAINING FOR PCNA In order to ascertain whether the increased expression of PCNA reactive cells in cerium exposed cultures of cardiac fibroblasts is mediated by free radicals, scavenging enzymes superoxide dismutase (100 U/ml) and catalase (120 U/ml) were included in the culture medium simultaneously with 0.5 μM concentration of cerium. As is

evident from Fig. 4-9, catalase had no effect but superoxide dismutase lowered the proportion of PCNA reactive cells. NAC (20 mM) when included along with cerium was found to lower (16.4%±0.22 *vs* 37.5%±0.1.2) the percentage of immunoreactive cells. Cell viability was not affected by the presence of the scavengers.

DETERMINATION OF CELL DENSITY Cardiac fibroblasts were exposed to cerium (0.5 μM) alongwith the scavenging enzymes SOD (100 U/ml) and catalase (120 U/ml) for 96 hours. There was a significant decrease in the cell number in cultures exposed to SOD ($2.8x10^{-5} \pm 0.10$ *vs* $4.7x10^{-5} \pm 0.22$) ($p < 0.0005$). Catalase had no effect on the cells (Fig. 4-10).

Exposure to NAC (20 mM) simultaneously with cerium (0.5 μM) for 96 hours showed a significant decrease ($p<0.0005$) in the cerium stimulated increase in cell number. The degree of inhibition was higher compared to that seen for extracellular scavenging enzyme SOD (100 U/ml). The cell density was significantly lower ($p< 0.005$) than that observed in untreated cultures. The decrease in cell density was not the result of decreased viability as assessed by trypan blue staining.

MEASUREMENT OF SUPEROXIDE ANION CONTENT Cardiac fibroblasts were exposed to cerium (0.5 μM) along with the scavenging enzymes SOD (100 U/ml) and catalase (120 U/ml). Nitroblue tetrazolium reduction assay was done to measure the superoxide content after an

exposure period of 1 hour. SOD significantly reduced ($p < 0.0005$) the formazan production whereas catalase had no such effect on the cardiac fibroblasts (Fig. 4-11).

A significant decrease (0.008 ± 0.0006 *vs* 0.048 ± 0.0008) in the formazan production was seen when cardiac fibroblasts were exposed to NAC for 1 hour along with cerium (0.5 μM). The inhibition was higher compared to the effect of scavenging enzyme SOD. The level compared to control was significantly low ($p < 0.0005$).

The effect of scavengers could also be visualized as evident from Fig. 4-12. Extent of reduction of NBT was lower compared to cerium exposed cultures (Fig. 4-6b).

Inference : The neutralization of the cerium induced stimulatory response on free radical generation and cell proliferation by superoxide dismutase and N-acetyl-L-cysteine fortifies the role of superoxide anions in cerium induced cardiac fibroblast proliferation. The exaggerated response to the cell permeant antioxidant NAC on cell growth with a positive association with the intracellular superoxide anion content suggests that these oxygen radicals may have a prominent role in the mediation of cell proliferation.

Response of cardiac fibroblasts to extracellular superoxide anion generating system

On exposure of cardiac fibroblasts to the exogenous superoxide anion generating system, Hyp (1 mM) +XO (10^{-6} U/ml) for 1 hour, the superoxide anion released was 1.03 nmoles/10^6cells as measured by SOD inhibitable cytochrome c reduction assay.

MEASUREMENT OF INTRACELLULAR SUPEROXIDE ANION CONTENT OF CARDIAC FIBROBLASTS EXPOSED TO Hyp + XO The intracellular content of superoxide anion was measured by NBT reduction assay following exposure to extracellular source of free radicals - Hyp (1 mM) and XO (10^{-6} U/ml). As depicted in the Fig.4-13, there was a significant increase in the content of superoxide anions in cells exposed to superoxide anion generating system. On inclusion of SOD (100 U/ml), the intracellular content of superoxide anion was comparable to that of the control as assessed by the formazan production. A significant reduction in the formazan content was observed in the presence of the cell permeant antioxidant NAC ($p < 0.0005$).

EFFECT OF Hyp + XO ON CELL PROLIFERATION Exposure to Hyp and XO for 3 hours led to a significant increase in the proportion of immunoreactive cells compared to control. The inclusion of enzymatic and non - enzymatic free radical scavengers reduced the proportion of PCNA

reactive cells thus confirming the role of superoxide anions in cell proliferation (Fig. 4-14).

EFFECT OF Hyp + XO ON CELL DENSITY Cardiac fibroblasts showed a significant increase in cell number when exposed to Hyp+XO for 96 hours. However, the treatment with SOD (100 U/ml) and NAC (20 mM) for the same duration was found to inhibit the trophic response to extracellular superoxide anions (Fig. 4-15).

Inference : This observation indicates that the intracellular superoxide anion content can increase consequent to a rise in the extracellular generation of superoxide anions and that the increase in superoxide anions is associated with stimulation of cell proliferation.

Response of skeletal muscle fibroblasts to cerium and superoxide anion generating system

Skeletal muscle fibroblasts were isolated following the same procedure as applied for cardiac fibroblasts. Morphological appearance was comparable to that of cardiac fibroblasts (Fig. 4-16). The cells were vimentin positive and negative to desmin and factor VIII on immunostaining (Fig. 4-17).

EFFECT OF CERIUM (0.5 μM) ON CELL PROLIFERATION AS MEASURED BY PCNA STAINING Exposure to cerium for 3 hours was found to increase the percentage of immunoreactive cells in skeletal muscle fibroblasts. The scavenging enzyme SOD and cell permeant antioxidant NAC lowered the stimulatory response significantly ($p < 0.005$) thus suggesting that superoxide anions mediate the cerium induced proliferative response (Fig. 4-18).

EFFECT OF CERIUM ON CELL DENSITY After 96 hours of exposure to cerium, cell number increased significantly ($p<0.005$). The inclusion of SOD, NAC and CAT however lowered the cerium induced increase in cell count (Fig. 4-19).

EFFECT OF CERIUM ON INTRACELLULAR GENERATION OF SUPEROXIDE ANIONS The intracellular superoxide anion content was significantly increased in skeletal muscle fibroblasts when exposed to cerium for 1 hour. This increase was however neutralized with the inclusion of scavenging enzyme SOD and cell permeant antioxidant NAC into the culture medium (Fig. 4-20).

MEASUREMENT OF INTRACELLULAR SUPEROXIDE CONTENT ON EXPOSURE TO Hyp + XO Exposure to extracellular free radical generating system for 1 hour led to a significant increase in the intracellular

superoxide anion content in skeletal muscle fibroblasts. In the presence of SOD and NAC, neutralization of this stimulatory effect was evident, thus suggesting that the increase in superoxide anion content is due to the presence of free radicals generated in the external medium (Fig. 4-21).

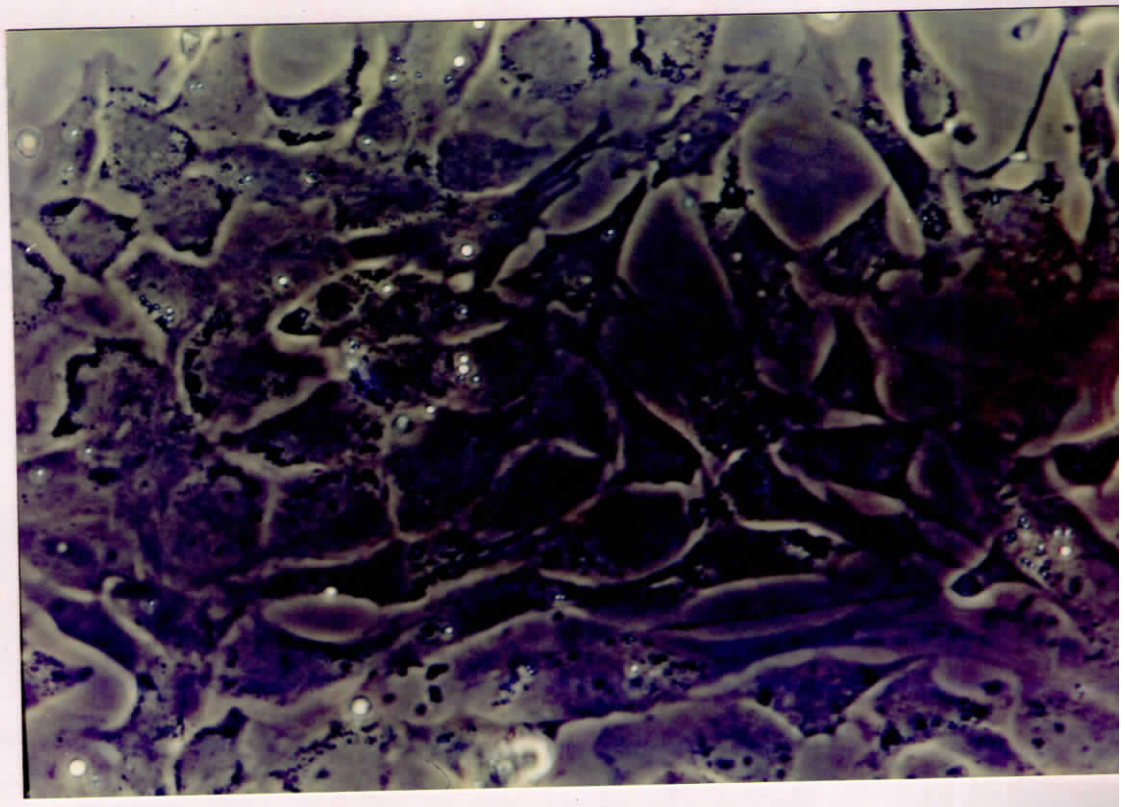
EFFECT OF Hyp + XO ON CELL PROLIFERATION AS MEASURED BY PCNA STAINING There was a significant increase in the proportion of PCNA reactive cells in the treated cultures compared to the control. The expression of PCNA was however lowered significantly with the inclusion of SOD and NAC in the medium (Fig. 4-22).

EFFECT OF Hyp + XO ON CELL DENSITY Exposure to Hyp+XO for 96 hours led to a significant increase in the number of skeletal muscle fibroblasts and neutralization of this stimulatory effect was observed by SOD and NAC (Fig. 4-23).

Inference : The response of skeletal muscle fibroblasts to the presence of cerium or extracellular superoxide anions is found to be similar to that of cardiac fibroblasts.

FIGURE 4-1

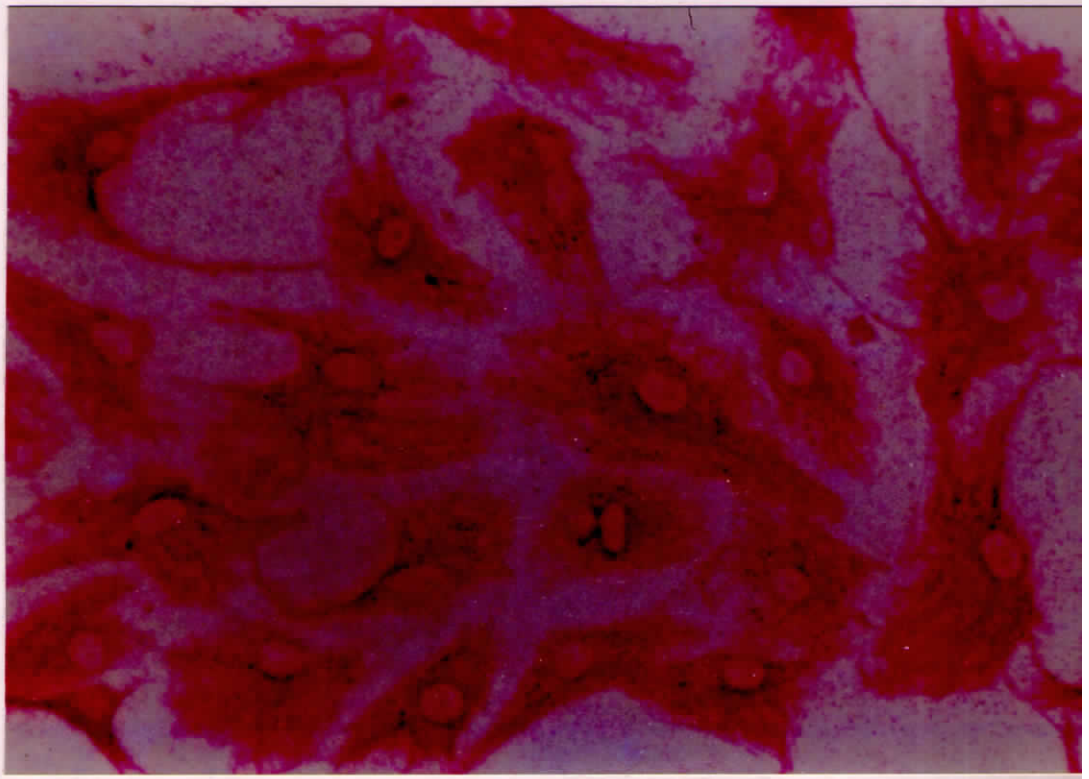
PHASE CONTRAST PICTURE OF NEONATAL RAT CARDIAC FIBROBLASTS



MAGNIFICATION (420 X)

FIGURE 4-2

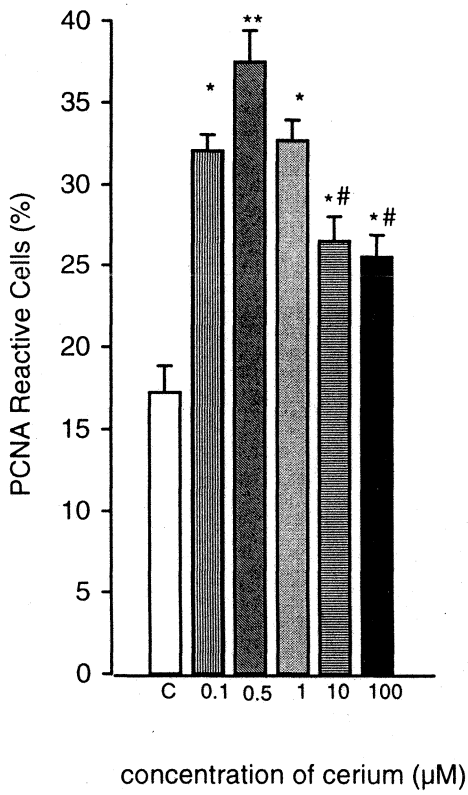
PHOTOMICROGRAPH OF CARDIAC FIBROBLASTS STAINED FOR VIMENTIN



MAGNIFICATION (420 X)

FIGURE 4-3

EFFECT OF DIFFERENT CONCENTRATIONS OF CERIUM ON CARDIAC FIBROBLAST PROLIFERATION AS MEASURED BY IMMUNOREACTIVITY FOR PROLIFERATING CELL NUCLEAR ANTIGEN



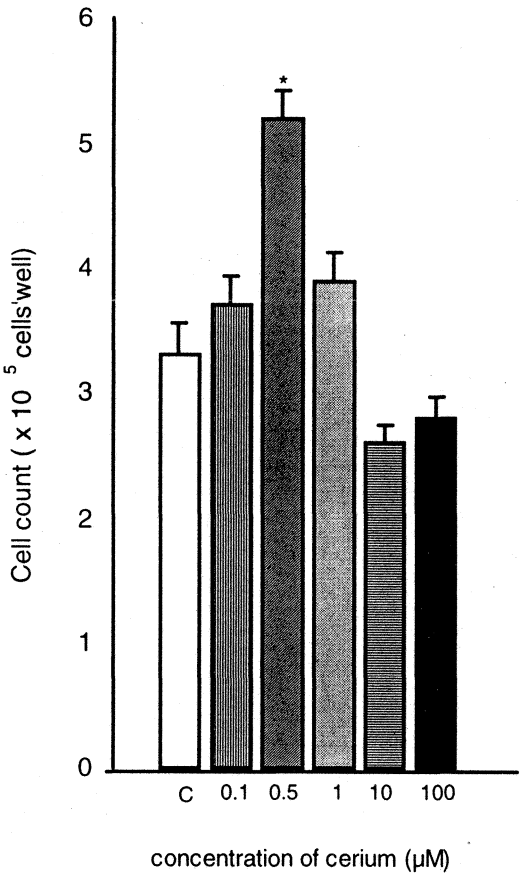
Values represent mean ± SEM; n = 4-5; ANOVA $p < 0.05$;

* $p < 0.05$ *vs* control (C); ** $p < 0.005$ *vs* control (C);

# $p < 0.005$ *vs* 0.5 μM cerium.

FIGURE 4-4

EFFECT OF DIFFERENT CONCENTRATIONS OF CERIUM ON GROWTH OF CARDIAC FIBROBLASTS AS DETERMINED BY TOTAL CELL COUNT

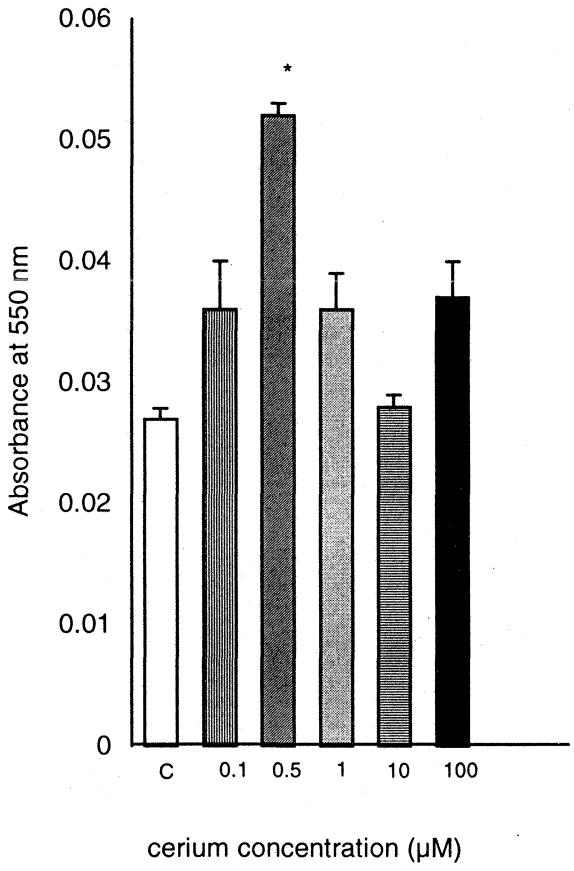


Values are mean ± SEM; n = 9; ANOVA $p < 0.05$;

* $p < 0.0005$ *vs* control (C);

FIGURE 4-5

EFFECT OF DIFFERENT CONCENTRATIONS OF CERIUM ON INTRACELLULAR GENERATION OF SUPEROXIDE ANIONS IN CARDIAC FIBROBLASTS AS ASSESSED BY REDUCTION OF NBT

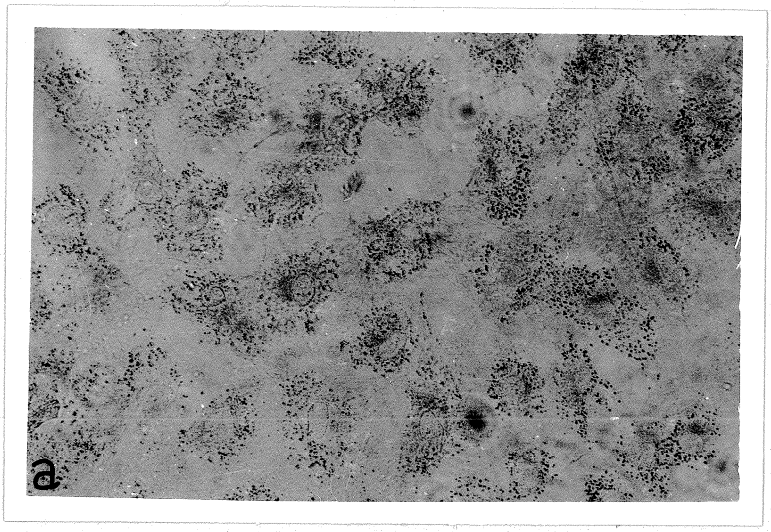


Values represent mean ± SEM; n= 4- 8; ANOVA $p < 0.05$;

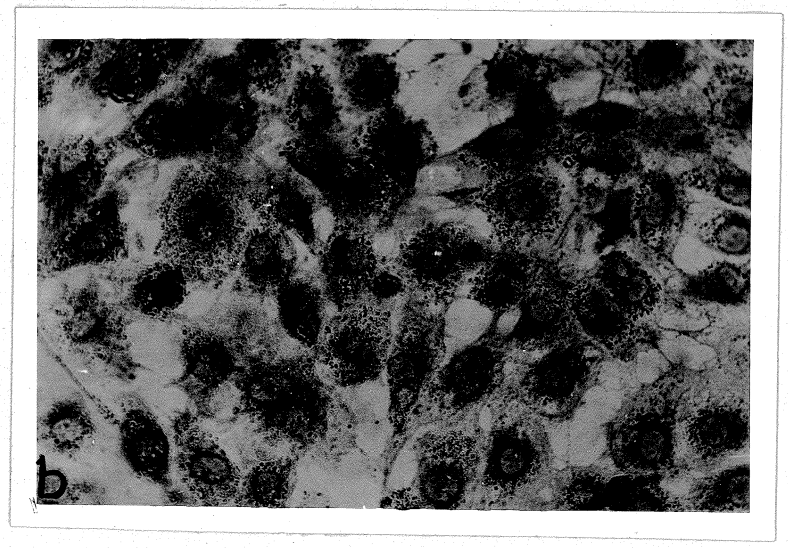
* $p < 0.0005$ *vs* control (C);

FIGURE 4-6

PHOTOMICROGRAPHS SHOWING SUPEROXIDE ANION DEPENDENT REDUCTION OF NBT IN CARDIAC FIBROBLASTS



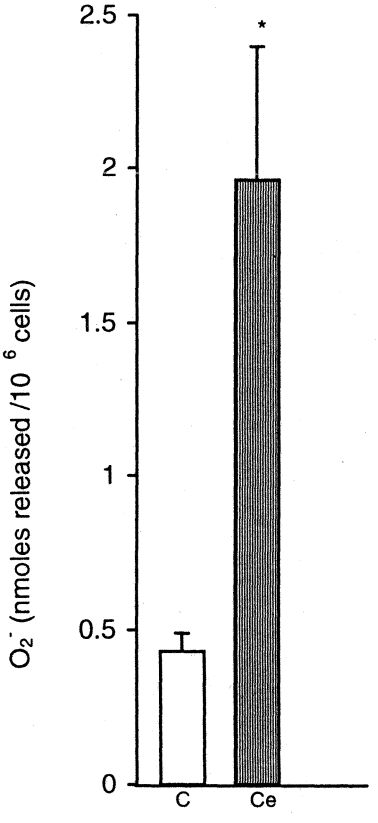
a) control MAGNIFICATION (280 X)



b) cerium (0.5 μM) treated cardiac fibroblasts
MAGNIFICATION (280 X)

FIGURE 4-7

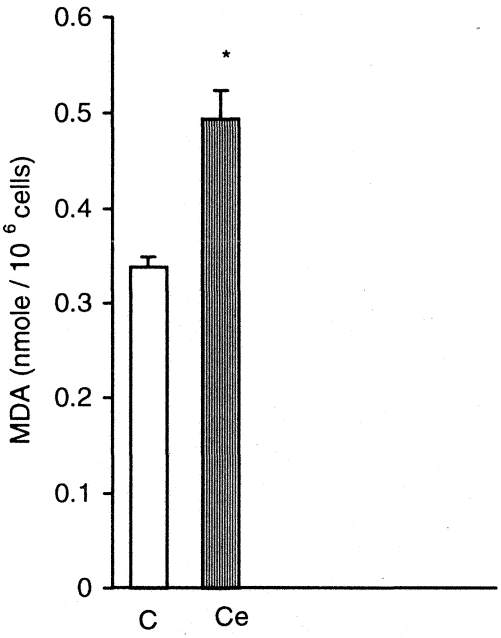
EFFECT OF CERIUM (0.5 μM) ON EXTRACELLULAR GENERATION OF SUPEROXIDE ANIONS BY CARDIAC FIBROBLASTS MEASURED BY SUPEROXIDE DISMUTASE INHIBITABLE REDUCTION OF CYTOCHROME C



Values represent mean ± SEM; n = 12; * $p < 0.001$ *vs* control (C).

FIGURE 4-8

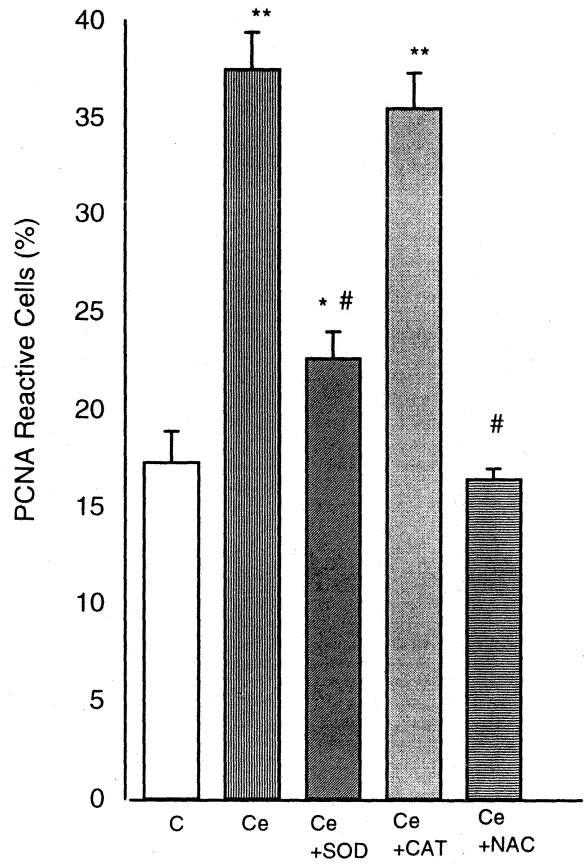
EFFECT OF CERIUM (0.5 μM) ON LIPID PEROXIDATION IN CARDIAC FIBROBLASTS AS MEASURED BY TBARS LEVELS



Values represent mean ± SEM; n = 10 - 16; * $p < 0.005$ *vs* control (C).

FIGURE 4-9

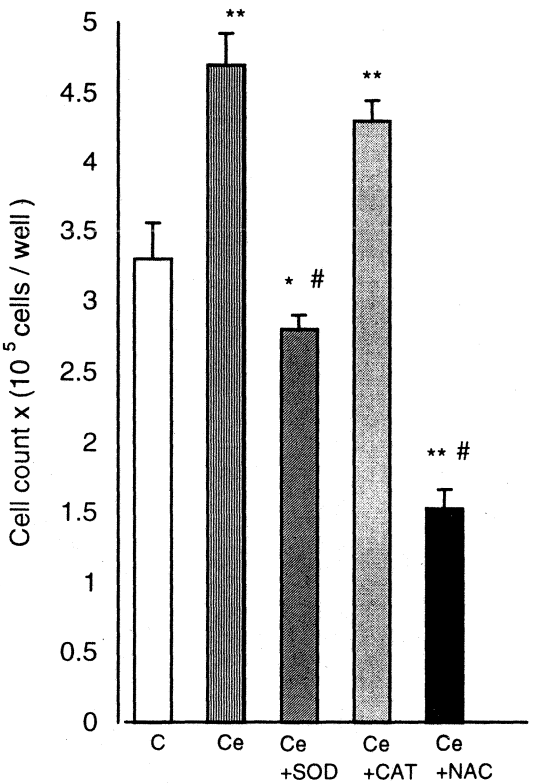
EFFECT OF FREE RADICAL SCAVENGERS ON CERIUM (0.5 μM) INDUCED PROLIFERATION OF CARDIAC FIBROBLASTS MEASURED BY PCNA REACTIVE CELLS



Values represent mean ± SEM; n = 4 - 12; * $p < 0.005$ *vs* control (C); ** $p < 0.0005$ *vs* control (C); # $p < 0.005$ *vs* cerium; SOD - 100 U/ml; CAT - 120 U/ml; NAC -20 mM

FIGURE 4-10

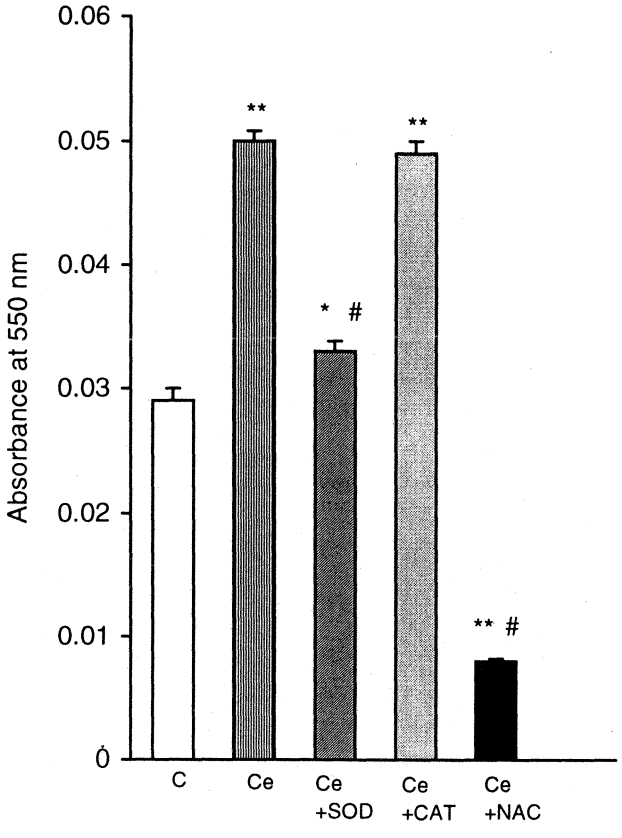
EFFECT OF FREE RADICAL SCAVENGERS ON CERIUM (0.5 μM) INDUCED GROWTH OF CARDIAC FIBROBLASTS ASSESSED BY TOTAL CELL COUNT



Values represent mean ± SEM; n=9 - 10; * $p < 0.05$ *vs* control (C); ** $p < 0.005$ *vs* control (C); # $p < 0.0005$ *vs* cerium; SOD - 100 U/ml; CAT - 120 U/ml; NAC - 20 mM.

FIGURE 4-11

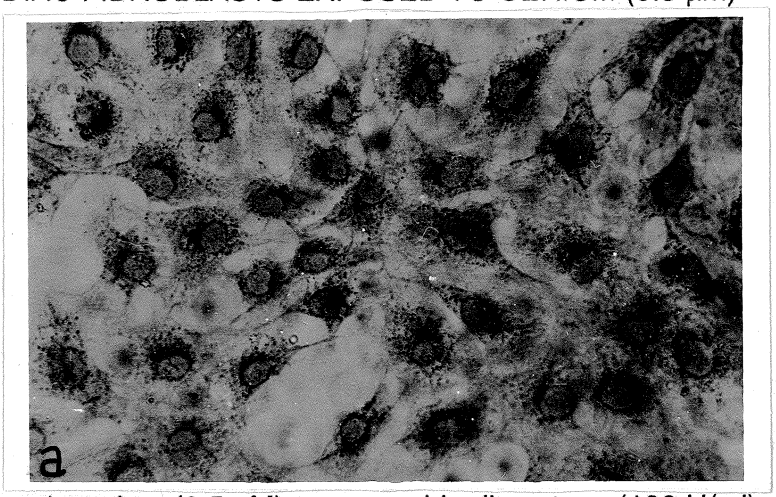
EFFECT OF FREE RADICAL SCAVENGERS ON CERIUM (0.5 μM) INDUCED INTRACELLULAR GENERATION OF SUPEROXIDE ANIONS IN CARDIAC FIBROBLASTS ASSESSED BY REDUCTION OF NBT



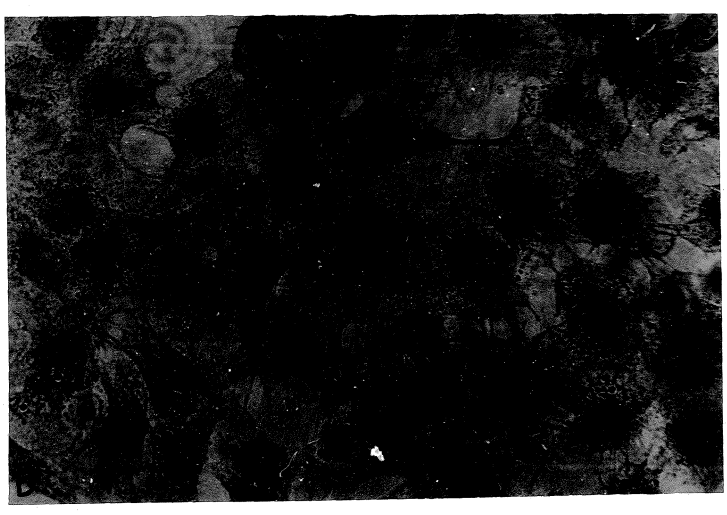
Values represent mean ± SEM; n = 5 - 12; * $p < 0.01$ *vs* control (C); ** $p < 0.0005$ vs control; # $p < 0.0005$ *vs* cerium; SOD - 100 U/ml; CAT - 100 U/ml; NAC - 20 mM.

FIGURE 4 -12

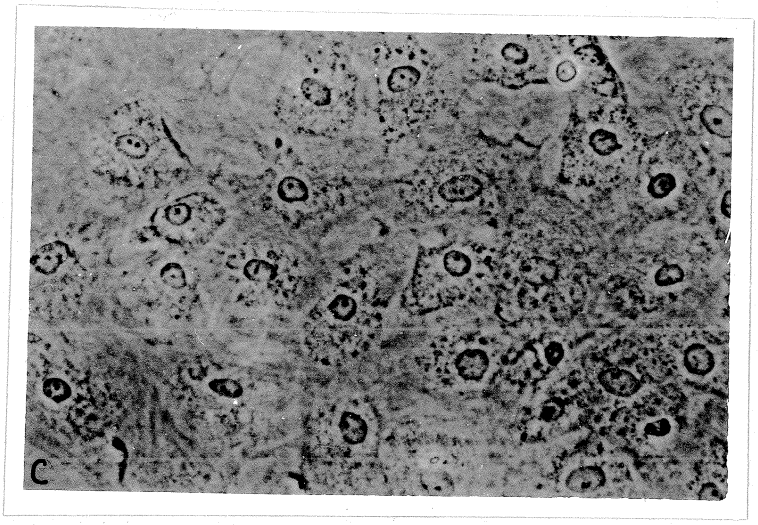
PHOTOMICROGRAPHS SHOWING THE EFFECT OF FREE RADICAL SCAVENGERS ON SUPEROXIDE ANION INDUCED NBT REDUCTION IN CARDIAC FIBROBLASTS EXPOSED TO CERIUM (0.5 μm)



a) cerium (0.5 μM) + superoxide dismutase (100 U/ml)
MAGNIFICATION (280 X)



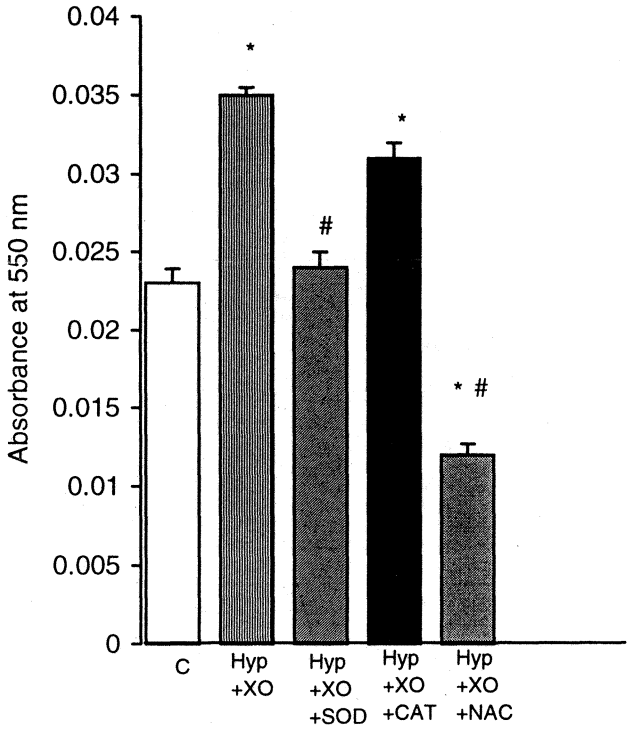
b) cerium (0.5 μM) + catalase (120 U/ml)
MAGNIFICATION (280 X)



c) cerium (0.5 μM) + N-acetyl - L-cysteine (20 mM)
MAGNIFICATION (280 X)

FIGURE 4-13

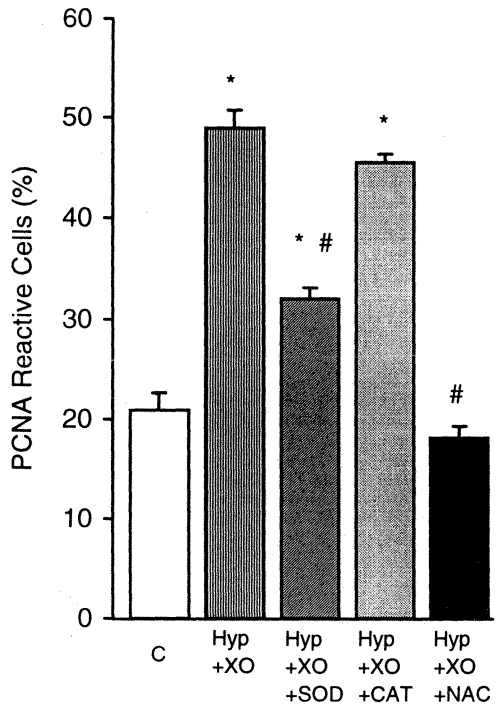
INTRACELLULAR CONTENT OF SUPEROXIDE ANIONS IN CARDIAC FIBROBLASTS EXPOSED TO Hyp (1 mM) + XO (10^{-6} U/ ml) ASSESSED BY REDUCTION OF NBT



Values represent mean ± SEM; n = 12; * $p < 0.0005$ *vs* control (C);
# $p < 0.0005$ *vs* Hyp+XO; SOD - 100 U/ml; CAT - 120 U/ml; NAC - 20 mM.

FIGURE 4-14

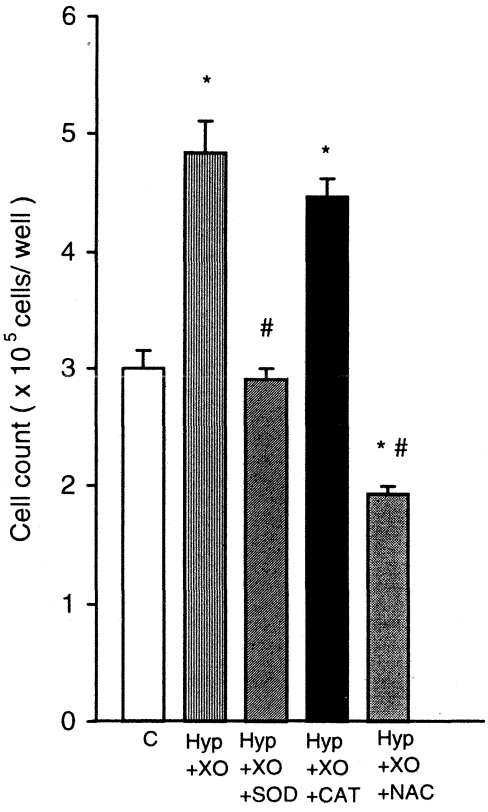
EFFECT OF Hyp (1 mM) + XO (10^{-6} U/ ml) ON PROLIFERATION OF CARDIAC FIBROBLASTS AS MEASURED BY PCNA STAINING



Values represent mean ± SEM; n = 9; *p < 0.0005 *vs* control (C);
#p < 0.0005 *vs* Hyp + XO; SOD - 100 U/ml; CAT - 120 U/ml; NAC - 20 mM.

FIGURE 4-15

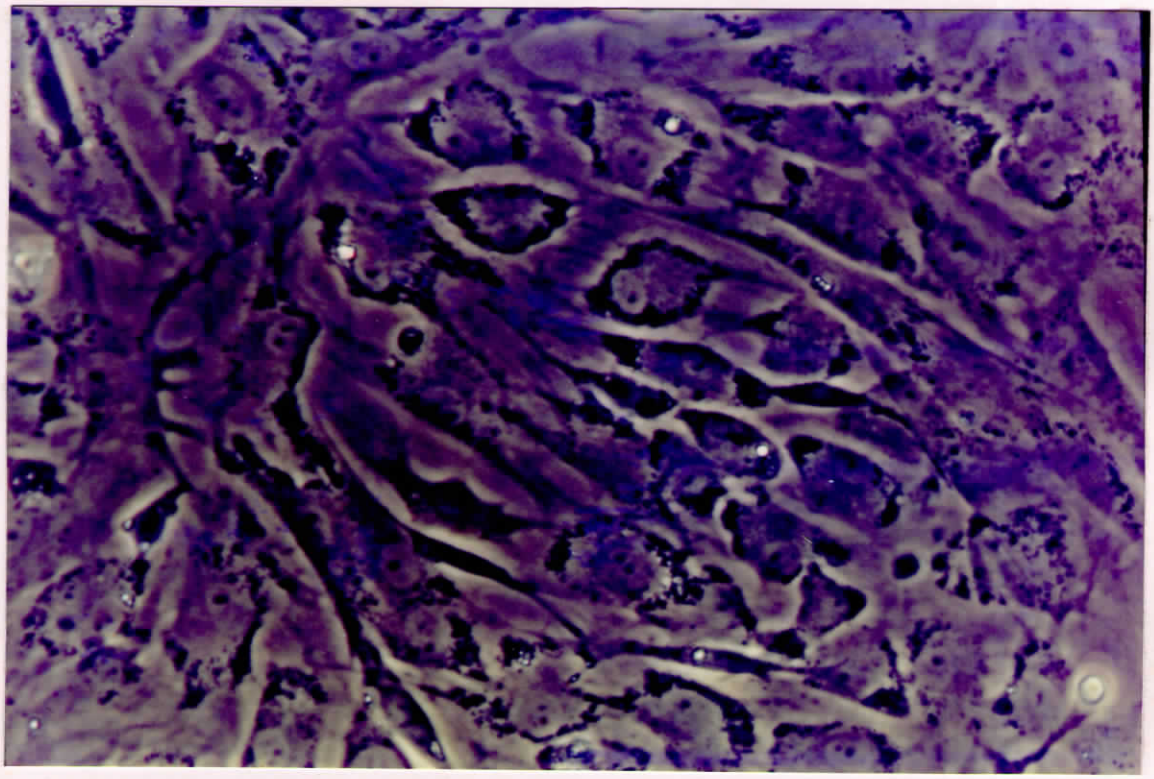
EFFECT OF Hyp (1 mM) +XO (10^{-6} U/ ml) ON GROWTH OF CARDIAC FIBROBLASTS DETERMINED BY TOTAL CELL COUNT



Values represent mean ± SEM; n = 15; * $p < 0.0005$ *vs* control (C);
# $p < 0.0005$ *vs* Hyp + XO; SOD - 100 U/ml; CAT - 120 U/ml; NAC - 20 mM.

FIGURE 4-16

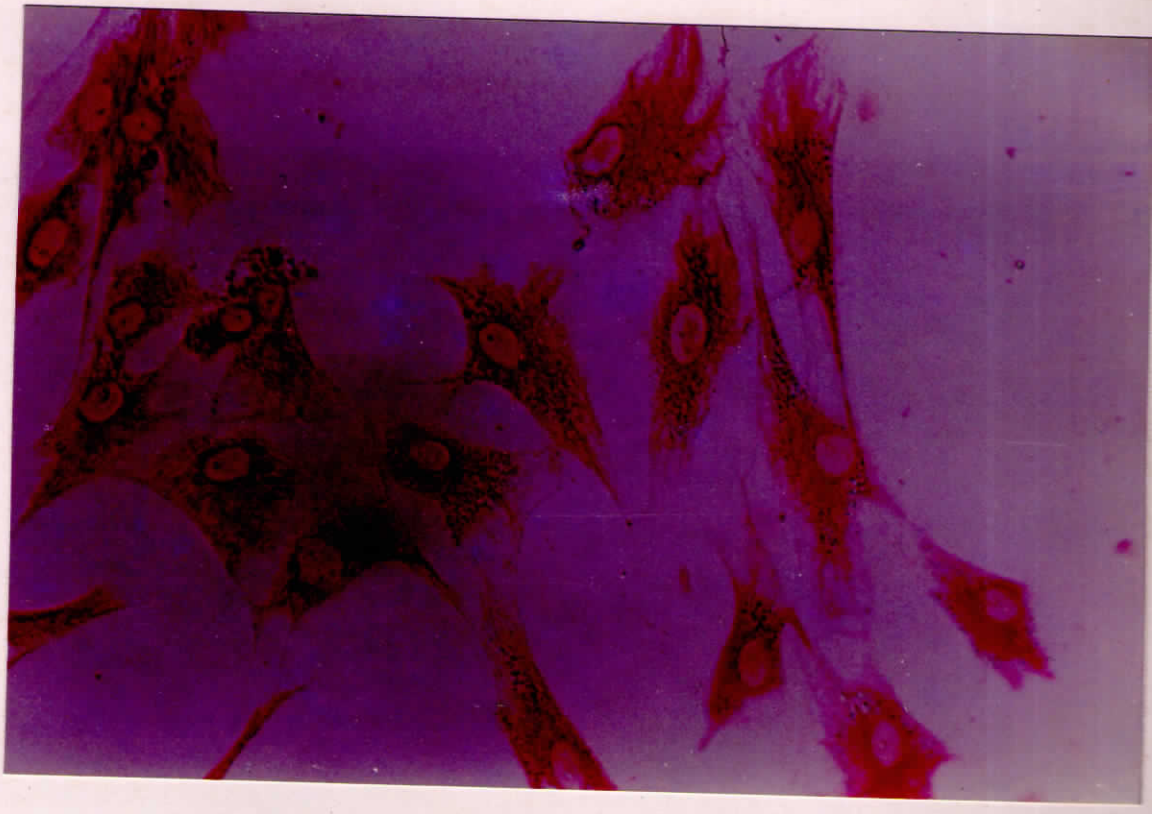
PHASE CONTRAST PICTURE OF NEONATAL RAT SKELETAL MUSCLE FIBROBLASTS



MAGNIFICATION (420 X)

FIGURE 4-17

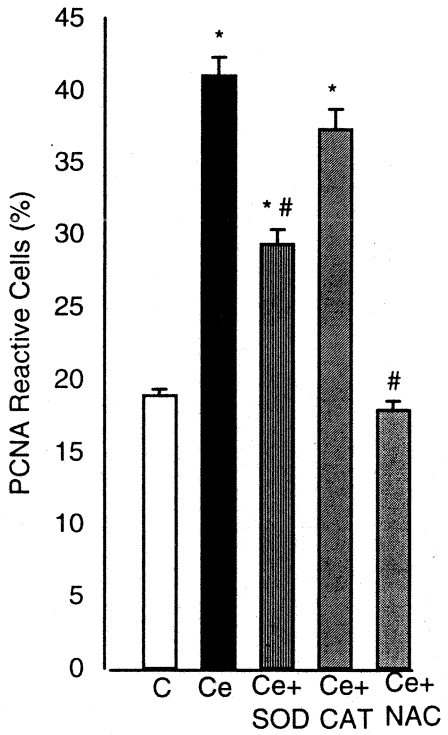
PHOTOMICROGRAPH SHOWING SKELETAL MUSCLE FIBROBLASTS STAINED FOR VIMENTIN



MAGNIFICATION (420 X)

FIGURE 4-18

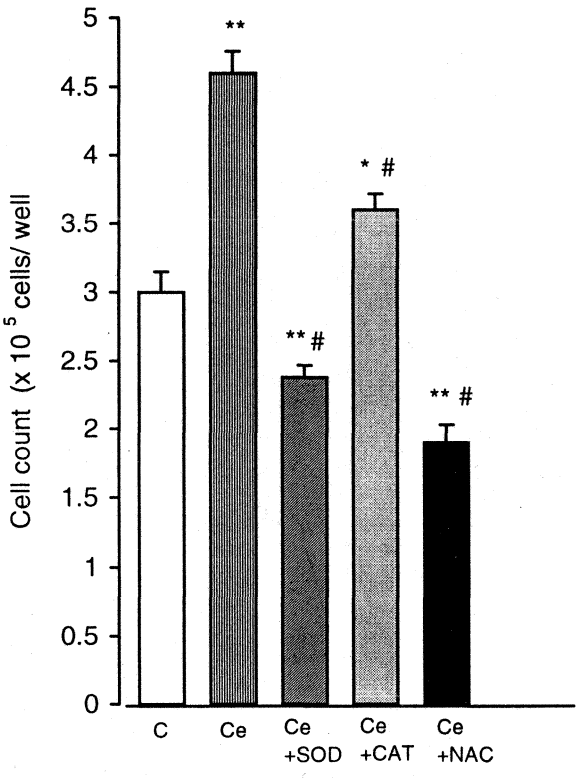
EFFECT OF CERIUM (0.5 μM) ON PROLIFERATION OF SKELETAL MUSCLE FIBROBLASTS AS MEASURED BY IMMUNOREACTIVITY FOR PCNA



Values represent mean ± SEM; n = 9; *p < 0.0005 *vs* control (C);
# p < 0.005 *vs* cerium; SOD - 100 U/ml; CAT - 120 U/ml; NAC - 20 mM.

FIGURE 4 -19

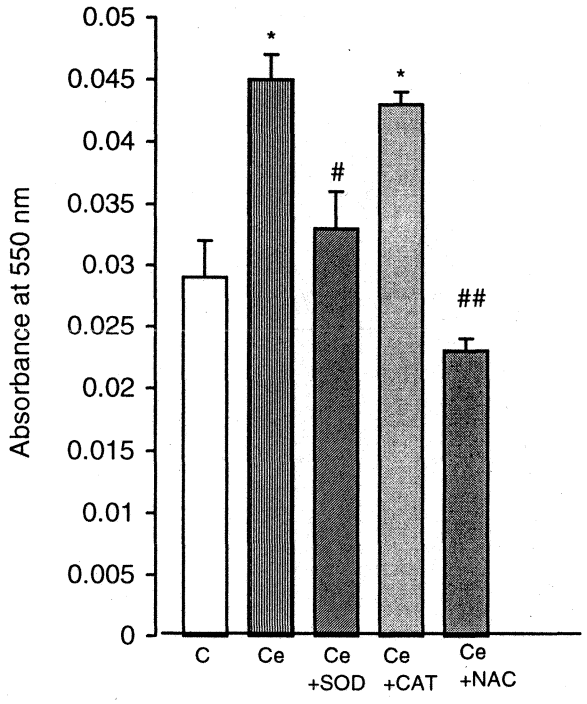
EFFECT OF CERIUM (0.5 μM) ON GROWTH OF SKELETAL MUSCLE FIBROBLASTS DETERMINED BY TOTAL CELL COUNT



Values represent mean ± SEM; n = 14 -20; *p < 0.005 *vs* control (C); **p < 0.0005 *vs* control (C); #p < 0.0005 *vs* cerium; SOD - 100 U/ml; CAT - 120 U/ml; NAC - 20 mM.

FIGURE 4 -20

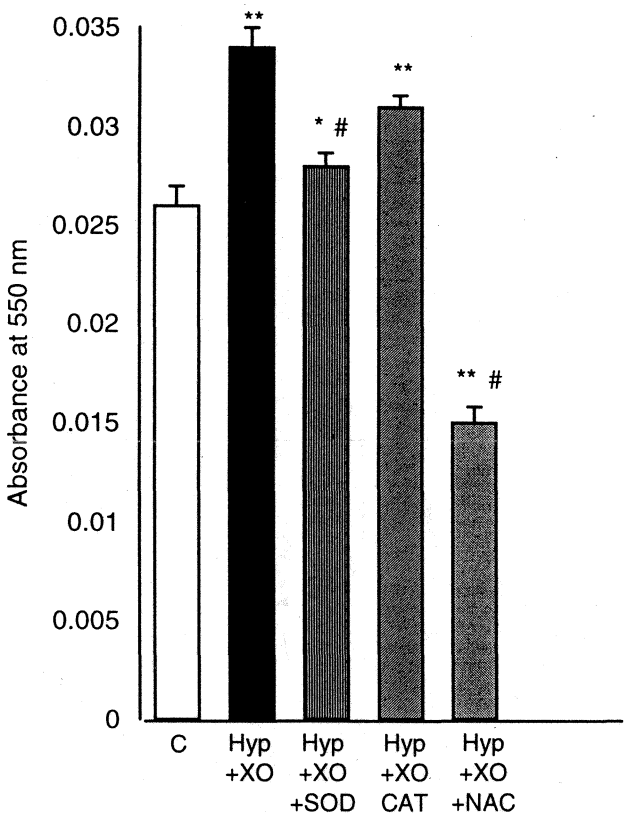
EFFECT OF CERIUM (0.5 μM) ON INTRACELLULAR GENERATION OF SUPEROXIDE ANIONS IN SKELETAL MUSCLE FIBROBLASTS AS ASSESSED BY REDUCTION OF NBT



Values represent mean ± SEM; n = 9 -13; * $p < 0.0005$ *vs* control (C); # $p < 0.005$ *vs* cerium; ## $p < 0.0005$ *vs* cerium; SOD - 100 U/ml; CAT - 120 U/ml; NAC - 20 mM.

FIGURE 4-21

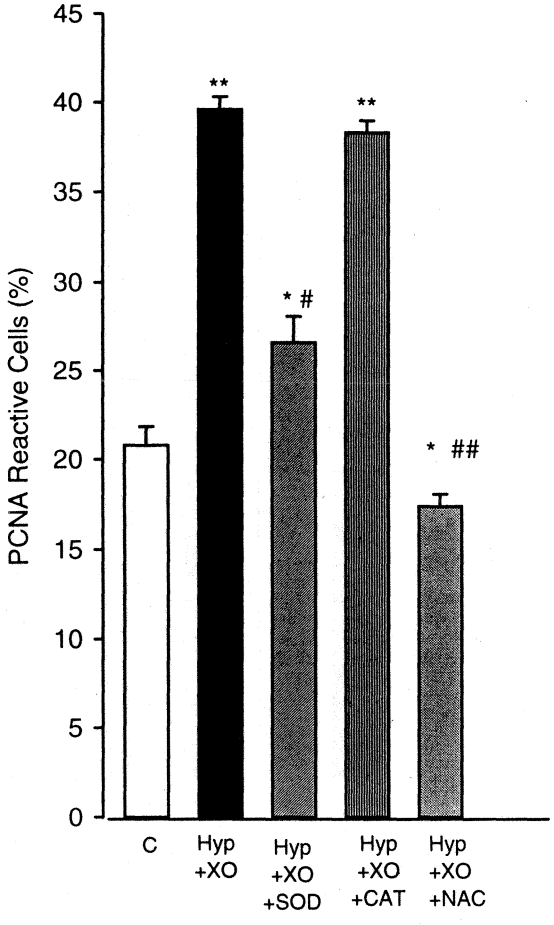
INTRACELLULAR CONTENT OF SUPEROXIDE ANIONS IN SKELETAL MUSCLE FIBROBLASTS EXPOSED TO Hyp (1 mM) + XO (10^{-6} U/ ml) ASSESSED BY REDUCTION OF NBT



Values represent mean ± SEM; n = 9; * $p < 0.05$ *vs* control (C); ** $p < 0.0005$ *vs* control (C); # $p < 0.0005$ *vs* Hyp + XO; SOD - 100 U/ml; CAT - 120 U/ml; NAC - 20 mM.

FIGURE 4-22

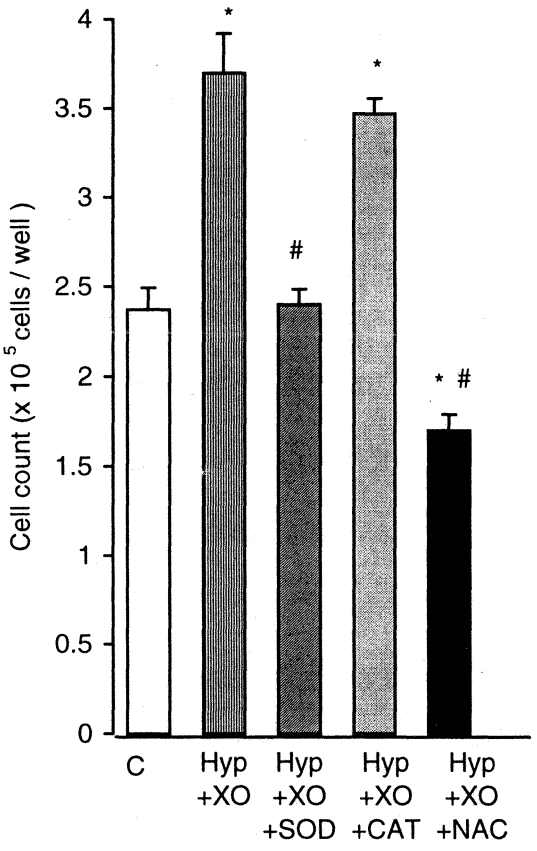
EFFECT OF Hyp (1 mM) + XO (10^{-6} U/ ml) ON PROLIFERATION OF SKELETAL MUSCLE FIBROBLASTS AS MEASURED BY PCNA STAINING



Values represent mean ± SEM; n = 9; * $p < 0.01$ *vs* control (C); ** $p < 0.0005$ *vs* control (C); # $p < 0.05$ *vs* Hyp + XO; ## $p < 0.0005$ *vs* Hyp + XO; SOD - 100 U/ml; CAT - 120 U/ml; NAC - 20 mM.

FIGURE 4 -23

EFFECT OF Hyp (1 mM) + XO (10^{-6} U/ ml) ON GROWTH OF SKELETAL MUSCLE FIBROBLASTS DETERMINED BY TOTAL CELL COUNT



Values represent mean ± SEM; n = 10 - 26; * $p < 0.0005$ *vs* control (C);
# $p < 0.0005$ *vs* Hyp+XO; SOD - 100 U/ml; CAT - 120 U/ml; NAC - 20 mM.

DISCUSSION

A fibroproliferative response could be initiated by cytokines, growth factors, mechanical stretch, low oxygen potential or exposure to metals. It has been reported that silica (Holian et al, 1997), asbestos (Robledo and Mossman, 1999) and zinc (Ishiyama et al, 1997) induce excessive proliferation of the connective tissue cells culminating in fibrosis. Lanthanides too have been associated with this pathological phenomenon. Cerium, belonging to the subgroup of light lanthanides, is the most plentiful element of rare earth and is about 100 times more abundant than other metals. It is normally trivalent and has the highest solubility among all rare earth metals. It resembles aluminium in chemical properties and pharmacological action. Rare earth metals such as neodymium and cerium were used therapeutically long ago for their anticoagulant properties until their toxicity became known. It is reported that cerium is a potent antiseptic drug for gram negative bacteria and fungi (Monafo et al, 1976). Cerium is used in the manufacture of ceramics, catalysts, glass additives etc. (Ito, 1985; Ohmachi, 1988). Cerium has been identified as the cause for pneumoconiosis associated with pulmonary fibrosis in industrial workers due to the inhalation of dust particles containing the element (Heuck and Hoschek, 1968). Tropical endomyocardial fibrosis is a pathological phenomenon, characterised by extensive thickening of the endocardium that extends into the inflow tracts of either or both the

ventricles, where cerium has been implicated as the cause for the disease (Valiathan et al, 1989). Based on the analysis of cardiac tissue of patients with EMF a geochemical hypothesis was postulated suggesting that cerium may have a role to play in the pathogenesis of the extensive thickening of the subendocardial tissue (Valiathan et al, 1989 and 1990). Investigations carried out in pursuance of the postulation provide histological evidence of cerium induced proliferative response in the heart of rats (Kumar et al, 1996) and rabbits (Kartha et al, 1998) on prolonged administration of cerium. In vivo experiments therefore show that cerium can induce a proliferative response on cardiac fibroblasts. A wound healing response was implicated in studies where enhanced lipid peroxidation and cell proliferation was observed in the cardiac tissue of rats on a cerium enriched diet (Kumar and Shivakumar, 1998).

Lanthanides have been reported to have a proliferative response on living systems. Apart from the studies carried out in pursuance of the geochemical hypothesis, no reports are available on the cardiac response to cerium. However, there are reports on the effect of lanthanides and other metals on the induction of cell proliferation. In man after intradermal injection of small amounts of lanthanum to volunteers, a delayed non - inflammatory whitish fibrotic change was noticed at the site of injection (Shelley, 1955). Intradermal administration of Ln_2O_3 was found to induce a proliferative reaction in rats and mice (Evans, 1990) and in vitro experiments have shown that concentrations as low as 1 μM La^{3+} stimulate murine fibroblasts (Evans,

1990). Several studies have reported the stimulatory response of metals at low concentrations. Low levels of lead (0.5 -10 μM) and cadmium has been reported to stimulate the proliferation of cultured smooth muscle cells in a dose- dependent manner (Fujiwara et al, 1995; Fujiwara, 1998). It has also been shown that at levels of 0.5 to 1.5 mMol/l these elements could inhibit growth of cancer cells and change their morphology (Xiao et al, 1997).

The reports show that low levels can be stimulatory and high levels inhibitory. This study was carried out to assess the effect low levels of the lanthanide cerium on cardiac fibroblast proliferation. Synchronised cardiac fibroblast cultures exposed to different concentrations of cerium showed a significant increase in the proportion of PCNA reactive cells (Fig. 4-3) and also in cell number (Fig. 4-4). At low levels, cerium was found to have a stimulatory effect with a peak at 0.5 μM concentration.

The level of cerium that induces a stimulatory response as observed in this study is comparable to that observed in the cardiac tissue and sera of patients with EMF (Valiathan et al, 1989; Eapen et al, 1997). Having established that low levels of cerium induces a fibroproliferative response, the next step was to elucidate the mechanism that stimulates cell proliferation.

Metals initiate lipid peroxidation once they get bound to cell membrane and generate an array of oxy radicals. Oxygen radicals have both beneficial as well as a deleterious effect on the biological systems. These highly active oxygen species produced under normal physiological conditions are the

primary means to shield microbial attack (Babior, 1978a,b; Klebanoff, 1980). Oxygen radicals released during ischemic preconditioning has been reported to contribute to cardioprotection in the rabbit myocardium (Baines et al, 1997). Free radicals are now considered as true signal molecules that are able to transfer information from outside of the cell to inside thus modulating diverse biological activities.

Exposure of cardiac fibroblasts to different concentrations of cerium showed an increase in the intracellular generation of free radicals as assessed by NBT reduction assay (Fig. 4-5). The concentration dependent effect of cerium on cell proliferation and intracellular generation of superoxide anions was similar, with a stimulatory response at lower concentrations reaching a peak at 0.5 μM concentration of cerium. The fibroblasts however remained morphologically normal and viable at higher concentrations of cerium. Using enzymatic (SOD - 100 U/ml; CAT - 120 U/ml) and non enzymatic (NAC - 20 mM) scavengers, cerium induced stimulatory response on intracellular generation of free radicals was studied. Superoxide dismutase and NAC lowered the generation of superoxide anions whereas catalase had no effect (Fig. 4-11).

Independent experiments have therefore shown that cerium stimulates cell proliferation as well as superoxide anion generation. It remained to be tested whether both the phenomena are interrelated. Therefore using 0.5 μM concentration of cerium, effect of enzymatic and non- enzymatic free radical scavengers on cell proliferation was studied. Enzymatic extracellular free

radical scavenger SOD inhibited the cerium induced increase in the proportion of PCNA reactive cells (Fig. 4-9). There was also a significant reduction in cell number (Fig. 4-10). Catalase, however did not have a significant effect on cerium exposed cultures. This observation fortifies the role of superoxide anions in the mediation of cerium induced cardiac fibroblast proliferation. An observation in this study that merits attention is that the inhibition of cerium induced stimulatory response on cardiac fibroblast proliferation in terms of PCNA reactive cells (Fig. 4-9) and cell number (Fig. 4-10) by NAC was higher compared to the inhibition by superoxide dismutase. The intracellular superoxide anion content was also measured following inclusion of NAC and it was found that the free radical levels are also reduced in the presence of the antioxidant (Fig. 4-11). Sekharam et al (1998) studied the modulation of cell cycle progression by NAC in mouse fibroblasts NIH3T3 cells and found that NAC blocked the cell cycle in the G_1 phase. The significant reduction of superoxide anion generation by NAC associated with significant reduction in cell growth suggests the prominence of intracellular superoxide anions in the mediation of proliferation of cardiac fibroblasts. To corroborate the postulation that superoxide anions mediate trophic effects, cardiac fibroblasts were also exposed to exogenous superoxide anions. A significant increase was seen in the content of superoxide anions in cells on exposure to superoxide anion generating system (Fig. 4-13). The intracellular levels as determined by NBT reduction assay is comparable to that induced by cerium. On inclusion of superoxide dismutase (100 U/ml),

intracellular content of superoxide anions was significantly low indicating that the rise in intracellular superoxide anion content was consequent to the extracellular generation of superoxide anion. A significant reduction in formazan content was also observed in the presence of the cell permeant antioxidant NAC (Fig. 4-13). In the presence of Hyp+XO, a significant increase in the proportion of PCNA reactive cells (Fig. 4-14) and cell density (Fig. 4-15) was also observed. Reduction in cell number on addition of SOD and NAC suggests that extracellular anion mediated rise in intracellular superoxide anion level was responsible for the stimulatory effect on cell proliferation.

Growing experimental evidence suggests that the generation of reactive oxygen species participates in cellular activation and intracellular signal transduction but the mechanism of activation remains a matter of debate. Superoxide anion and hydrogen peroxide have been implicated in the activation of phospholipase D, p42/p44 MAPK, p38 MAPK and Ras induced cell cycle progression (Huot et al, 1997; Baas and Berk, 1995; Natarajan et al, 1993; Irani et al, 1997). Lee and others (1998) in their study found that superoxide anion acted as an intermediate signal for serotonin stimulated mitogenesis in bovine pulmonary artery smooth muscle cells. Increased generation of hydrogen peroxide and superoxide anion have been found to stimulate vascular smooth muscle cell growth and DNA synthesis associated with the induction of several growth related proto-oncogenes including c-myc and c-fos and the induction of proto-oncogene mRNA expression in a protein

kinase C dependent manner (Rao, 1992; Rao and Berk, 1992; Rao, 1993a; Rao, 1993b). Other investigators have demonstrated that hydrogen peroxide and superoxide anion stimulate growth related events such as cell alkalinization and proto - oncogene induction (Shibanuma, 1988a; Shibanuma, 1988b). Active oxygen species may act as growth factors by direct oxidation of sulfhydryl groups, leading to activation of growth regulatory factors, or by formation of transition metal complexes, which may inhibit protein phosphatases. Examples of active oxygen species acting in this fashion include dimerization of Fos - Jun proteins (Abate et al, 1990), activation of NF - κB (Schreck et al, 1991), activation of endoplasmic reticulum tyrosine kinases (Bauskin et al, 1991) and stimulation of kinases involved in growth related signal transduction (Devary et al, 1992). These studies indicate that active oxygen species share properties with growth factors. These species at submicromolar levels thus act as novel intra and intercellular messengers capable of promoting growth response in culture. The mechanisms may involve direct interaction with molecules such as protein kinases, protein phosphatases, transcription factors or transcription inhibitors. Drawing support from the above reports it is reasonable to envisage that superoxide anions can stimulate a proliferative response in cardiac fibroblasts. The result of this study indicate that superoxide anions can function as second messengers translating the chemical stimulus induced by cerium into a biochemical response.

The increase in superoxide anion generation at 100 μM concentration of cerium following a decrease at 10 μM concentration of cerium is enigmatic. Metallothionein - a cysteine - rich low molecular weight metalloprotein - is thought to be involved in normal metal homeostasis and it binds to a number of important toxic metals with high affinity. Exposure of cells to metal ions induces the synthesis of metallothionein, providing an extracellular sink of cysteine sulfhydryl groups, capable of sequestering metal ions (Heck and Costa, 1986). It is speculated that superoxide anion generation increases with increasing concentration of cerium. However at higher concentrations of cerium, the cells are induced to express metalloprotein, which would explain the observation of reduced free radical stress at certain concentrations. Metallothionein synthesis is likely to be induced when the concentration of the metal reaches a threshold. This can be responsible for the decrease in superoxide anion at 10 μM concentration of cerium. The metallothionein levels may not increase beyond a point, and that explains the observed increase of superoxide anions at 100 μM concentration of cerium, where it exceeds the buffering capacity of the protein. As cell viability was not affected by higher levels of cerium, the decrease in cell proliferation at these concentrations could be due to the antimitogenic effects of the metal at higher concentrations. An earlier report on the paradoxical effect of cerium on protein synthesis supports this phenomenon where decrease of protein synthesis was observed at higher concentrations of cerium (Shivakumar et al, 1992).

Due to its large ionic radius cerium ions usually do not permeate the cell. Hence it was assumed that cerium causes membrane lipid peroxidation. The generation of superoxide anions by cardiac fibroblasts is found to be associated with membrane lipid peroxidation as confirmed by the significant increase in TBARS levels in cerium exposed cardiac fibroblast cultures.

Fibroblasts are known to be morphologically and functionally heterogenous. Endomyocardial fibrosis being a cardioselective disease, it was decided to examine whether the proliferative response to cerium and superoxide anions is cardiospecific. The behaviour of cardiac fibroblasts was compared with that of skeletal muscle fibroblasts. Skeletal muscle comprises about 35% of our body weight making it the largest single type of tissue in the human body. Fibroblasts constitute about 16% of total skeletal muscle cell population (Yablonka - Reuveni and Nameroff, 1987) as observed by in vitro studies and this is said to closely reflect the status of cells in the intact muscle. As the skeletal muscle cells are vulnerable to oxidative stress, the effect of cerium was studied on skeletal muscle fibroblasts. The concentration chosen for the experiments was 0.5 μM cerium, as this was the concentration that gave the maximal stimulation in cardiac fibroblasts.

The pattern of cell proliferation (Fig. 4-18, 4-19) and intracellular generation of superoxide anions (Fig. 4-20) on exposure to the lanthanide cerium was similar to that observed in cardiac fibroblast cultures. Exogenous free radicals also exerted a stimulatory response on the skeletal muscle

fibroblasts (Fig. 4-22, 4-23) thus suggesting that superoxide anions at sub toxic levels can induce a proliferative response.

The experimental observations indicate that cerium has similar action on both cardiac and skeletal muscle fibroblasts. Presuming that cerium has a significant role in the induction of fibrotic changes in the heart, cardioselectivity in EMF can be attributed to the differential accumulation of cerium. Experimental studies in rats have also shown preferential deposition of cerium in cardiac tissue relative to skeletal muscle (Eapen et al, 1996).

CONCLUSION This study has shown conclusively that low levels of cerium has a stimulatory effect on cardiac fibroblast proliferation. The increase in superoxide anion content in cerium exposed cardiac fibroblasts and the subsequent neutralization of the cerium induced stimulatory effect on both cell proliferation as well as superoxide generation by both enzymatic and non enzymatic scavengers (SOD and NAC) confirms the hypothesis that cerium induced cardiac fibroblast proliferation is mediated by superoxide anions. Extracellular superoxide anion generating system (Hyp+XO) was found to reproduce the effects induced by cerium. The important outcome of this study is the role of superoxide anions as a biologic mediator or as second messengers in the transduction of cellular growth responses. It has so far been assumed that fibroblast proliferation in tissues is mediated by pro -inflammatory communications and perivascular inflammatory cell mobilization leading to fibroblast activation. It is observed that even in the

absence of profibrogenic signals from activated cells, an autogenic reaction of fibroblasts can stimulate a proliferative response.

SUMMARY AND CONCLUSION

CHAPTER - 5

SUMMARY AND CONCLUSION

Inappropriate proliferation of connective tissue cells and their hyperfunction are implicated in fibrotic disorders. The triggering factors are many but it is the mechanism that contributes to proliferation of fibroblasts that remains enigmatic. A reactive fibrous tissue response which is due to the pathologic accumulation of fibrillar collagen in the perivascular and contiguous interstitial space, distorts tissue architecture and function. Therefore mechanisms contributing to this inappropriate proliferation of fibroblasts has been of considerable interest to many workers.

This study was carried out with the aim to elucidate the mechanism of action of the lanthanide - cerium in the induction of a fibroproliferative response in cardiac fibroblasts. The reason for selecting cerium as the metal for examining the tissue metal interaction is the observation of the element in the cardiac tissue of patients with EMF, a condition prevalent in Kerala. It was hypothesised that low levels of cerium could stimulate cardiac fibroblast proliferation and that the cerium induced proliferative response could be mediated by superoxide anions.

Therefore the main objectives of the study were :

1. To ascertain whether low levels of cerium could induce a stimulatory response on cardiac fibroblast proliferation

2. To examine whether superoxide anions could mediate the cerium induced stimulatory response on cardiac fibroblast proliferation
3. To assess the tissue dependent variations in response to cerium

Description of the procedure

The experiments were carried out on cardiac fibroblasts isolated and cultured from the heart of newborn rats (Wistar strain). For delineating the mechanism of action leading to a pathological state, it is essential to have a system devoid of a number of interfering factors. Such a controlled system helps in understanding whether the changes observed in vivo is primary to the factor under consideration or secondary to an intermediate reaction.

Cardiac fibroblasts were isolated from 2-3 day old Wistar rats by the enzymatic dispersion method. The isolated cells were dispersed in culture medium (M199 with 10% fetal calf serum) and seeded in polystyrene culture bottles and incubated at 37°C in a humidified incubator (99% humidity with 5% CO_2) for 90 minutes to allow the selective adhesion of cardiac fibroblasts. The supernatant containing the unattached myocytes was withdrawn after 90 minutes and the fibroblasts were re-incubated in fresh medium. Confluent cultures were passaged twice to get pure fibroblast cultures. In addition to its typical morphology fibroblasts were identified immunohistochemically where vimentin positive and desmin and factor VIII negative cells were characterised as fibroblasts. Before carrying out the experiments the cultures

were synchronised by serum deprivation for 24 hours. Serum concentration in the medium used for experiments was nominal (0.4%).

Cardiac fibroblasts were treated with different concentrations of cerium (μM) so as to determine the concentration that stimulates growth and generation of superoxide anions. The mitogenic effect of cerium was assessed by immunohistochemical staining for proliferating cell nuclear antigen (PCNA) and by determining the total cell count.

The intracellular generation of superoxide anions on exposure to different concentrations of cerium, was measured by nitroblue tetrazolium reduction test. The formazan production was visualized directly and it was also quantitated spectrophotometrically at 550 nm following extraction with propanol. To study the effect of cerium on extracellular generation of superoxide anions, SOD inhibitable cytochrome c reduction assay was adopted. Reduction of ferricytochrome c occurs from superoxide anions generated by the cells. Superoxide anion mediated reduction of cytochrome c was then measured spectrophotometrically by increase in absorbancy at 550 nm. It was postulated that the oxygen radical generation could be mediated by lipid peroxidation. Hence lipid peroxidation was studied by measuring TBARS levels in cardiac fibroblasts exposed to the lanthanide cerium.

To ascertain the role of oxygen radicals on cell proliferation, cultures were exposed to free radical scavengers superoxide dismutase, catalase and NAC alongwith cerium. The proportion of PCNA reactive cells and cell

count was determined. The stimulatory effect of the oxygen radicals on cell proliferation was also verified by using known generators of superoxide anions (Hyp+XO).

To examine tissue specific response to cerium or to oxygen radical stress, fibroblasts from a comparable tissue, the skeletal muscle was isolated and the response to cerium and superoxide anion generating system was examined.

Major findings

1. Low levels of cerium (0.5 μ M) was found to have a stimulatory response on cardiac fibroblast proliferation.
2. The same concentration of cerium was found to increase the intracellular superoxide anion content.
3. Neutralization of the cerium induced fibroproliferative response by superoxide dismutase but not by catalase affirms the role of superoxide anions as a biologic mediator in the cerium induced stimulatory response in cardiac fibroblasts.
4. The higher inhibitory effect of cell permeant antioxidant N-acetyl -L-cysteine on cerium induced cell proliferation and superoxide anion content reaffirms the prominence of intracellular superoxide anions in the mediation of growth response in cardiac fibroblasts.
5. A finding that further supports the postulation that superoxide anions have mitogenic potential is the observation that extracellular source of

superoxide anions stimulates intracellular generation of superoxide anions and induces a fibroproliferative response which were again neutralised by the presence of free radical scavengers.

6. The response of skeletal muscle fibroblasts both in terms of superoxide anion generation and cell proliferation was similar to that of cardiac fibroblasts thus ruling out the possibility that the reaction is specific to cardiac fibroblasts.

Conclusion

From the experimental data, it is inferred that low levels of cerium can instigate the proliferation of cardiac fibroblasts and the observation that superoxide anions mediate the cerium induced stimulatory response strengthens the emerging role of superoxide anions as second messengers in evoking growth responses. The study opens up new vistas for the study of fibrosis.

Future recommendations

1. A better understanding of the intracellular cascade of signalling pathways activated by cerium and superoxide anions in the evocation of growth responses is essential to delineate the mechanism of fibrosis.
2. The mechanism of apoptosis counterbalances the effect of cell proliferation by mitotic division. Therefore the possibility that both proliferation and apoptosis can regulate the rate of fibroblast growth in fibrotic diseases cannot be ruled out. Investigations on these lines would provide additional information on the mechanism of fibrotic disorders.
3. The cell has an inherent defensive mechanism to combat the oxidative stress. Free radical stress occurs either when free radicals are produced excessively or when the defence mechanism is suppressed. An increased generation of superoxide anions in the fibroblasts on exposure to low levels of cerium was observed but the antioxidant status of the cell on exposure to different levels of cerium remains to be studied.
4. Future studies may be directed at understanding the age and tissue dependent response of fibroblasts on exposure to low levels of cerium and free radical stress.
5. Investigations on the response of adult heart fibroblasts and also on the alterations in collagen turnover would provide a better understanding of cardiac fibrosis on exposure to low levels of cerium and free radical stress.

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APPENDIX

ANOVA OF THE PROPORTION OF PCNA REACTIVE CARDIAC FIBROBLASTS FOLLOWING EXPOSURE TO CERIUM

Data written to the working file.
2 variables and 25 cases written.
Variable : GROUP Type: Number Format: F11.2
Varibale : BK2VAR Type: Number Format: F11.2

- - Description of Subpopulations - -

Summaries of BK2VAR
By levels of GROUP

Variable	Value Label	Mean	Std Dev	Cases
For Entire Population		28.6640	7.1640	25
GROUP	1.00	17.2500	3.2016	4
GROUP	2.00	32.0800	2.4570	5
GROUP	3.00	37.5000	4.4347	4
GROUP	4.00	32.7250	2.9523	4
GROUP	5.00	26.0500	3.5180	4
GROUP	6.00	25.5250	3.1669	4

Total Cases = 25

----- ONEWAY -----

Variable BK2VAR
By Variable GROUP

Analysis of Variance

Source	D.F.	Sum of Squares	Mean Squares	F Ratio	F Prob.
Between Groups	5	1024.4746	204.8949	18.7829	.0000
Within Groups	19	207.2630	10.9086		
Total	24	1231.7376			

----- ONEWAY -----

Variable BK2VAR
By Variable GROUP

Multiple Range Tests: Modified LSD (Bonferroni) test with significance level .05

The difference between two means is significant if
MEAN(J)-MEAN(I) >= 2.3354 * RANGE * SQRT(1/N(I) + 1/N(J))
with the following value(s) for RANGE: 4.74

(*) Indicates significant differences which are shown in the lower triangle

Mean	GROUP	Grp 1	Grp 6	Grp 5	Grp 2	Grp 4	Grp 3
17.2500	Grp 1						
25.5250	Grp 6	*					
26.0500	Grp 5	*					
32.0800	Grp 2	*					
32.7250	Grp 4	*					
37.5000	Grp 3	*	*	*			

ANOVA OF CELL DENSITY OF CARDIAC FIBROBLASTS IN RESPONSE TO CERIUM

----------ONE WAY ANOVA----------

ANALYSIS OF VARIANCE

Source	D.F	Sum of Squares	Mean of Squares	F Ratio	F Prob.
Between Groups	5	38.0015	7.6003	16.1916	0.0000
Within Groups	48	22.5311	0.4694		
Total	53	60.5326			

Group	Count	Mean	Standard Deviation	Standard Error
Grp 1	9	3.3111	0.8253	0.2751
Grp 2	9	3.7444	0.7748	0.2583
Grp 3	9	5.2333	0.7194	0.2398
Grp 4	9	3.9222	0.7362	0.2454
Grp 5	9	2.6889	0.4540	0.1513
Grp 6	9	2.8778	0.5191	0.1730
Total	54	3.6296	1.0687	0.1454

GROUP	MINIMUM	MAXIMUM
Grp1	2.6000	5.0000
Grp 2	2.9000	5.2000
Grp 3	4.2000	6.2000
Grp 4	2.9000	4.8000
Grp 5	2.1000	3.5000
Grp 6	2.1000	3.6000
TOTAL	2.1000	6.2000

----------ONE WAY ----------

MULTIPLE RANGE TESTS: MODIFIED LSD (BONFERRONI) TEST WITH SIGNIFICANCE

LEVEL 0.05

MEAN	GROUP	G r p 5	G r p 6	G r p 1	G r p 2	G r p 4	G r p 3
2.6889	GRP 5						
2.8778	GRP 6						
3.3111	GRP 1						
3.7444	GRP 2	*					
3.9222	GRP 4	*	*				
5.2333	GRP 3	*	*	*	*	*	*

ANOVA OF SUPEROXIDE ANION GENERATION BY CARDIAC FIBROBLASTS IN RESPONSE TO CERIUM

Data written to the working file.
2 variables and 45 cases written.
Variable: GROUP Type: Number Format: F11.2
Variable: BK3VAR Type: Number Format: F11.2

20 Sep 99 SPSS for MS WINDOWS Release 6.1
- - Description of Subpopulations - -

Summaries of BK3VAR
By levels of GROUP

Variable	Value Label	Mean	Std Dev	Cases
For Entire Population		.0364	.0122	45
GROUP	1.00	.0270	.0025	5
GROUP	2.00	.0369	.0146	10
GROUP	3.00	.0529	.0040	8
GROUP	4.00	.0289	.0030	8
GROUP	5.00	.0314	.0090	7
GROUP	6.00	.0371	.0104	7

Total Cases = 45

----- ONEWAY -----

Variable BK3VAR
By Variable GROUP

Analysis of Variance

Source	D.F.	Sum of Squares	Mean Squares	F Ratio	F Prob.
Between Groups	5	.0032	.0006	7.7865	.0000
Within Groups	39	.0033	.0001		
TOTAL	44	.0065			

20 Sep 99 SPSS for MS WINDOWS Release 6.1
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Variable BK3VAR
By Variable GROUP

Multiple Range Tests: Modified LSD (Bonferroni) test with significance level .05

The difference between two means is significant if
MEAN(J)-MEAN(I) >= .0065 * RANGE * SQRT(1/N(I) + 1/N(J))
with the following value(s) for RANGE: 4.42

(*) Indicates significant differences which are shown in the lower triangle

Mean	GROUP	Grp 1	Grp 4	Grp 5	Grp 2	Grp 6	Grp 3
.0270	Grp 1						
.0289	Grp 4						
.0314	Grp 5						
.0369	Grp 2						
.0371	Grp 6						
.0529	Grp 3	*	*	*	*	*	