"OXIDATIVE STRESS, ANTIOXIDANT STATUS AND CALCIUM, PHOSPHOROUS LEVELS IN RHEUMATOID ARTHRITIS- A CASE CONTROL STUDY"

By

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Dissertation

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DR. P. B. DESAI. M.D.

DEPARTMENT OF BIOCHEMISTRY

JAWAHARLAL NEHRU MEDICAL COLLEGE,

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MAY - 2010

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LIST OF ABBREVIATIONS USED

Anti-CCP	-	Anti-Cyclic Citrullinated Peptide antibodies
BUN	-	Blood urea nitrogen
Ca	-	Calcium
CSF	-	cerebro spinal fluid
CRP	-	C- reactive protein
CCR	-	Carbon centered radicals
CYP P 450	-	Cytochrome P 450 system
cAMP	-	Cyclic adenosine mono phosphate
CCl_4	-	Carbon tetrachloride
$CCl_3^{\bullet}$	-	Trichloromethyl radical
CH_2	-	Methylene
Cu	-	Copper
DNA	-	Deoxy ribonucleic acid
D/W	-	Distilled water
Da	-	Dalton
ESR	-	Erythrocyte sedimentation rate
ECF	-	Extra cellular fluid
Fe	-	Iron
Fe^{2+}	-	Ferrous iron
Fe^{3+}	-	Ferric iron
FAD	-	Flavin adenine dinucleotide
g or gm	-	Gram
GSSG	-	Oxidized glutathione
GSH	-	Reduced glutathione

GR	-	Glutathione reductase
GSH-Px	-	Glutathione peroxidase
Glu	-	Glucose
H_2O_2	-	Hydrogen peroxide
$HCOO^{\bullet}$	-	Hydroperoxyl radical
HOCl	-	Hypochlorus acid
HCl	-	Hydrochloric acid
Hb	-	Hemoglobin
H_2O	-	Water
IU	-	International units
K	-	Potassium
KDa	-	Kilo Daltons
Kg	-	Kilogram
L	-	Litre
M	-	Molar
mEQ	-	Milli equivalents
mg	-	Milligram
mol. wt	-	Molecular weight
MDA	-	Malondialdehyde
mmol	-	Milli mol
μmol	-	Micro mol
mg/dl	-	Milligram/deci litre
Min	-	Minute
Ml	-	Milli litre
Mn	-	Manganese

MPO	-	Myeloperoxidase
MRI	-	Magnetic resonance imaging
$NO^{\bullet}$	-	Nitric oxide radical
NADPH	-	Nicotinamide adenine dinucleotide phosphate
NADH	-	Reduced nicotinamide adenine dinucleotide
nmol	-	Nano mol
$O_2CCl_3^{\bullet}$	-	Peroxyl radicals
$ONOO^-$	-	Peroxynitrite anion
O_2	-	Singlet oxygen
$OH^{\bullet}$	-	Hydroxyl radical
$O_2^{\bullet -}$	-	Superoxide anion radical
PUFA	-	Polyunsaturated fatty acid
PAI-1	-	Plasminogen Activator Inhibitor-1
PGI_2	-	Prostaglandin I_2
PDGF	-	Platelet derived growth factor
RBC	-	Red blood cells
$RS^{\bullet}$	-	Thiyl radical
$R^{\bullet}$	-	Lipid radical
$ROO^{\bullet}$	-	Lipid peroxide radical
RA	-	Rheumatoid Arthritis
RDA	-	Recommended daily allowance
ROS	-	Reactive Oxygen species
TBA	-	Thiobarbituric acid
TBAS	-	Thiobarbituric acid reactive substances
Tc	-	Technicium

TXA_2	-	Thromboxane A_2
UV	-	Ultraviolet
vit	-	Vitamin
Zn	-	Zinc

ABSTRACT

Background and Objectives:

Rheumatoid Arthritis(RA) is a chronic multisystem disease of unknown cause. RA affects approximately 1-2% of the total population. The characteristic feature of RA is non-specific inflammation of the peripheral joints with joint swelling, morning stiffness, destruction of articular tissues and joint deformities.

Formation of reactive oxygen species and lipid peroxides as a result of disease activity may play an important role in RA. Oxidative stress and decreased antioxidant status are the hallmarks in patients of Rheumatoid Arthritis as observed in recent years. The objectives of the study was to compare lipid peroxidation, antioxidant status and evaluate calcium, phosphorous levels in patients of Rheumatoid Arthritis and controls.

Methods:

The present study comprises of 40 cases of RA clinically diagnosed and confirmed by laboratory tests attending the OPD of KLE Society's Dr Prabhakar Kore Hospital and Research Centre, Belgaum. This study was undertaken between September 2007 to August 2008.

All the patients were in the age group of 40-60 years including both the sexes. Biochemical parameters like malondialdehyde, enzymatic antioxidants : superoxide dismutase, glutathione reductase and non enzymatic antioxidants like vitamin A vitamin E, vitamin C were estimated in cases and controls. Calcium and phosphorous levels were also measured in healthy controls and the cases.

Results:

The mean level of blood malondialdehyde (in nmol/ml) in controls was 6.19 ± 0.96 and 11.48 ± 0.76 in cases. The mean level of superoxide dismutase (IU/g of Hb) in controls was 948.32 ± 99.88 and 443.68 ± 111.69 in the cases. The mean glutathione reductase level (in IU/g of Hb) in controls was 8.91 ± 1.04 and 2.96 ± 0.79 in the cases. The mean level of vitamin A (in µg/dl) in controls was 35.13 ± 5.25 and 11.60 ± 3.03 in the cases. The mean level of vitamin E (in mg/dl) in controls was 0.92 ± 0.10 and 0.53 ± 0.14 in the cases. The mean vitamin C level (in mg/dl) in controls was 0.88 ± 0.12 and 0.48 ± 0.09 in the cases.

The mean value of Calcium (in mg/dl) in controls was 10.21 ± 0.65 and 7.33 ± 0.93 in the cases. The mean value of phosphorous(in mg/dl) in controls was 2.89 ± 0.57 and 4.28 ± 0.81 in the cases.

Interpretation and conclusion:

Our study revealed that there was increased oxidative stress and decreased antioxidant defense in patients of Rheumatoid Arthritis.

Our study also revealed that there was altered serum calcium and phosphorous levels in patients of Rheumatoid Arthritis..

Supplementation of natural antioxidants to the individuals who are diagnosed as Rheumatoid Arthritis may alleviate the morbidity associated with Rheumatoid Arthritis to a certain extent.

Key words: Rheumatoid Arthritis; lipid peroxidation; antioxidants; Calcium; Phosphorous.

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INTRODUCTION

Rheumatoid Arthritis(RA) is a chronic multisystem disease of unknown cause. Although there are a variety of systemic manifestations , the characteristic feature of established RA is a persistent inflammatory synovitis usually involving peripheral joints in a symmetric distribution.[1] Rheumatoid Arthritis is not only a polyarthritis, and the name rheumatoid disease is preferable since it directs attention to the whole patients and not just the joints.[2]

RA affects approximately 1-2% of the total population. Annual incidence rate of RA between 0.5% to 1% of total population is reported every year in both developed and developing countries. Women are affected more than men. The onset is more frequent during the fourth and fifth decades of life , with 80% of all patients developing the disease between the ages 35 and 50 years.

The cause of RA remains unknown. Genetic risk factors do not fully account for the incidence of RA. Multiple factors which doubtlessly are responsible for amplifying and perpetuating the initial pathology are now recognized.

It may well be that there is no single etiologic factor and that many similar or diverse stimuli brought to bear upon the susceptible host may trigger reactions leading to the clinical syndrome which we now recognize as RA.

An exogenous infection or molecular components of an infectious agent are likely candidates for the primary etiologic agent. The puzzle of whether antibodies to host components have a casual relationship to disease or whether they are simply another manifestation of it remains unsolved.

The earliest abnormality in RA is an increase in blood flow to the synovium . Increased permeability of vessels leads to increased accumulation of synovial fluid and superficial synovial lining cells proliferate . The proliferative synovitis however can be silent and destroy joints without outward signs of inflammation.

Exact reason behind bone erosion and joint deformities is not fully understood. Many investigators have focused on oxidative stress since last few years and suggest that RA patients are more prone to lipid peroxidation. Lipid peroxidation occurs as a result of free radicals generated in the body. Free radicals and free radical derived oxidants play important roles in biological system and have been implicated in the pathology of many diseases.

A free radical, in contrast, is a molecule or molecular fragment that contains one or more unpaired electrons in the outer orbital.[3] Free radicals are produced in the body due to leak in the electron transport chain, inflammatory conditions , ionizing radiations, drugs, chemical toxins etc.

Oxidation reactions ensure that molecular oxygen is completely reduced to water . The products of partial reduction of oxygen are highly reactive and make havoc in the living systems. Hence they are also called reactive oxygen species (ROS) ex.superoxide anion radical($O_2^{\cdot-}$), hydroperoxy radical($HOO^{\cdot}$), hydroxyl radical($OH^{\cdot}$) etc.

Important characteristics of the ROS are:

1. Extreme reactivity
2. Short life span
3. Generation of new ROS by chain reaction
4. Damage to various tissues.

Lipid peroxidation and consequent degradation products such as malondialdehyde (MDA) are a result of free radicals generated in the body.

The damage produced by ROS may be prevented by antioxidants . There are two types

1. Preventive antioxidants : which will inhibit the initial production of free radicals e.g. Catalase.
2. Chain breaking antioxidants : once the peroxy radicals are generated they can inhibit the propagative phase.ex superoxide dismutase (SOD),vit E etc.

It is possible that , generation of ROS may be particularly important factor for bone resorption in inflammatory process. Hypoxic conditions also disrupt an intracellular ionic environment and alter calcium and phosphorous level.

Human selectins mediate the inflammatory responses in Rheumatoid Arthritis, asthma, psoriasis, multiple sclerosis and the rejection of transplanted organs and thus there is great interest in developing drugs that inhibit selectin mediated cell adhesion.[4]

In recent years it has been shown that oxidative stress and antioxidants play an important role in the disease process of RA. So the present study was undertaken to assess oxidative stress by measuring malondialdehyde and antioxidant status by estimating superoxide dismutase , glutathione reductase ,vitamin A , vitamin E, vitamin C and calcium , phosphorous levels in Rheumatoid Arthritis patients.

OBJECTIVES OF THE STUDY

1. To determine oxidative stress by estimating blood malondialdehyde levels, hemolysate superoxide dismutase(SOD), glutathione reductase, plasma Vitamin A, vitamin E and Vitamin C levels in Rheumatoid Arthritis patients.
2. To estimate serum calcium and phosphorous levels in the selected patients.
3. To correlate these values with normal healthy individuals.

REVIEW OF LITERATURE

Rheumatoid Arthritis(RA) is a chronic multisystem disease of unknown cause. Although there are a variety of systemic manifestations , the characteristic feature of established RA is a persistent inflammatory synovitis usually involving peripheral joints in a symmetric distribution.[1] The potential of the synovial inflammation to cause cartilage damage and bone erosions and subsequent changes in joint integrity is the hallmark of the disease. Rheumatoid Arthritis is not only a polyarthritis, and the name rheumatoid disease is preferable since it directs attention to the whole patients and not just the joints.[2]

The recognition that RA is not simply a disease of painful joints but is associated with disability and increased mortality has changed the therapeutic management of affected patients. To prevent irreversible tissue damage aggressive treatment should be initiated in the early stages of the disease.

Epidemiology

RA affects approximately 1-2% of the total world population.[5] Annual incidence rate of RA between 0.5% to 1% of total population is reported every year in both developed and developing countries.[6] Women are affected more than men. RA is seen throughout the world and affects all races. The onset is more frequent during the fourth and fifth decades of life , with 80% of all patients developing the disease between the ages 35 and 50 years. In the absence of a known primary cause of RA , epidemiologic studies have strived to identify environmental factors important in disease cause. These studies have failed to provide conclusive evidence of environmental risk factors and have not given any clues about the cause of the disease . Hence the cause of RA remains unknown.

PATHOLOGY AND PATHOGENESIS

It is believed that RA is an autoimmune disease triggered by exposure of a genetically susceptible host to an unknown arthritogenic antigen.[7] Microvascular injury and an increase in the number of synovial lining cells appear to be the earliest lesion in rheumatoid synovitis . Subsequently an increased number of synovial lining cells is seen along with perivascular infiltration with mononuclear cells. As the process continues synovium becomes edematous and protrudes into the joint cavity as villous projections . The rheumatoid synovium is characterized by the presence of a number of secreted products of activated lymphocytes , macrophages and fibroblasts. The local production of these cytokines and chemokines appear to account for many of the pathologic and clinical manifestations of RA. These effector molecules include those that are derived from T lymphocytes, those originating from activated myeloid cells, and those secreted by other cell types in the synovium, such as fibroblasts and endothelial cells. The activity of these chemokines and cytokines appears to account for many of the features of Rheumatoid synovitis, including the synovial tissue inflammation, synovial fluid inflammation, synovial proliferation, and cartilage and bone damage, as well as the systemic manifestations of RA. In addition to the production of effector molecules that propagate the inflammatory process, local factors are produced that tend to slow the inflammation, including specific inhibitors of cytokine action and additional cytokines, such as transforming growth factor β (TGF-β), which inhibits many of the features of rheumatoid synovitis including t cell-activation and proliferation, B cell differentiation, and migration of cells into the inflammatory site, and may be involved in generating a population of regulatory T cells, as a means to control inflammation. These findings have suggested that the

propagation of RA is an immunologically mediated event, although the original initiating stimulus has not been characterized.

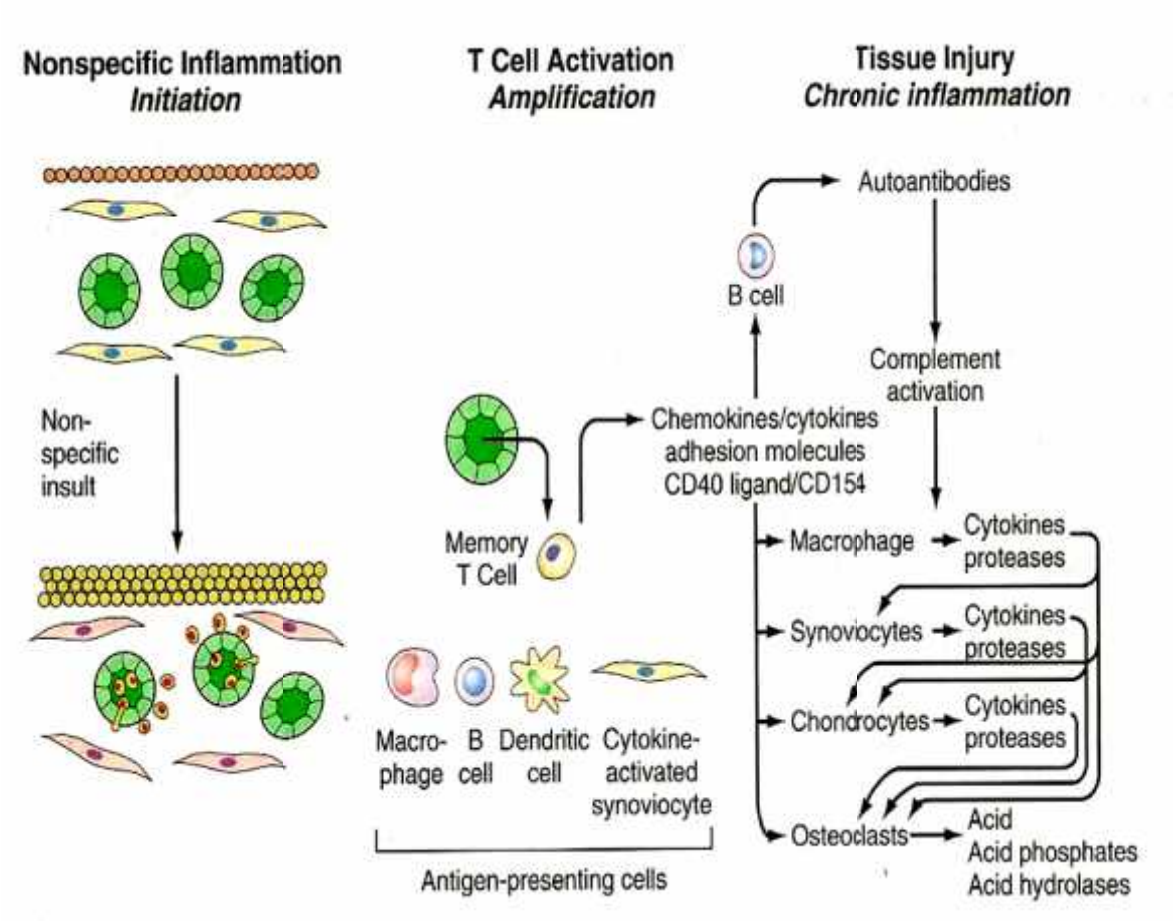


Figure 1: The progression of rheumatoid synovitis

CLINICAL MANIFESTATIONS

ONSET:

Characteristically, RA is a chronic polyarthritis. In approximately two-thirds of patients , it begins insidiously with fatigue, anorexia, generalized weakness and vague musculoskeletal symptoms until the appearance of synovitis becomes apparent. Later joints become affected in a symmetric manner.

SIGNS AND SYMPTOMS

Pain , swelling and tenderness may initially be poorly localized to the joints. Pain in affected joints , aggravated by movement is the most common manifestation of established RA. Morning stiffness of more than one hour duration is an almost invariable feature of inflammatory arthritis. The majority of patients will experience constitutional symptoms such as weakness , easy fatigability , anorexia and weight loss. Although inflammation can affect any diarthrodial joints , RA most often causes symmetric arthritis with characteristic involvement of certain specific joints such as the proximal interphalangeal and metacarpophalangeal joints . With persistent inflammation a variety of characteristic joint changes develop .Characteristic changes of hand include

1) Radial deviation at the wrist with ulnar deviation of the digits often with palmar subluxation of the proximal phalanges(“Z” deformity);
2) Hyperextension of the proximal interphalangeal joints , with compensatory flexion of the distal interphalangeal joints (swan-neck deformity):
3) Flexion contracture of the proximal interphalangeal joints and extension of the distal interphalangeal joints (boutonniere deformity);

4) Hyperextension of the first interphalangeal joint and flexion of the first metacarpophalangeal joint with a consequent loss of thumb mobility and pinch.

Typical joint changes may also develop in the feet, including eversion of the hindfoot (subtalar joint), plantar subluxation of the metatarsal heads, widening of the forefoot, hallux valgus, and lateral deviation and dorsal subluxation of the toes.

EXTRA ARTICULAR MANIFESTATIONS.

RA is a systemic disease with a variety of extraarticular manifestations. It is estimated that as many as 40% of patients may have extraarticular manifestations and in approximately 15% these are severe.

Rheumatoid nodules : these may develop in 20-30% of persons with RA. They are usually found on periarticular structures, extensor surfaces or other areas subjected to mechanical pressure , but they can develop elsewhere, including the pleura and meninges.

Rheumatoid vasculitis : which can affect nearly any organ system is seen patients with severe RA and high titres of circulating rheumatoid factor.

PLEUROPULMONARY MANIFESTATIONS: Which are more commonly observed in men include pleural disease, interstitial fibrosis , pleuropulmonary nodules , pneumonitis and arthritis.

FELTY'S SYNDROME consists of chronic RA , spleenomegaly , neutropenia and on occasion anemia and thrombocytopenia.

Osteoporosis secondary to rheumatoid involvement is common and may be aggravated by glucocorticoid therapy.

RA might be associated with an increased incidence of lymphoma, especially large B cell lymphoma.

RA in the elderly: the incidence of RA continues to increase past age 60 years. It has been suggested that elderly onset RA might have a poorer prognosis and more rapid functional decline. Aggressive disease is largely restricted to those patients with high titers of rheumatoid factor. By contrast, elderly patients who develop RA without elevated titers of rheumatoid factor (seronegative disease) generally have less sever, often self-limited disease.

LABORATORY FINDINGS:

No tests are specific for diagnosing RA.

Rheumatoid factor(RF) :RFs which are autoantibodies reactive with Fc portion of IgG ,are found in more than two thirds of adults with the disease and have clinically been used to evaluate patients with RA . Widely utilized tests largely detect IgM RFs.

The presence of RF can be of prognostic significance because patients with high titres tend to have more and progressive disease with extraarticular manifestations.

Antibodies to CCP(designated anti-CCP) can also be used to evaluate patients with RA. In addition, the anti-CCP test has a similar sensitivity and a better specificity for RA than does rheumatoid factor, and, therefore some have advocated its use to evaluate RA patients instead of rheumatoid factor.

ESR(Erythrocyte Sedimentation Rate): ESR is increased in nearly all patients with active RA .

Synovial Fluid analysis confirms the presence of inflammatory arthritis , although none of the findings are specific.

Radiographic evaluation

Early in the disease, radiographic evaluation of the affected joints are usually not helpful in establishing a diagnosis. Other means of imaging bones and joints including 99mTc bisphosphonate bone scanning and MRI may be capable of detecting early inflammatory changes that are not apparent from standard radiography but are rarely necessary in the routine evaluation of patients with RA.

Calcium, phosphorous levels

For the formation of bones , calcium and phosphorous are very important. About 99% of calcium in body is part of bone.[8]Extracellular calcium is needed for bone mineralization, blood coagulation and other functions. Inorganic phosphate is major component of hydroxyapetite in bone.[9] Eighty percent of it is laid down in bone matrix as insoluble calcium salts.[10] It is possible that generation of ROS (Reactive Oxygen Species) may be particularly important factor for bone resumption in inflammatory process.[11] Hypoxic conditions also disrupt an intracellular ionic environment and alter calcium phosphorous level.

DIAGNOSIS OF RA

The diagnosis of RA is easily made in persons with typical established disease. The typical picture of bilateral symmetric inflammatory polyarthritis involving small and large joints in both the upper and lower extremities with sparing of the axial skeleton except the cervical spine suggests the diagnosis. Constitutional

features indicative of the inflammatory nature of the disease, such as morning stiffness, support the diagnosis. Demonstration of subcutaneous nodules is a helpful diagnostic feature. Additionally, the presence of rheumatoid factor, anti-CCP antibodies, inflammatory synovial fluid with increased numbers of PMNLs, and radiographic findings of juxtaarticular bone demineralization and erosins of the affected joints substantiate the diagnosis.

The diagnosis is somewhat more difficult early in the course when only constitutional symptoms or intermittent arthralgias or arthritis in an asymmetric distribution may present. A period of observation may be necessary before the diagnosis can be established. A definitive diagnosis of RA depends predominantly on characteristic clinical features and the exclusion of other inflammatory processes. The isolated finding of a positive test for rheumatoid factor, anti-CCP antibody, or an elevated ESR or CRP, especially in an older person with joint pains, should not itself be used as evidence of RA.

In 1987, the American College of Rheumatology developed revised criteria for the classification of RA. These criteria demonstrate a sensitivity of 91-94% and a specificity of 89% when used to classify patients with RA compared with control subjects with rheumatic diseases other that RA. Although these criteria were developed as a means of disease classification for investigational purposes, they meet these criteria, however, especially during the early stages of the disease, does not exclude the diagnosis. Indeed, these criteria do not effectively differentiate patients with new-onset RA from those with a variety of other forms of early inflammatory arthritis. Moreover, in patients with early arthritis, the criteria do not discriminate effectively between patients who subsequently develop persistent, disabling, or erosive disease and those who do not.

TABLE No: 1

THE 1987 REVISED CRITERIA FOR THE CLASSIFICATION OF RA

1. Guidelines for classification

    a. Four of seven criteria are required to classify a patient as having rheumatoid arthritis(RA)

    b. Patients with two or more clinical diagnoses are not excluded.

2. Criteria*

a. Morning stiffness: Stiffness in and around the joints lasting 1 hour before maximal improvement

b. Arthritis of three or more joint areas: at least three joint areas, observed by a physician simultaneously, have soft tissue swelling or joint effusions, not just bony overgrowth. The 14 possible joint areas involved are right or left proximal interphalangeal, metacarpophalangeal, wrist, elbow, knee, ankle, and metatarsophalangeal joints.

c. Arthritis of hand joints: Arthritis of wrist ,metacarpophalangeal joint or proximal interphalangeal joint.

d. Symmetric arthritis: Simultaneous involvement of the same joint areas on both sides of the body.

e. Rheumatoid nodules: Subcutaneous nodules overbony prominences , extensor surfaces, or juxtaarticular regions observed by a physician.

f. Serum rheumatoid factor: Demonstration of abnormal amounts of serum rheumatoid factor by any method for which the result has been positive in less than 5% of normal control subjects.

g. Radiographic changes: Typical changes of RA on posteroanterior hand and wrist radiographs that must include erosions or unequivocal bony decalcification localized in or most marked adjacent to the involved joints.

(*Criteria a-d must be present for atleast 6 weeks. Criteria b-e must be observed by a physician)

CLINICAL COURSE AND PROGNOSIS

The course of RA is quite variable and difficult to predict in an individual patient. A number of features are correlated with a greater likelihood of developing joint abnormalities or disabilities . These include the presence of >20 inflamed joints, a markedly elevated ESR, radiographic evidence of bone erosions, the presence of rheumatoid nodules, high titres of rheumatoid factor or anti CCP antibodies, the presence of functional disability, persistent inflammation, advanced age at onset, presence of co-morbid conditions, low socioeconomic status or educational level or the presence of HLA-DRβ1*401 or –DRβ*0404.

Medical management of RA patients include drugs, substitution of omega-3 fattyacids in diet, substitution of vitamins, plant and animal extracts, hormones and topical preperations of various sorts. The treatment of patients with inflammation involves two primary goals: first the relief of pain, which is often the presenting symptom and the major continuing complaint of the patient; and second , the slowing or –in theory-arrest of the tissue damaging process.[12]

FREE RADICALS

A free radical is any species capable of independent existence that contains one or more unpaired electrons. An unpaired electron is one that is alone in an orbital.[13] The unpaired electron gives certain characteristic properties to the free

radical, such as para magnetism. The chemical reactivity of free radicals is usually high. They may be positively charged, negatively charged or electrically neutral.[14] A free radical is conventionally represented by a superscript dot ($R^{\bullet}$).

A compound becomes a free radical by gaining an additional electron, as in the case of reduction of molecular oxygen to superoxide anion radical ($O_2^{\bullet -}$). Other free radicals are hydroperoxyl radical ($HOO^{\bullet}$), hydroxyl radical ($OH^{\bullet}$), lipid peroxyl radical ($ROO^{\bullet}$). The sequential univalent reduction steps of oxygen may be represented as,[15]

$$O_2 \xrightarrow{(+)\ e^-} O_2^{\bullet -} \xrightarrow{(+)\ e^{-},\ 2H} H_2O_2 \xrightarrow[-H_2O]{(+)\ e^{-},\ H^+} OH^{\bullet} \xrightarrow{(+)\ e^{-},\ H^+} H_2O$$

Generation of Free Radicals:

Free radicals may be formed by;

a. Cleavage of a covalent bond of a normal molecule.

b. Loss of a single electron from a normal molecule and

c. Addition of single electron to a normal molecule.

Sources of Free Radicals:

Oxidants related to human disease are derived from three sources.

- Those generated via normal intracellular biologic processes but in an exaggerated, inappropriate fashion or in a milieu where the normal defenses that serve to protect tissues are inadequate.
- Those released by inflammatory cells into their local environment.

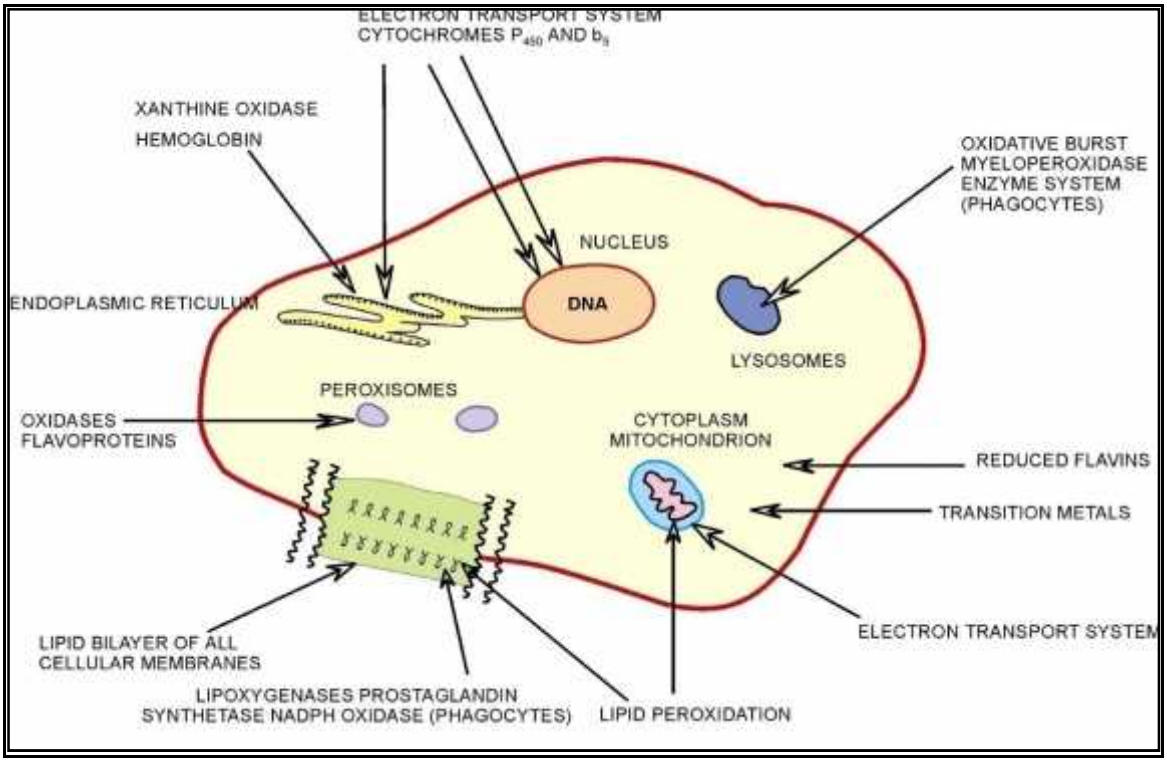


Fig. No.2. The Cellular Sources of free Radicals

Those secondary to xenobiotics, either because the xenobiotics includes oxidants (e.g. inhaled oxidant gases) or because these xenobiotics induce oxidant generation within cells, e.g. drugs that injure tissues through oxidant mechanisms.[16]

i) Endogenous Sources:

a) Endogenous Sources of free radicals include those that are generated and act intracellularly as well as those that are formed within the cells and are released

into the surrounding area[17] (Fig No.3). Oxidation-reduction reactions generate free radicals constantly within the body. These can be mediated by the action of enzymes or non-enzymatically, often through the redox chemistry of transition metal ions[18].

Oxidases and electron transport systems are prime, continuous sources of intracellular reactive oxygen free metals. Electron transfer from transition metals such as iron to oxygen can initiate free radical reactions[17]. An important source of superoxide anion radical is the "univalent leak" of superoxide anion radical from the mitochondrial electron transport system[19].

b) Intracellular free radicals are generated from the auto oxidation and consequent inactivation of small molecules such as reduced flavins and thiols, and from the activity of certain oxidases, cycloxygenases, lipoxygenases, dehydrogenases and peroxidases[17] (Fig No.3). A variety of enzyme systems catalyze the univalent reduction of molecular oxygen to superoxide anion radical. Such univalent reduction of molecular oxygen also occurs invivo in non-enzymatic electron-transfer oxidation-reduction reactions. (E.g. hydroquinone + $O_2 \longrightarrow$ semiquinone + $O_2^{\bullet -}$ + H^+) and during auto-oxidation reactions including those that involve catecholamines, flavins and reduced ferridoxins[20].

c) Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in the inflammatory cells (neutrophils, eosinophils, monocytes and macrophages) produces superoxide anion by the process of respiratory burst during phagocytosis. The superoxide is converted to hydrogen peroxide and then to hypochlorous acid (HOCI) with the help of superoxide dismutase (SOD) and

Myeloperoxidase (MPO). The superoxide and hypochlorous ions are the final effectors of bactericidal action. The gene for myeloperoxidase is on chromosome 17. The enzyme myeloperoxidase has a molecular weight of 156 Kilo Daltons (KD); and contains two iron atoms per molecule. This is deliberate production of free radicals by the body. About 10% of the oxygen uptake by macrophage is used for free radical generation. Along with the activation of macrophages, the consumption of oxygen by the cell is increased drastically; this is called respiratory burst[21] (Fig No.4).

d) Hydroxyl radicals are formed in the "Fenton reaction" whenever hydrogen peroxide comes into contact with ferrous or cupric ions. An iron catalyzed Haber-weiss type of reaction may also form this radical. 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 hydroxyl radical. Finally, the $OH^{\bullet}$ radical is also a product of ionizing radiation[20, 22].

$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$ (Fenton reaction)

$O2^{\bullet -} + H_2O_2 \xrightarrow{\text{Fe salt catalyst}} O_2 + OH^{\bullet} + OH^{-}$.

(Haber Weiss reaction)

The chelated Fe^{3+} can be reduced to Fe^{2+} by thiols, ascorbate and most of the other reductants. Fe^{2+} can then auto-oxidize, producing $O_2^{\bullet -}$

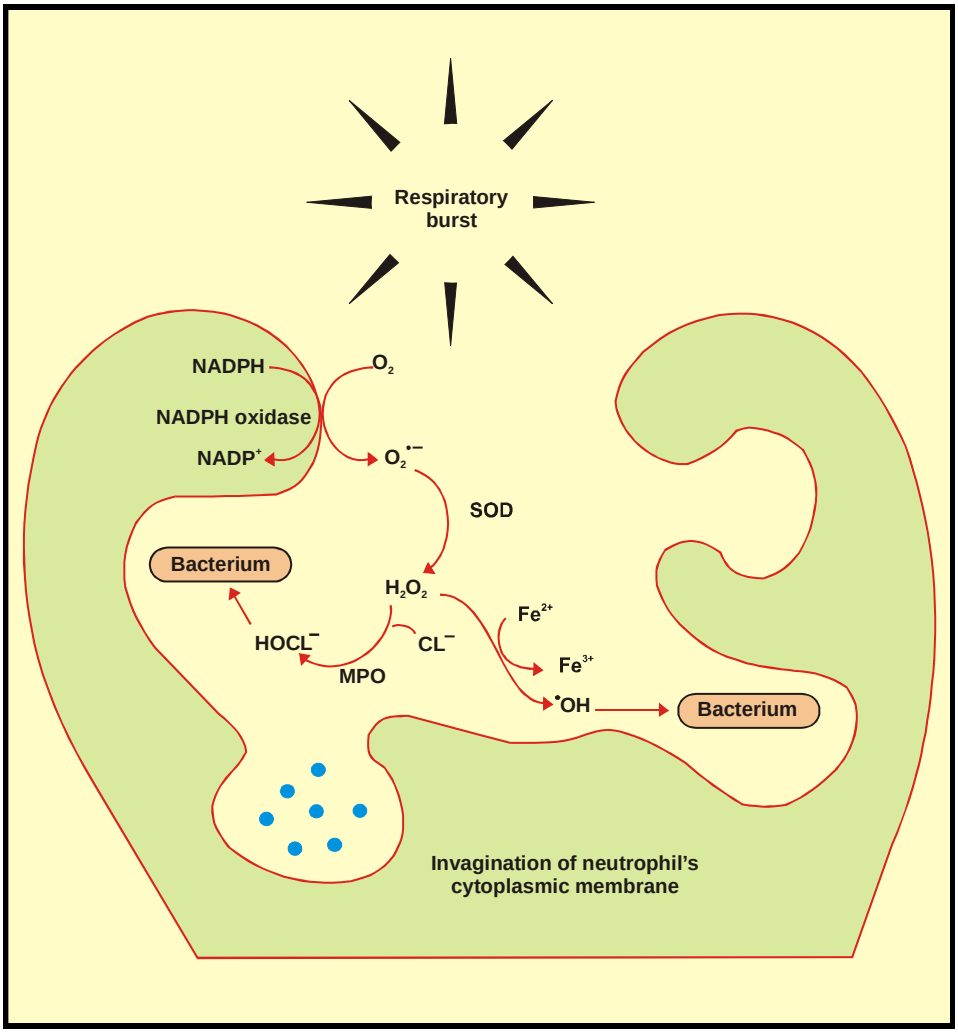


Fig. No. 3. Generation of Oxygen Free Radicals in Respiratory Burst

ii) *Exogenous Sources:*

a) Cigarette smoke contains high concentrations of various free radicals. Other toxic compounds such as carbon tetrachloride, drugs and inhalation of air pollutants, anaesthetics, organic solvents, hypertoxic environments and pesticides will increase the production of free radicals[17].

b) Ionising radiation damages tissues by producing hemolytic fission of background water.

$$H_2O \longrightarrow (\text{gamma, UV radiation}) \longrightarrow H^{\bullet}+OH^{\bullet}$$

c) Light of appropriate wavelengths can cause excitation of oxygen in presence of photosensitizers to produce singlet oxygen.

Sites of Free Radical Generation:

Main sites of free radical generation are mitochondria, lysosomes, peroxisomes, nuclei, endoplasmic reticulum, plasma membranes and the cytosol[17].

i) Endoplasmic Reticulum, Nuclear Membrane and Electron Transport Systems:

Free radicals produced by the endoplasmic reticulum and nuclear membrane can undergo both intraorganelle and cytosolic reactions. In case of nuclear membrane generated radicals DNA would be particularly susceptible to free radical damage.

ii***) Plasma Membrane:***

Plasma membrane is a site of action of extracellularly generated free radicals. They must cross the plasma membrane before reacting with other cell components and may initiate toxic reactions at the membrane. The unsaturated fatty acids present in membrane and transmembrane proteins containing oxidizable aminoacids are susceptible to free radical damage. Increased membrane permeability caused by lipid peroxidation or oxidation of structurally important proteins can cause breakdown of transmembrane ion gradients, resulting in loss of secondary functions and inhibition of integrated cellular metabolic processes.

The interior of biological membranes is hydrophobic and $O_2^{\bullet -}$ produced in the environment could be extremely damaging. Much of the $O_2^{\bullet -}$ generated within cells comes from membrane bound systems and it is certainly possible that some of it is formed in the membrane interior[23].

iii) Peroxisomes:

Peroxisomes are potent sources of cellular hydrogen peroxide because of high concentrations of oxidases.

REACTIVE OXYGEN SPECIES (ROS)

The oxygen derived free radicals and related non-radical species are collectively known as reaction oxygen species (ROS). Several reactive oxygen species (ROS) are known, among them the most prominent are:

Superoxide Radical ($O_2^{\bullet -}$):

This ROS is formed, when oxygen takes up one electron and as leaks in the mitochondrial electron transport but its formation is easily increased when exogenous components (redox cycling compounds) are present. Its first production site is the internal mitochondrial membrane (NADH ubiquinone reductase and ubiquinone cytochrome-c-reductase).[24] This species is reduced and forms hydrogen peroxide (H_2O_2). The production of superoxide radicals at the membrane level (NADPH oxidase) is initiated in specialized cells with phagocytic functions (macrophages) and contributes to their bactericidal action (oxidative burst) [25]. The flavin cytosolic enzyme xanthine oxidase found in quite all tissues and in milk fat globules generates superoxide radicals from hypoxanthine and oxygen and is supposed to be at the origin of vascular pathologies.

Hydrogen Peroxide (H_2O_2):

Hydrogen peroxide is mainly produced by enzymatic reactions. These enzymes are located in microsomes, peroxisomes and mitochondria. Even in normal conditions, the hydrogen peroxide production is relatively important and leads to a constant cellular concentration between 10^{-9} and 10^{-7} M. In plant and animal cells, superoxide dismutase is able to produce H_2O_2 by dismutation of $O_2^{\bullet -}$, thus contributing to the lowering of oxidative reactions. The natural combination of dismutase and catalase contributes to remove H_2O_2 and thus has a true cellular antioxidant activity. H_2O_2 is also able to diffuse easily through membranes.[26]

Hydroxy Radical ($OH^{\bullet}$):

In the presence of Fe^{2+}, H_2O_2 produces the very active species $OH^{\bullet}$ by the Fenton reaction.

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^{\bullet} + OH^{-}$$

The iron-catalyzed decomposition of oxygen peroxide is considered the most prevalent reaction in biological systems and the source of various deleterious lipid peroxidation products.

Nitric Oxide ($NO^{\bullet}$):

Nitric Oxide is produced in vascular endothelium. This species is not too reactive (poorly oxidizing function), it reacts readily with $O_2^{\bullet -}$ and gives the extremely reactive peroxynitrite ($ONOO^{-}$). This ROS is naturally formed in activated macrophages[27] and endothelial cells[28] and is considered as an active agent in several pathologies based on inflammation, organ reperfusion and also may play an important role in atherosclerosis.

Single Oxygen (O_2):

This chemical form of oxygen is not a true radical but is reported to be an important ROS in reactions related to ultraviolet exposition (UVA 320-400 nm). Its toxicity is reinforced when appropriate photoexcitable compounds (sensitizers) are present with molecular oxygen[29]. Several natural sensitizers are known to catalyze oxidative reactions such as tetrapyrroles (bilirubin), flavins, chlorophyll, hemoproteins and reduced pyridine nucleotides (NADH). Some of these sensitizers are also found in foods and cosmetics. Some others are used for therapeutic purposes (anti-cancer treatments) and are sensitive to visible light. The presence of metals contributes to increase in the production of singlet oxygen, as well as anion superoxide, and thus accelerates the oxidation of unsaturated lipids generating hydroperoxides. It has been suggested that singlet oxygen may be formed during the degradation of lipid peroxides and thus may cause the production of other peroxide molecules. This singlet oxygen formation may account for the chemiluminescence observed during lipid peroxidation.[30]

Thiyl Radicals ($RS^{\bullet}$):

Thiol compounds (RSH) are frequently oxidized in the presence of iron or copper ions.

$$RSH + Cu^{2+} \longrightarrow RS^{\bullet} + Cu^{+} + H^{+}$$

These thiyl radicals have strong reactivity in combining with oxygen.[31, 32]

$$RS^{\bullet} + O_2 \longrightarrow RSO^{\bullet}_2$$

Furthermore, they are able to oxidize NADH into NAD, ascorbic acid and to generate various free radicals ($^{\bullet}OH$ and $O2^{\bullet-}$). These thiyl radicals may also be formed by hemolytic fission of disulfide bonds in proteins.

Carbon Centered Radicals:

The formation of this reactive free radical is observed in cells treated with carbon tetrachloride (CCl_4). The action of the cytochrome P_{450} systems generates the trichloromethy1 radical ($^{\bullet}CCl_3$), which is able to react with oxygen to give several peroxy1 radicals (i.e. $^{\bullet}O_2\,CCl_3$).[33]

DAMAGE PRODUCED BY FREE RADICALS

Free radicals are extremely reactive. Their mean effective radius of action is only $30A^0$. Their half-life is only a few milliseconds. When a free radical reacts with a normal compound, other free radicals are generated. This chain reaction leads to thousands of events. The peroxidation of polyunsaturated fatty acids (PUFA) severely damages the cell membrane leading to loss of membrane functions like absorption, secretion etc. Almost all biological macromolecules are damaged by the free radicals e.g.

a) peroxidation of PUFA in plasma membranes,

b) Oxidative inactivation of sulfhydry1 containing enzymes,

c) Polysaccharide depolymerization and DNA breaks.

d) DNA damage may directly cause inhibition of protein and enzyme synthesis, indirectly it also cause cell death or mutation and carcinogenesis.

e) Lipid peroxidation and consequent degradation product such as MDA seen in biological fluid. Their effect in the serum is often employed to assess the oxidant stress.[34]

Proteins:

Protein molecules undergo substantial modifications through reactive reactions with free radicals. Proteins containing tryptophan, tyrosine, phenylalnine histidine, methionine and cysteine can undergo free radical mediated amino acid modification. Free radical promote sulfhydryl mediated cross-linking of such labile amino acids as well as cause fragmentation of polypeptide chains. Oxidative modifications enhance degradation of critical enzymes by cytosolic neutral proteases[35]. Enzymes undergo cross-linking with resulting increase in molecular weight, such enzymes cross-link with their neighbours in a random destructive reaction. The normal precision arrangement of protein and enzymes in subcellular membranes and organelles is badly disrupted and their biological properties are lost or impaired.[36]

Carbohydrates:

Advances in free radical chemistry indicate that no biological substance is impervious to free radical attack. Therefore, it is not surprising that glucose and other related monosaccharides undergo oxidation but conditions are appropriate.[35] Hyaluronic acid undergoes polymer fragmentation following exposure to free radical system, which leads to destabilization of connective tissue and loss of synovial fluid viscosity.

Nucleic Acid:

DNA is readily attacked by oxidizing radicals if they are formed in its vicinity has been clearly demonstrated by radiation biologists. It must therefore be considered as a vulnerable and important target. Cell mutation and death from ionizing radiation is primarily due to free radical reactions with DNA. Cell death and mutations arising

from free radicals generated during normal metabolism have also been ascribed to reactions with DNA[37].

Lipids:

All of the major classes of biomolecule may be attacked by free radicals but lipids are probably the most susceptible[2]. Cell membranes are rich sources of polyunsaturated fatty acids. Biomembrane and organelles are the major sites of lipid peroxidation damage. Major constituents of biological membranes are lipids and proteins. Lipid peroxidation can damage membrane proteins as well as lipids.[23] The membrane fluidity is due to the presence of polyunsaturated fatty acid side chain in many membrane lipids, which lower the melting point of the interior membrane. Lipid peroxidation decreases membrane fluidity. Conditions, which favour lipid peroxidation are:

i) A high degree of unsaturation in the lipid substrate.

ii) A rich supply of oxygen and

iii) The presence of transitional metal catalysts.[38]

LIPID PEROXIDATION

Lipid peroxidation is defined as "oxidative deterioration of polyunsaturated lipids". Lipid peroxidation is particularly damaging because it proceeds as a self perpetuating chain reaction.[39]

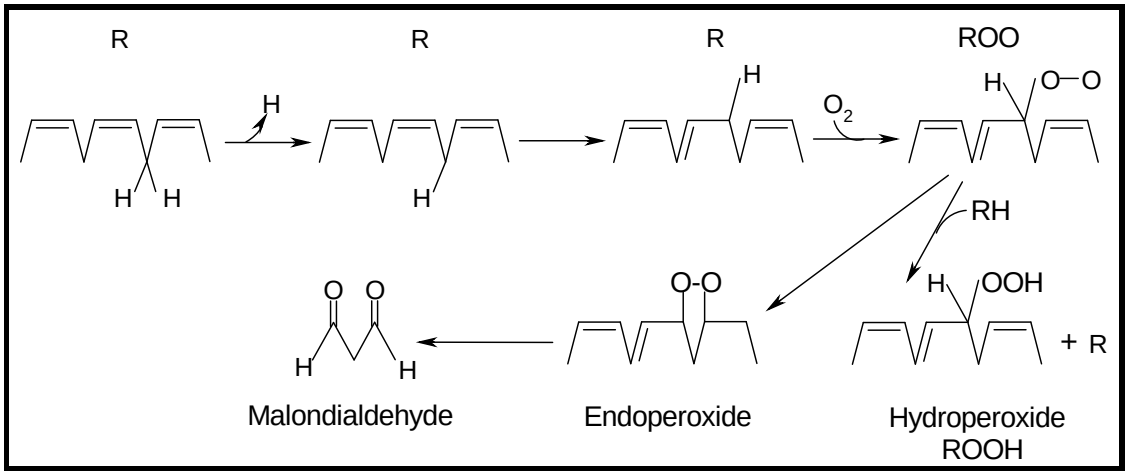


Fig. No.4: The lipid peroxidation process

Figure 5 Illustrate lipid peroxidation. The reaction is initiated by an existing free radical ($X^{\bullet}$), by light, or by metal ions. Malondialdehyde is only formed by fatty acids with three or more double bonds, and is used as a measure of lipid peroxidation together with ethane from the terminal two carbon of ω3 fatty acids and pentane from the terminal five carbon of ω6 fatty acids.[40]

Peroxidation of polyunsaturated fatty acids usually involves three operationally defined processes.[23]

Initiation Phase:

During this phase, the primary event is the abstraction of hydrogen atom from bisallylic site of PUFA. Initiation of a peroxidation sequence in membrane or PUFA[35] is due to the attack of any species that has sufficient reactivity to abstract a hydrogen atom from a methylene (CH_2) group. This leaves behind an unpaired electron on the carbon, -CH-. The carbon radical tends to be stabilized by a molecular rearrangement to produce a conjugated diene, which then easily reacts with an oxygen molecule to give peroxy radical, R-$OO^{\bullet}$. The presence of the redox active metals such as iron or copper can facilitate the initiation process.[23]

$ROOH + Metal\ (n)^{+} \longrightarrow ROO^{\bullet} + Metal^{(n-1)+} + H^{+}$

$X^{\bullet} + RH \longrightarrow R^{\bullet} + XH$

Propagation Phase:

During this phase lipid peroxidation relies on the interaction of molecular oxygen with carbon-centered free radicals to form lipid hydroperoxides.[35] The peroxy radical abstract a hydrogen atom from another lipid molecule and once the process begins it tends to continue. The peroxy radical combines with the hydrogen atom that it abstracts to give a lipid hydroperoxides R-OOH. A probable alternative fate of peroxy radicals is to form cyclic peroxides. With the help of metal catalysts, the decomposition of hydroperoxides result in the formation of alkoxyl or peroxyl radicals. These radicals are capable of further reactions and thus the propagation of lipid peroxidation continues.[23, 35]

$R^{\bullet} + O_2 \longrightarrow ROO^{\bullet}$

$ROO^{\bullet} + RH \longrightarrow ROOH + R^{\bullet}$ etc

Termination Phase:

The propagation reactions of lipid peroxidation will not proceed very far before they meet a protein molecule, which can then be attacked and damaged, in addition aldehyde can attack amino groups on the protein molecule to form both intramolecular cross links and also cross links between different protein molecules. eg. malondialdehyde. Any kind of lipid free radical can react with a lipid peroxyl radical to give non-initiating and non-propagating species.

$ROO^{\bullet} + ROO^{\bullet} \longrightarrow RO\text{-}OR + O_2$

$ROO^{\bullet} + R \longrightarrow ROOR$

$R^{\bullet} + R^{\bullet} \longrightarrow RR$

TOXIC EFFECTS OF LIPID PEROXIDATION

The uncontrolled peroxidation of bio-membranes can lead to profound effects on membrane structure and function and may be sufficient to cause cell death. The toxic products generated during lipid peroxidation may be involved in damage to specific protein and transport systems critical to cell function.[41] Malondialdehyde produced by lipid peroxidation can cause cross linking and polymerization of membrane components. This can alter the intrinsic membrane properties such as ion transport, enzyme activity. Because malondialdehyde is diffusible, it will also react with nitrogenous bases of DNA[42] lipid hydroperoxides can directly inhibit enzymes.[41]

ANTIOXIDANT DEFENSE SYSTEMS

Detoxification of reactive oxygen species is one of the prerequisite of aerobic life. Many defense systems have evolved by providing an important antioxidant defense system of prevention, interception and repair consisting of non-enzymatic and enzymatic scavengers and quenchers (Fig. No. 5).[43]

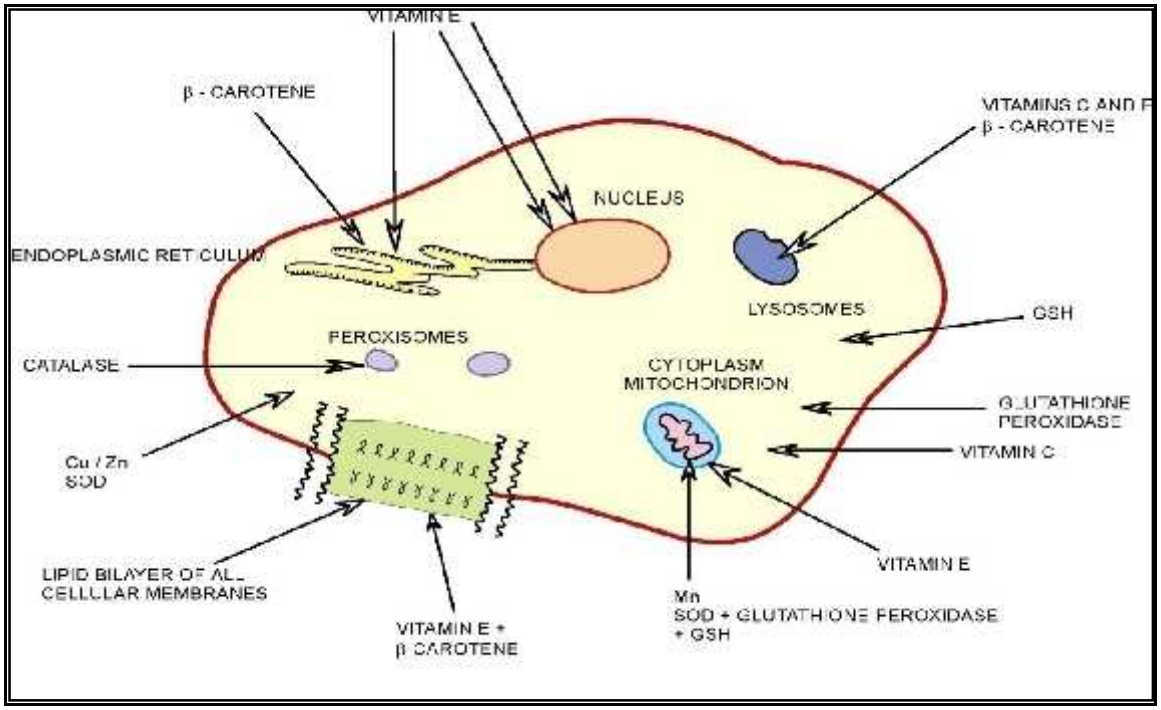


Fig No.5. Antioxidant protection within the cell

Prevention:

The important function of these antioxidant defenses is to prevent the generation of free radicals. Antioxidants defense remove peroxides that are reacting with transition metal ions to produce reactive free radicals. These peroxides include both hydrogen peroxide and also the lipid hydroperoxides, which are produced during lipid peroxidation. Catalase and glutathione peroxidases are the enzymes, their role is to safely decompose peroxides.[23] Transferrin and iron binding protein lactoferrin also function as preventive antioxidant by binding iron and stopping it from participating in radical reactions. Uric acid, albumin, haptoglobin and hemopexin have also been shown to inhibit various free radical reactions.[44,45] Cerulloplasmin an important extracellular antioxidant [46] which oxidizes Fe^{2+} to Fe^{3+} that swiftly binds to transferrin and any iron mobilized from serum ferritin.[47]

Interception:

The free radical scavenging enzyme is superoxide dismutase whose substrate is a free radical; other scavengers include lipid soluble vitamin E, vitamin A, ubiquinol and the aqueous phase compounds such as free radical scavengers like ascorbic acid glutathione, uric acid etc.[18]

Repair:

Another category of natural antioxidant defense is repair processes, which remove damaged biomolecule before their presence, which alter cell metabolism or viability. Specific enzymes repair oxidatively damaged nucleic acids. Oxidized proteins are removed by proteolytic systems and oxidized membrane lipids acted upon by lipases, peroxidases and acyl transferases.

Antioxidants oppose the toxic effect of lipid peroxides and oxygen radicals, and they limit the amount of lipid peroxides that are formed[48]. To counteract the free radical damage, the tissues have an effective antioxidant defense system. Under normal circumstances, the defense system is able to cope up with a free radical in the tissues by the antioxidant donating an electron to stabilize the free radical. In doing so it can harmlessly decay itself, or later regenerate by other antioxidants.

Kirnskey defined antioxidants are "compounds that protect biologic systems against the potentially harmful effects of processes or reactions that can cause excessive oxidation".[49] With this definition we can describe various types of biologic antioxidants, their locations within and outside of cells and mechanism of actions.

Antioxidants can be classified depending on the mechanism of action as the preventive or the chain breaking. In the former category are metal chelators and enzymes like superoxide dismutase, catalase and glutathione peroxidase. In the second category is the chain breaking agents such as alpha tocopherol, ubiquinone, beta-carotene, bilirubin and water-soluble substances such as ascorbate, GSH and urate. The defense of living eukaryotic cells against the damage caused by the activated oxygen is a complex process which involves a interrelated protective agencies for activated oxygen targets like DNA, proteins and polyunsaturated fatty acids. Peroxidation of polyunsaturated fatty acids will result in a disruption of membrane. So, the antioxidants lie functionally at the heart of this protective mechanism.

Intracellular defense system is largely dependent on the antioxidant enzymes such as GSH-Px, SOD, and catalase requiring the antioxidant micronutrients like

selenium, copper, zinc and iron. Riboflavin functions as an antioxidant cofactor (FAD) needed for the glutathione reductase.

ENZYMATIC ANTIOXIDANTS

SUPEROXIDE DISMUTASE

McCord and Fridorich described the metalloenzyme superoxide dismutase.[50] Superoxide dismutase is the major intracellular antioxidant enzyme, which is essential for the survival of aerobic cells. It catalytically scavenges the superoxide radical, which appears to be important agent for toxicity of oxygen and thus provides a defense against oxygen toxicity.

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \longrightarrow H_2O_2 + O_2$$

Superoxide dismutase catalyzes the dismutation of the superoxide anion free radical ($O_2^{\bullet -}$) to hydrogen peroxide and molecular oxygen at a rate 104 times faster than spontaneous dismutation at physiological pH[51] resulting in no superoxide anion available to react with hydrogen peroxide to from hydroxy1 radical through the iron catalyzed reactions. Superoxide dismutase enzyme exists in several forms and is present in mitochondrial matrix, the cytoplasm and the extracellular fluid.

Superoxide dismutase is broadly classified into distinct classes depending on the metal ion content.

1) Cu and Zn containing SOD found in cytosol.

2) Mn containing SOD found in mitochondria.

1) <u>Cu/Zn containing superoxide dismutase</u>: Superoxide dismutase is present in cytosol of eukaryotic cells having a molecular weight of 32,000, and is made up of two identical subunits one Cu^{2+} and one Zn^{2+} per subunit. In the Cu/Zu SOD copper is

catalytically active which oxidizes cupric to the cuprous state, while Zn appears, primarily to play a structural role.[52]

2) Manganese containing superoxide dismutase: Manganese superoxide dismutase has been isolated from human liver mitochondria.[52] It contains four subunits and has a molecular weight of 80,000.

GLUTATHIONE REDUCTASE

Glutathione reductase is present in the liver, kidney, pancreas, heart, thyroid and erythrocytes. It has a molecular weight of 44000. It catalyzes the reduction of oxidized glutathione (GSSG) to glutathione (GSH) in the presence of reduced coenzyme II ($NADPH_2$)

Glutathione reductase is NADPH dependent and in erythrocytes the main source of NADPH2 is from Hexose monophosphate shunt activity.[53] The ratios of GSH/GSSG in normal cells are kept high so there must be a mechanism for reducing GSSG back to GSH, which is achieved by glutathione reductase.

Glutathione reductase contains the FAD at its active site. NADPH reduces the FAD, which then passes its electrons on to a disulfide bridge (S-S) between two cysteine residues in the protein. The two-sulphydryl groups so formed then interact with GSSG and reduce it to 2GSH, reforming the protein disulfides.[23] Glutathione reductase is present in two forms.

1. An active form associated with FAD
2. An inactive form not bound to FAD.

Reduction of GSSG to GSH mediated by glutathione reductase whose substrate is NADPH and Glucose -6-Phosphate dehydrogenase as a source of NADPH guarantees the reduction of GSSG and maintenance of constant pool of GSH.

RETINOL (VITAMIN-A)

Vitamin-A is a member of fat-soluble vitamin, function as a non-enzymatic antioxidant, acts as free radical scavenger. Carotenoids have long been considered antioxidants because of their capacity to scavenge free radicals. β-Carotene a pigment found in all plants is the most efficient quencher of singlet oxygen known in nature and can also function as an antioxidant. β-Carotene is the major carotenoid precursor of vitamin-A. Vitamin-A, however cannot quench singlet oxygen and has a very small capacity to scavenge free radicals. β Carotene has been found in cellular membranes, including those of lysosomes. Carotenoids protect lipids against peroxidation by quenching free radicals and other reactive oxygen species, notably singlet oxygen. The structural arrangement of β-carotene with their long chain of conjugated double bonds suggests that they are excellent scavengers for reactive free radicals.

VITAMIN – E (α– TOCOPHEROL)

Vitamin E or Alpha tocopherol is a fat-soluble antioxidant widely distributed in both plant and animal kingdom with many diverse functions in the body. The generic term vitamin E refers to at-least eight structural isomers of α-tocopherol. Among these, α-tocopherol is the well known isomer and possesses the most potent fat soluble antioxidant at high oxygen tension. It is believed to be the first line of defense offering protection to the membrane phospholipids PUFA against peroxidative damage.

Vitamin-E is one of the most important chain breaking antioxidant, it protects the polyunsaturated fatty acids from peroxidative damage by donating hydrogen to the lipid peroxyl radical. Because of the lipophilic property of the tocopherol molecule, vitamin E is the major free radical chain terminator in the lipophilic environment. High levels of tocopherol are found in selected mammalian tissues (e.g. adrenal glands, heart, testes and liver), and this preferential distribution may result from its high lipid solubility. Intracellularly, vitamin E is associated with lipid rich membranes such as mitochondria and endoplasmic reticulum. Thus the antioxidant action of tocopherol is expected to be highly effective in protecting against membrane lipid peroxidation by reacting with lipid peroxyl and alkoxyl radicals.[35]

α– Tocopherol is known as a chain breaking antioxidant because it functions to intercept lipid peroxyl radicals ($LOO^{\bullet}$) and so terminates lipid peroxidation chain reactions. Peroxyl and alkoxyl radicals generated during lipid peroxidation preferentially combine with the antioxidant[23]

$LOO^{\bullet}$ + α – tocopherol OH → LOOH + α – tocopherol+ $O^{\bullet}$

α-tocopherol quenches and reacts with singlet oxygen and could therefore protect the membrane against this species. It also reacts with the superoxide radical.[23]

The chromanol head group of α-tocopherol is very close to the surface of the membrane while the flexible phytyl tail is believed to be approximately aligned with the acyl chains in the interior of the membrane. The close proximity of the chromane head group to the membrane surface is consistent with the acyl chains in the interior of the membrane. The close proximity of the chromane head group to the membrane surface is consistent with the synergistic antioxidant behavior of alpha tocopherol and ascorbic acid.[59] Although the two vitamins are completely sequestered and separated

in their respective lipid and aqueous phases, a very significant extent of inhibition of peroxidation is obtained when both are present.[35]

ASCORBIC ACID (VITAMIN-C)

Vitamin-C or Ascorbic Acid is a water-soluble non-enzymatic antioxidant. Its role as an antioxidant is indicated by its known free radical scavenging action. As a reducing and antioxidant agent, it directly reacts with superoxide and hydroxyl radical and various lipid hydroperoxides. In addition it can restore the antioxidant properties of oxidized vitamin-E, suggesting that a major function of vitamin-C is to recycle the vitamin-E radicals. Ascorbic acid is widely distributed in mammalian tissues, but it is present in relatively high amounts in the adrenal and pituitary glands, lesser amounts are found in the liver, spleen, pancreas and brain. Ascorbic acid serves as both an antioxidant and a proxidant. As an antioxidant, vitamin-C exerts a sparing effect on the antioxidant actions of vitamin-E and selenium. On the other hand, excess amount (≅ mM) may act as a prooxidant in the presence of transition metals Fe^{3+} or Cu^{2+}. Many studies have shown that ascorbate's, Pro-oxidant action, which induces lipid peroxidation, resides in its ability to reduce Fe^{3+} to the Fe^{2+} state. Fe^{2+} is known to be a potent free radical inducer.[35]

Ascorbic acid appears to trap virtually all peroxyl radicals in the aqueous phase before they can diffuse into the plasma lipids. The ability of ascorbate to show antioxidant properties is related to its fast reaction with many reactive oxygen species (peroxyl radicals) and to the fact that the resulting semidehydroascorbate radical is poorly reactive. Enzymatic systems exist invivo to reduce semidehydroascorbate back to ascorbate at the expense of NADH (NADH-semidehydroascorbate reductase enzyme).[60]

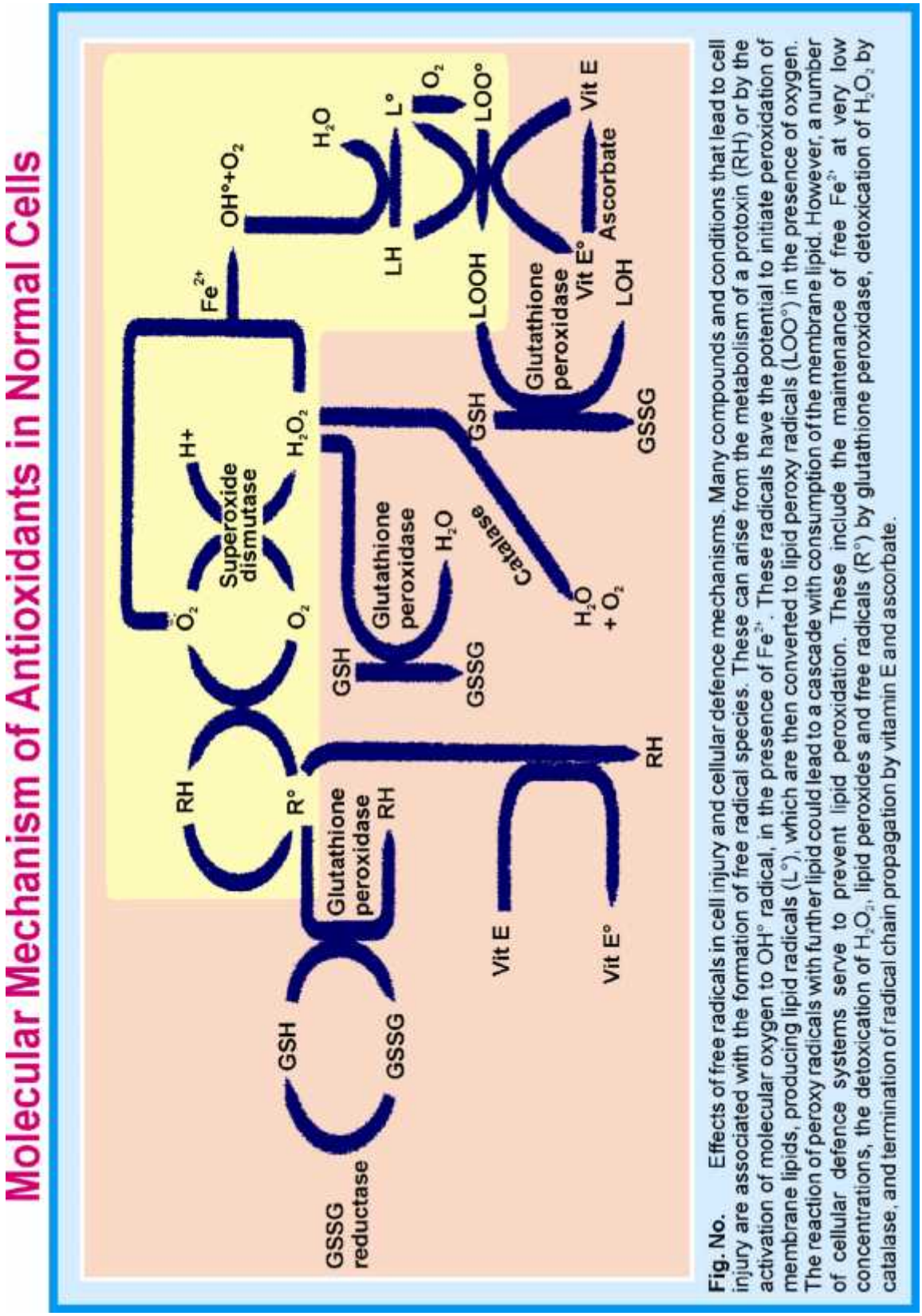


Fig. No. Effects of free radicals in cell injury and cellular defence mechanisms. Many compounds and conditions that lead to cell injury are associated with the formation of free radical species. These can arise from the metabolism of a protoxin (RH) or by the activation of molecular oxygen to OH° radical, in the presence of Fe^{2+}. These radicals have the potential to initiate peroxidation of membrane lipids, producing lipid radicals (L°), which are then converted to lipid peroxy radicals (LOO°) in the presence of oxygen. The reaction of peroxy radicals with further lipid could lead to a cascade with consumption of the membrane lipid. However, a number of cellular defence systems serve to prevent lipid peroxidation. These include the maintenance of free Fe^{2+} at very low concentrations, the detoxication of H_2O_2, lipid peroxides and free radicals (R°) by glutathione peroxidase, detoxication of H_2O_2 by catalase, and termination of radical chain propagation by vitamin E and ascorbate.

MATERIALS AND METHODS

Source of data:

The present study comprises of 40 patients , from KLE Society's Dr. Prabhakar Kore Hospital and Medical Research Centre, Belgaum who were clinically diagnosed as RA and confirmed by laboratory tests. The study was undertaken between September 2007 to August 2008. All the patients were in the age group of 40-60 years and were not on any nutritional supplements. Age and sex matched 40 healthy individuals served as controls.

Criteria for the selection of the cases:

1) Inclusion criteria: Clinically diagnosed cases of Rheumatoid arthritis and confirmed by laboratory tests..

2) Exclusion criteria:
   a) Osteoarthritis
   b) Tubercular arthritis
   c) Infective arthritis
   d) Rheumatic fever
   e) Pulmonary tuberculosis, pneumonia,
   f) Costochondritis (Tietze's syndrome)
   g) Arthritis other than RA fitting into any syndromes
   h) Chronic smokers and alcoholics
   i) Any other systemic disorder

Informed consent was taken from all the cases and the study was approved by the ethical and research committee of JNMC, Belgaum.

Collection and storage of blood sample:

10ml of blood was collected from the patients immediately after admission. Samples were also collected from the controls, under aseptic precautionary measures by using disposable syringe. Out of this 1ml of whole blood was used for estimation of malondialdehyde and 1ml was used for the preparation of hemolysate. Enzymes like glutathione reductase and superoxide dismutase were analysed from the hemolysate within one hour. 4ml of blood was centrifused immediately and plasma was obtained for estimating vitamin A,vitamin E and vitamin C. Remaining 4ml of blood was allowed to stand for sometime and the serum was separated and used for estimating calcium and phosphorous..

Methods of assay

A) Whole blood

- Malondialdehyde (Thiobarbituric acid method)

B) Hemolysate

I) Haemoglobin (Drabkin's method)

II) Enzymatic antioxidants

a. Superoxide dismutase (Misra and Fridorich)

b. Glutathione reductase (Beutler's E method)

C) Plasma

I. Vitamin A(Retinol):(Bessay et al method)

II. Vitamin E (Quafie et al, Baker and Frank)

III. Vitamin C (Evelyn and Melloy method)

D) Serum

I. Calcium (Trinder method)

II. Phosphorous (Delsal and Manhouri method)

STATISTICAL ANALYSIS:

Statistical analysis was carried out by using student's t – test (unpaired).

ESTIMATION OF MALONDIALDEHYDE (MDA) IN WHOLE BLLOD:[63]

Principle:

The reaction depends on the formation of pink coloured complex between malondialdehyde and thiobarbituric acid (TBA), having an absorption of maximum at 532 nm.

Thiobarbituric acid reagent:

- 75 ml thiobarbituric acid
- 15 gm trichloroacetic acid
- 2.08 ml – 0.2 N HCI

All were mixed and volume made up to 100 ml with distilled water.

Procedure:

	Blank (ml)	Test (ml)
Whole blood	-	0.75
Distilled water	0.75	-
Thiobarbituric acid reagent	3	3

- Keep in boiling water bath for 15 minutes
- Cool, centrifuge for 10 minutes at 10,000 r.p.m.
- Read absorbance of supernatants of blank and test immediately at 535 nm

Calculation:

Malondialdehyde (nano moles / ml)

$$= \frac{\text{Absorbance of test} \times \text{total volume}}{\text{Nanomolar extension coefficient} \times \text{Sample volume} \times 100}$$

$$= \frac{\text{Absorbance of test} \times 3.75}{1.56 \times 10^5 \times 0.75 \times 100}$$

$$= \frac{\text{Absorbance of test} \times 3205}{100}$$

PREPARATION OF (RBC) HEMOLYSATE

Isolation of Red Blood Cells: [64]

Most of the enzyme activities in red cells are lower than those in white blood cells and platelets, and hence it was of extreme importance to remove virtually all platelets and WBCs. In order to isolate RBCs the whole blood was filtered through a column of α-cellulose and microcrystalline cellulose mixture.

α – Cellulose and microcrystalline cellulose in 1:1 (W/W) was mixed with isotonic (9.0 gm/L) sodium chloride solution. 5ml plastic disposable syringe without barrel was taken. It was placed in vertical position with the outlet pointing downwards. A small piece of filter paper was placed at the bottom of the syringe. The well – mixed cellulose slurry was poured to the 2-ml mark. The bed was washed with 5-ml isotonic sodium chloride and 1 ml of whole blood was allowed to flow through the column. To ensure efficient removal of WBCs and platelets, the volume of cellulose mixture used was at least twice as that of the blood sample.

The effluent was collected into a centrifuge tube. The saline suspended red cells were washed twice in at least 10 volumes of ice-cold isotonic sodium chloride. After washing, the packed cells were resuspended in isotonic sodium chloride to give an approximately 50% suspension (1:1) dilution). This suspension was subjected to hemolysis.

LYSING OF THE RBCS:[65]

Reagents:

Stabilizing solution: 2.7 mM EDTA (pH 7.0) and 0.7mM β- mercaptoethanol: This solution was prepared by dissolving 100 mg of disodium salt of EDTA in D/W and

5µl of β- mercaptoethanol (Merck) were added to it. Final volume was made to 100 ml with D/W.

Procedure:

In order to prepare the hemolysate, 1 volume of the RBC suspension was mixed with 9 volumes of the stabilizing solution. The hemolysate was frozen rapidly at – 20^0C to – 25^0C in a freezer. Then it was thawed in a water bath at 20^0C to 25^0C. This hemolysate was then ready for the assay. The hemoglobin estimation was performed on hemolysate using Drabkin's reagent, in order to express the enzyme activities per gram of hemoglobin of the hemolysate. The red cell suspension and its hemolysate were prepared on the day of the assay.

The hemolysate was used for the following enzyme activities-

a) Superoxide dismutase (SOD)

b) Glutathione reductase (GR)

DETERMINATION OF HEMOGLOBIN[66]

Principle:

Drabkins reagent contains potassium cyanide and potassium ferricyanide Hemoglobin reacts with ferricyanide to form methemoglobin, which is converted to stable cyanmethemoglobin (HiCN) by the cyanide. The intensity of the colour is proportional to hemoglobin concentration and is compared with a known cyanomethemoglobin standard at 540 nm (green filter).

Reagents:

1. Drabkin's reagent: The prepared reagent was purchased (Span Diagnostics).
2. Cyanmethemoglobin standard, (cyanmeth-Hb standard) 15g%: The prepared standard was purchased (Span Diagnostics)

Procedure:

A set of tubes was prepared as follows:

	Bank (ml)	**Standard (ml)**	**Test (ml)**
Drabkin's reagent	5.0	---	5.0
Cyanmethemoglobin standard	----	5.0	----
Unknown	----	----	0.02

The contents in the test were mixed thoroughly and optical density of test and standard (15g %) were measured at 540 nm against blank (Drabkin's reagent)

Calculation:

$$\text{Hemoglobin g\%} = \frac{\text{ODtest}}{\text{ODstd}} \times 15$$

PERFORMANCE OF THE ENZYME ASSAYS FROM 1:20 HEMOLYSATE

SUPEROXIDE DISMUTASE[67]

Principle:

Epinephrine can be auto-oxidized to adrenochrome by superoxide radicals. Maximum auto-oxidation of epinephrine takes place at pH-10.2. The ability of superoxide dismutase to inhibit the auto-oxidation of epinephrine to adrenochrome at pH 10.2 has been used as the basis for the assay of this enzyme. In this method epinephrine acts both as the source of superoxide radical ($O_2^{\bullet -}$) and as the detecting system giving adrenochrome which can be monitored at 480 nm.

Reagents:

1) Chloroform.

2) Ethanol.

3) Sodium carbonate/ bicarbonate buffer. 0.05 M, pH 10.2;

This was prepared as follows.

a) Sodium carbonate (Na_2CO_3).0.2 M: 2.12g of anhydrous Na_2CO_3 was dissolved in small quantity of D/W and volume made to 100ml with D/W.

b) Sodium bicarbonate ($NaHCO_3$), 0.2M: 1.68g of $NaHCO_3$ were dissolved in small quantity of D/W and volume was made to 100ml with D/W

Solution (a) 33ml was mixed with solution (b) 17ml in small quantity of D/W, pH of the solution was adjusted to 10.2 and volume was made to 200 ml with D/W.

4) DL-(α) Epinephrine, 3 x 10^{-2} M: 55 mg of Dl-(α) Epinephrine were dissolved in 3ml of D/W. To dissolve epinephrine completely minimum amount of 1NHC1 was added and volume was made to 10ml with D/W.

5) EDTA (Na_2EDTA), 15 x 10^{-3} M: 49 mg of Na_2EDTA were dissolved in minimum quantity of D/W and volume was made to 10ml with D/W.

Procedure: Preparation of hemoglobin free filtrates:

Chloroform, ethanol and water were chilled before use. In a test tube, chloroform (0.125ml), ethanol (0.25ml) and D/W (0.8ml) were added. To this mixture, 0.2ml of cold hemolysate was transferred. The suspension was subjected to vortex agitation for 2 minutes by transferring the tube intermittently to ice bath. The precipitate was separated by centrifugation at 15,000 rpm at 4^0C for 10 minutes and the supernatant was used for the assay.

SOD Enzyme assay:

For the assay the final reaction mixture in control-contained epinephrine ($3x10^{-4}$M), EDTA ($1x10^{-4}$M) and carbonate buffer (0.05M). In a series of tests, in addition to the above reagents, varying amounts of chloroform-ethanol extracts were added.

Control and tests were setup as under:

	Control	$Test_1$	$Teast_2$	$Test_3$	$Test_4$	$Test_5$	$Test_6$
Carbonate buffer, 0.05M pH(10.2) (ml)	2.95	2.90	2.85	2.80	2.75	2.70	2.65
Chloroform-ethanol extract (µl)	----	50	150	150	200	250	300
EDTA, 15 x 10^{-3}M (ml)	0.02	0.02	0.02	0.02	0.02	0.02	0.02

To control tube 0.03ml of epinephrine ($3x10^{-2}M$) was added and after 90 secs (the lag phase) the auto-oxidation of epinephrine was assayed by measuring the optical densities at 0 minute and after 3 minutes, at 480nm.

To $Test_1$ 0.03ml of epinephrine was added and after 90 seconds, optical densities were measured at 0 minute and after 3 minute at 480 nm.

In a similar fashion the assay was performed by adding 0.03 ml of epinephrine one by one to each tube and after lag phase of 90 seconds optical densities were measured at 0 minute and after 3 minutes at 480 nm.

Calculations:

SOD activity was determined in terms of its inhibition of auto oxidation of epinephrine to adrenochrome.

One enzyme unit is the amount of protein required to inhibit the auto-oxidation of epinephrine by 50% under standard conditions of assay.

Percent inhibition was calculated from the optical density of each tube with varying amounts of enzyme (red cell extract) and optical density of the control without enzyme.

$$\text{Percent inhibition} = 100 - \frac{\Delta ODs}{\Delta ODc} \times 100$$

Where ΔODs: Difference in optical density of sample in 3 minutes.

ΔODc: Difference in optical density of control in 3 minutes.

A graph percent inhibition against amount of red cell extract was plotted as shown in the figure 16, to determine 50% inhibition of epinephrine oxidation. The

amount of extract, which gave 50% inhibition, as determined from the graph corresponds to 1 unit of SOD activity.

V µl = 1 unit of SOD activity

Units of SOD activity per 100 ml of red cell extract / X gm of Hb = $\frac{100 \times 1000}{V\ \mu l}$ units

Units of SOD activity / gm of hemoglobin = $\frac{100 \times 1000}{V\ \mu l} \times \frac{1}{x}$

NB:for each sample the graph was plotted to determine the 50% inhibition as shown below.

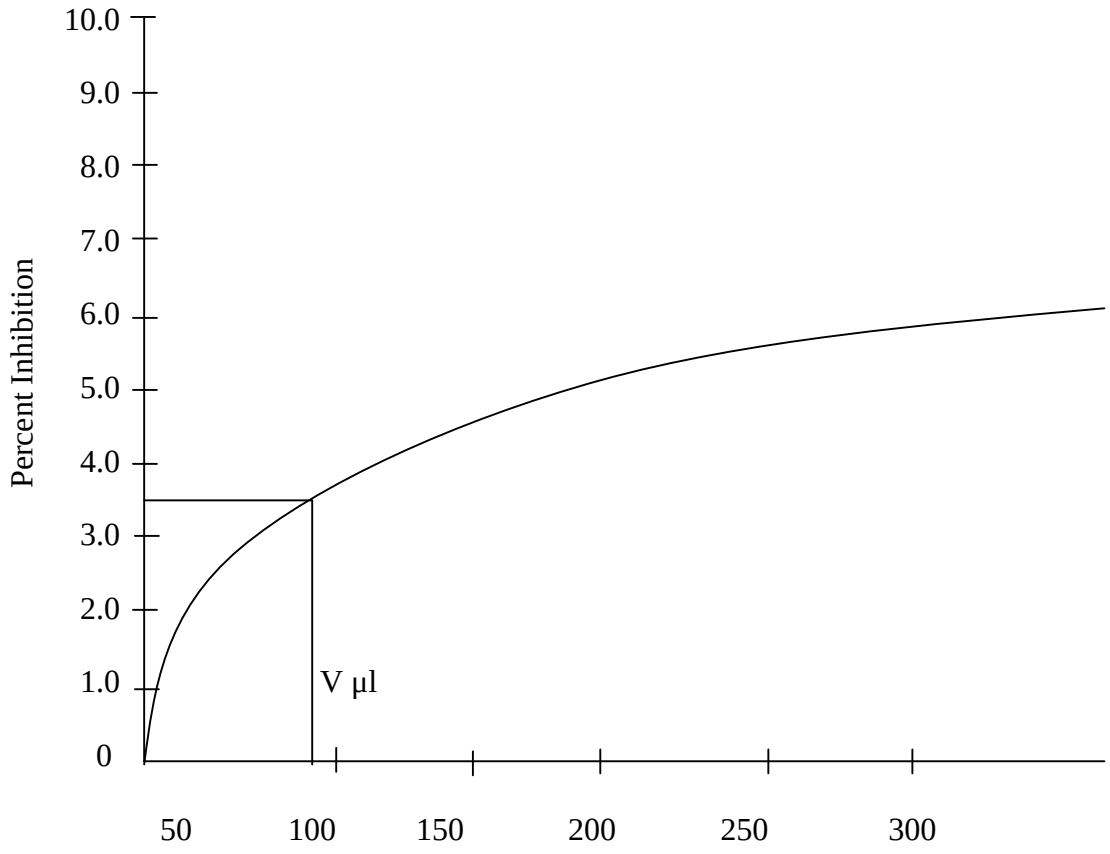


Fig. No 7: Determination of SOD Activity Red cell extract (rl)

Calculation:

The number of enzyme units per ml:

$$A = \frac{\Delta OD \times Vc}{\varepsilon \times N \times V_H}$$

Where

ΔOD: Change in optical density per minute

$$\Delta OD: \frac{Test - (Blank_1 + Blank\ )}{10 \text{ minutes}}$$

Vc : The volume of the cuvette in ml = 1ml

ε : The millimolar extinction coefficient of the NADPH = 6.22

N: The number of molecules (1) of NADPH converted per molecule of t-BHP consumed.

V_H : The volume of hemolysate added to the cuvette in ml = 0.01ml

The enzyme activity in international unit / g Hb

$$E = \frac{A \times 100}{Hb}$$

A: The number of enzyme units /ml

Hb: The grams of hemoglobin per 100ml of the hemolysate.

GLUTATHIONE REDUCTASE[68]

Principle:

Glutathione reductase (GR) catalyzes the reduction of oxidized glutathione (GSSG) by NADPH to reduced glutathione.

$$NADPH\ (NADH) + H^{+} + GSSG \xrightarrow{GR} NADP^{+}\ (NAD^{+}) + 2GSH$$

The activity of this enzyme is measured by following the oxidation of NADPH (NADH) spectrophotometrically at 340nm. Glutathione reductase is a flavin enzyme and it has been found that it is not fully activated by FAD in normal hemolysates. Complete activation of apoenzyme, requires the preincubation of the enzyme with FAD. This must be done before GSSG or NADPH is added to the reaction system, since these seem to interfere with activation of the enzyme by FAD.

Reagents:

1) Tris-HCI; 1M, EDTA: 5mM, pH 8.0: It was prepared as described earlier.
2) Flavin adenine dinucleotide (FAD), 10 μM: 0.8 mg of sodium salt of FAD (SRL, Mwt-829.52) was dissolved in 100 ml of D/W.
3) Glutathione oxidized (GSSG), 0.033M: 20.2 mg of GSSG (SRL, Mwt-612.7) were dissolved in 1ml of D/W.
4) Nicotinamide adenine dinucleotide phosphate reduced (NADPH) 2nmM: It was prepared as described earlier.

Procedure:

The following reagents were added to the cuvettes volume of 1ml

	Blank (µl)	**Test (µl)**
Tris HCI; 1M EDTA, 5mM, pH 8.0	50	50
1:20 hemolysate	10	10
D/W	790	690
FAD 10 µM	100	100
Tubes were incubated at 37^0C for 10 minutes		
GSSG, 0.033M	--	100
Tubes were incubated at 37^0C for 10 minutes		
NADPH, 2Mm	50	50

The decrease of optical density was measured at 340 nm at 37^0C of the test against the blank.

Calculations:

The number of enzyme units per ml:

$$A = \frac{\Delta OD \times Vc}{\varepsilon \times N \times V_H}$$

Where

ΔOD: Change in optical density per minute

Vc : The volume of the cuvette in ml = 1ml

ε : The millimolar extinction coefficient of the NADPH = 6.22

N: The number of molecules (1) of NADPH converted per molecule of GSSG consumed.

V_H : The volume of hemolysate added to the cuvette in ml = 0.01ml

The enzyme activity in international units / g Hb

$$E = \frac{A \times 100}{Hb}$$

A: The number of enzyme units /ml

Hb: The grams of hemoglobin per 100ml of the Hemolysate.

ESTIMATIONS FROM PLASMA

RETINOL[69]

Principle:

Proteins get precipitated on addition of ethanol and concentration of Retinol can be determined by reading extinction of heptane extract of retinol at 327nm.

Reagents:

1. Absolute Ethanol
2. N- Heptane
3. Retinol stock standard: 10-mg %: 10 mg of retinol palmitate (Sigma) were dissolved in 100 ml of n- heptane.
4. Retinol working standard, 100 μg %: 1 ml of stock standard retinol solution was diluted to 100 ml with n- heptane.

Procedure:

In a clean dry test tube, 2.0-ml plasma was taken. To this 1.0 ml of D/W, 4.0 ml of each ethanol and n – heptane were added. Contents of tube were mixed for 15 minutes using a cyclo mixer and then centrifuged at 3000 rpm for 5 minutes. Upper heptane layer was then separated and read at 327 nm against heptane blank using a double beam spectrophotometer. Working standard of retinol was directly read at 327 nm.

Calculations:

Concentration of retinol is expressed as μg % of retinol palmitate

$$= \frac{\text{OD of sample}}{\text{OD of standard}} \times \text{concentration of standard (in μg \%)}$$

α- TOCOPHEROL[70]

Principle:

This method is based on the Emmerie Engel reaction. Xylene extract of plasma containing α-tocopherol when reacts with ferric chloride, reduces ferric ions to ferrous ions. The ferrous ions then react with α, α' – dipyridy1 to give a red colored complex which is measured at 520 nm. Carotenoids, which are also extracted into xylene, are estimated by their absorbance at 460 nm and a correction is applied at 520 nm. The carotenoid absorption at 520 nm is 29% of absorption of 460 nm.

Reagents:

1) Absolute ethanol, aldehyde free

2) Xylene

3) n-propanol

4) α, α' Dipyridy1 reagent: 120 mg %: 120 mg of dipyridy1 were dissolved in small quantity of n-propanol and volume was made to 100 ml with n-propanol.

5) Ferric chloride reagent 120 mg %: 120 mg of ferric chloride hexahydrate ($FeCl_3.6H_2O$) were dissolved in small quantity of absolute ethanol and volume was made to 100 ml with absolute ethanol.

6) α- Tocopherol, stock standard 280 mg %: 280 mg of α – tocopherol were dissolved in small quantity of absolute ethanol and volume was made to 100 ml with absolute ethanol.

7) α– Tocopherol. Working standard: 1.4 mg %: 0.1 ml of the stock standard of α – tocopherol was diluted to 20 ml with absolute ethanol

Procedure:

A set of test tubes was prepared as follows:

	Blank (ml)	Standard (ml)	Test (ml)
Distilled water	1.5	-	-
Std α – tocopherol	-	1.5	-
Plasma	-	-	1.5
Absolute ethanol	1.5	1.5	1.5
Xylene	1.5	1.5	1.5

Tubes were stoppered and mixed on a vortex mixer for 2 minutes. After centrifugation at 2000 rpm for 10 minutes, 1.0 ml of xylene layer was withdrawn from each tube into separate set of corresponding tubes. To all the tubes 1.0 ml of α, α' – dipyridy1 reagent was added. The contents of the tubes were mixed and absorbance of test and standard was read at 460 nm, against blank. Beginning with the blank, to all tubes 0.4 ml of ferric chloride reagent was added and contents were mixed for 30 seconds. The absorbance was read exactly after 90 seconds of the addition of ferric chloride reagent. The test and standard were read at 520 nm against blank.

Calculation:

Plasma levels of α- tocopherol are expressed as mg %

Concentration of α – tocopherol (mg %) =

$$\frac{ODT_{520} - (0.29 \times OD\ T_{460})}{OD\ S_{520} - (0.29 \times OD\ S_{460})} \times C =$$

Where

OD T_{520} : Optical density of test at 520 nm

OD T_{460} : Optical density of test at 460 nm

OD S_{520} : Optical density of standard at 520 nm

OD S_{460} : Optical density of standard at 460 nm

C : Concentration of standard α – tocopherol in mg %

ASCORBIC ACID[71]

Principle:

When ascorbic acid reacts with 2, 6-dichlorophenol indophenols, reduced 2, 6-dichlorophenol indophenol is formed which is colorless. Decrease in color is proportional to the concentration of ascorbic acid present in the solution. Decrease in the optical density is measured at 520nm and concentration is calculated from standards treated similarly.

Reaction:

2, 6-dichlorophenol indophenol (Oxidized form) + Ascorbic acid $\longrightarrow$

2, 6-dichlorophenol indophenol (Reduced form) + Dehydroascorbic acid

Reagents:

1) 10% sodium tungstate: 10 g of sodium tungstate were dissolved in D/W, and the volume was made up to 100 mL.

2) 2/3 N sulphuric acid. Take 18.7ml concentrated H_2SO_4. Mix with distilled water make the volume up to 1 liter with D/W.

3) Metaphosphoric acid solution; 5%: 5g of metaphosphoric acid (Robert Johnson) is dissolved in 100ml of D/W without heating. Solution should be prepared weekly. Store in refrigerator.

4) 2, 6 dichlorophenol indophenol solution: 13mg of 2, 6 dichlorophenol indophenol (Loba GR) and 3g of anhydrous sodium acetate trihydrate (Qualigens SQ), were dissolved in 1 liter of D/W. 9ml of this reagent and 1 ml of metaphosphoric acid (reagent 1) were mixed to check the pH which should be around 3.5-3.6 (pH can be adjusted by varying the amount of sodium acetate in the reagent).

5) Ascorbic acid stock standard, 100mg%: 100mg of L-Ascorbic acid was dissolved in 100ml of metaphosphoric acid.

6) Ascorbic acid working standard, 1 mg%: 1ml of the stock standard ascorbic acid was diluted to 100ml with metaphosphoric acid.

Procedure:

In a clean dry test tube, 1 ml of plasma, 3ml of 5% metaphosphoric acid, 0.5ml of sodium tungstate and 0.5ml of 2/3N sulfuric acid were taken. The contents were mixed and filtered after 5 minutes.

Three clean dry test tubes labeled as Blank, Standard and Sample were taken, and following reagents were added as shown as follows.

	Blank (ml)	Standard (ml)	Sample (ml)
5% Metaphosphoric acid	0.3	-	-
Ascorbic acid standard	-	0.3	-
Plasma	-	-	0.3
2,6-dichlorophenol indophenol	2.7	2.7	2.7

Reading was taken at 520nm against D/W using a double beam Spectrophotometer.

Calculations:

Amount of ascorbic acid in mg% =

$$\frac{\text{OD of Blank} - \text{OD of sample}}{\text{OD of Blank} - \text{OD of Standard}} \times \text{concentration of standard (in mg \%).}$$

Estimation of serum calcium and phosphorous[72]:

1) CALCIUM

Principle: By precipitation . Trinder has described the use of naphtholhydroxamic acid as a precipitating agent which is so efficient that only a small excess of reagent is required. This means that it is unnecessary to wash the precipitate to remove excess reagent and a very convenient and accurate method results.

Reagents

1. Calcium reagent:dissolve 250 mg. of naphthalhydroxine acid by warming in 100 ml. of water containing 5 ml. of ethanolamine and 2 g. of tartaric acid. Add 9g. of sodium chloride dissolved in 500 ml. of water and dilute to 1 litre with water. If aprecipitate forms, filter through a whatman No.40 or 43 paper.

2. EDTA solution: 2 g. of disodium EDTA per litre in 0.1N-sodium hydroxide.
3. Colour reagent: dissolve 60g. of ferric nitrate ($Fe(NO_3)_3.9H_2O$) in 500ml. of water, add 15 ml. of conc. Nitric acid and dilute to 1 litre with water.
4. Calcium standard: (5 mEq. Per litre). Dissolve 125 mg. of dry calcium carbonate in 40 ml of 0.1 N-hydrochloric acid and dilute to 500 ml with water.

Method:

Test: pipette 0.2 ml. of serum into a centrifuge tube. Add 5 ml. of calcium reagent and mix.

Standard: 0.2 ml.of calcium standard (5mEq. Per litre) and 5ml. of calcium reagent .

Blank: 5 ml of calcium reagent.

Allow the tubes to stand at room temperature for 30 minutes. Centrifuge hard (3500 r.p.m., 6 inch radius for 5 minutes) and decant the supernatant fluid carefully by slowly inverting the tubes and immediately placing them to drain mouth downwards on a pad of filter paper, without returning them to the upright position. After 5 minutes , wipe the mouths of the tubes and add 1 ml. of EDTA solution. Shake to suspend the precipitate , cover the mouth of the tube with an aluminium cap or a marble and heat in a boiling water bath for 10 minutes with occasional mixing to ensure complete solution of the precipitate . Cool, add 3 ml. of colour reagent and mix. Compare the colours at 450nm or using an Ilford 622 blue or chance OB 10 filter.

Serum calcium=T-B/S-B×5 (mEq. Per litre).

(To convert the result into mg. per 100 ml.,multiply by 2)

2) PHOSPHOROUS:

Principle: The method is a variant of the phosphomolybdic acid reaction. A substituted phenol is used as a reducing agent and the pH is controlled by an acetate buffer . Copper in the buffer hastens colour development which is complete after 5 minutes. The blue colour is stable for atleast 30 minutes.

Reagents:

Trichloroacetic acid: 10 g. per 100 ml. in water.

Stock phosphate standard: (100 mg. phosphate per 100ml). 219g of potassium dihydrogen phosphate (KH_2PO_4) per 500ml.in water. add a few drops of chloroform and keep at 4^0 .

Working phosphate standard: (1 mg. phosphate per 100 ml.). 1 ml. of stock standard per 100 ml. in 5 percent trichloroacetic acid . prepare freshly every few weeks.

Acetate buffer. pH 4.0 : Dissolve 2.5g of copper sulphate ($CuSO_4.5H_2O$) and 46 g of sodium acetate($CH_3.COONa.3H_2O$) in 1 litreof 2 n-acetic acid. Adjust the pH to 4.0.

Rhodol : dissolve 2g. of Rhodol (paramethylaminophenol sulphate) in 80 ml. of water. Add 10 g. of hydrated sodium sulphite($Na_2SO_3.7H_2O$), make up to 100 ml. and filter. Store ina dark bottle at 4^0.

Ammonium molybdate : 5.g per 100 ml in water.

Method:

Test: to a centrifuge tube containing 0.8 ml. of water, add 0.2 ml. of fresh plasma. Mix and add 1ml. of 10 per cent trichloroacetic acid. Shake well and centrifuge. Take 1 ml. of the supernatant into a test tube ; the concentration of phosphate is 1/10 of that in the original sample.

Standard: Into a test tube pipette 1 ml. of working phosphate standard. (1 mg. per 100 ml.)

Blank: 0.5 ml of 10 percent trichloroacetic acid plus 0.5 ml.of water.

To test Standard and Blank,add 3 ml. of acetate buffer , 0.5 ml of ammonium molybdate solution and 0.5 ml of Rhodol , mixing well after each addition. Allow to stand for at least 5 minutes and compare the colours at 880nm or using an Ilford 608 red filter.

Inorganic phosphate =T-B/S-B×10 .

(mg phosphate per 100 ml.)

RESULTS

The present study comprises of 40 clinically diagnosed cases of Rheumatoid arthritis and 40 normal healthy controls. The age group ranges from 40 to 60 years. Statistical analysis was carried out by using students 't' test (unpaired)

The mean level of MDA in controls was 6.19 ± 0.96 nmol/ml .The value in cases was 11.48 ± 0.76 nmol/ml . The level was significantly increased (P< 0.0001) in the cases compared to controls .

The value of SOD in controls was 948.32 ± 99.88 IU/g of Hb. The mean value of SOD was 443.68 ± 111.69 IU/g Hb in the total cases .The level of SOD was significantly decreased (P<0.0001) in cases compared to controls.

The mean glutathione reducatase level was 8.91± 1.04 IU/g of Hb in controls and the level in cases was 2.96 ± 0.79 IU/g of Hb .The level of glutathione reducatase was decreased significantly (P<0.0001) in all the cases compared to controls.

The mean value of vitamin A in controls was 35.13 ± 5.25 µg/dl. The level of vitamin A in cases was 11.60 ± 3.03 µg/dl .The level was significantly decreased (P<0.0001) in all the cases compared to controls.

The mean value of vitamin E in controls was 0.92 ± 0.10 mg/dl. The level of vitamin E in cases was 0.53 ± 0.14 mg/dl. The level was significantly decreased (P<0.0001) in all the cases compared to controls.

The mean vitamin C level in controls was 0.88 ± 0.12 mg/dl and in cases 0.48 ± 0.09 mg/dl. The level of vitamin C was significantly decreased (P<0.0001) in all the cases in comparison to controls.

The mean Calcium level in controls was 10.21 $\pm$ 0.65 mg/dl and in cases 7.33±0.93 mg/dl. The level of Calcium was significantly decreased (P<0.0001) in cases compared to controls.

The mean phosphorous level in controls was 2.89 $\pm$ 0.57 mg/dl and in cases 4.28±0.81 mg/dl. The level of phosphorous was significantly increased (P<0.0001) in cases Compared to controls.

Table No.2: MDA and Enzymatic Antioxidants in controls and cases

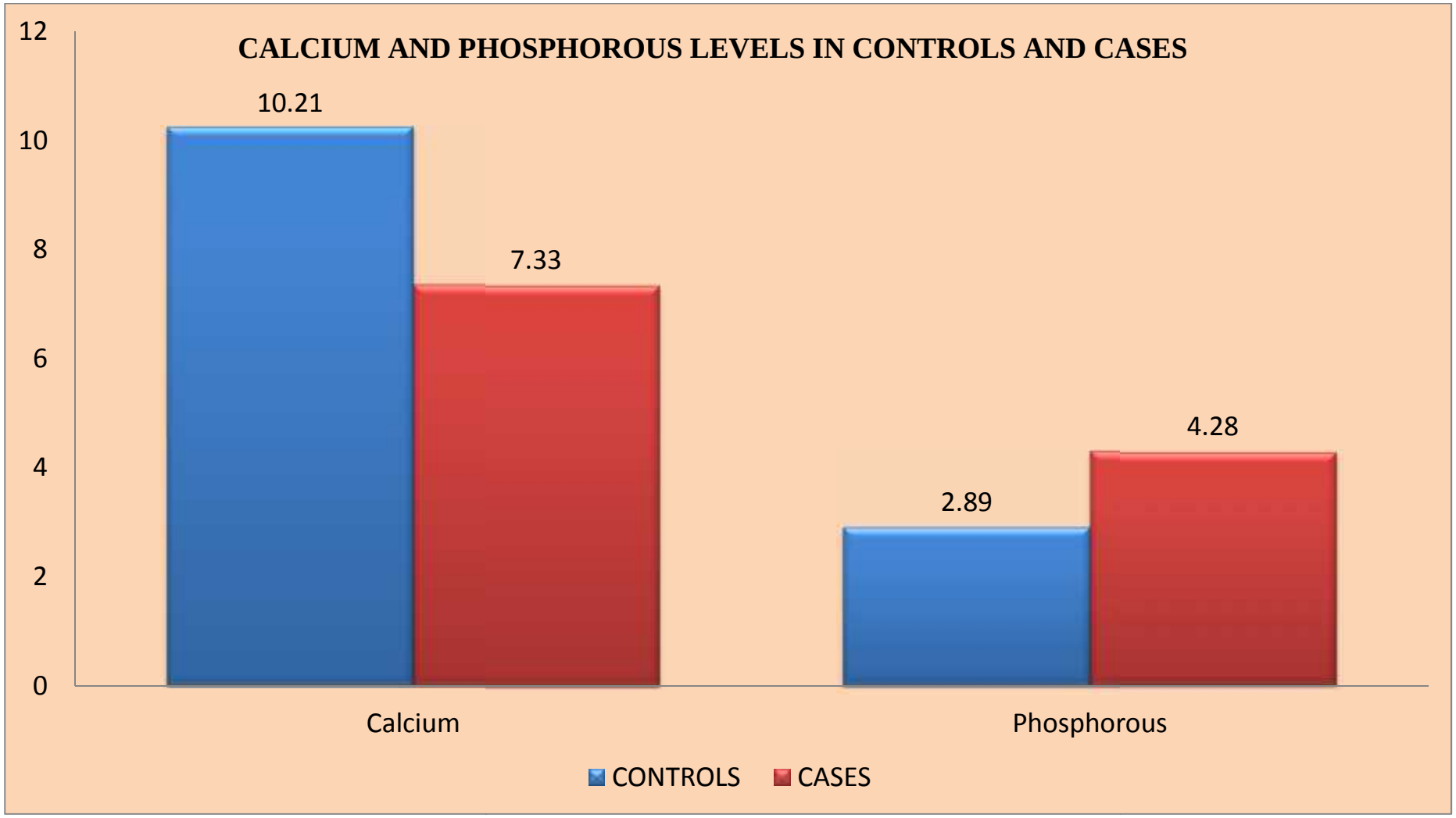
Parameters	MDA nmol/ml	SOD(IU/g of Hb)	Glut red (IU/g of Hb)
n=40 **CONTROLS** Mean ± S.D	6.19 ± 0.96	948.32 ± 99.88	8.91 ± 1.04
n=40 **CASES** Mean ± S.D	11.48 ± 0.76	443.68 ± 111.69	2.96 ± 0.79
P. value	<0.0001	<0.0001	<0.0001

Table No.3: MDA and Non-Enzymatic Antioxidants in controls and cases

Parameters	MDA nmol/ml	Vitamin A(µg/dl)	Vitamin E(mg/dl)	Vitamin C(mg/dl)
n=40 **CONTROLS** Mean ± S.D	6.19 ± 0.96	35.13 ± 5.25	0.92 ± 0.10	0.88 ± 0.12
n=40 **CASES** Mean ± S.D	11.48 ± 0.76	11.60 ± 3.03	0.53 ± 0.14	0.48 ± 0.09
P. value	<0.0001	<0.0001	<0.0001	<0.0001

Table No.4: Calcium and Phosphorous Levels in controls and cases

Parameters	Calcium(mg/dl)	Phosphorous(mg/dl)
n=40 **CONTROLS** Mean ± S.D	10.21 ± 0.65	2.89 ± 0.57
n=40 **CASES** Mean ± S.D	7.33 ± 0.93	4.28 ± 0.81
P. value	<0.0001	<0.0001


CALCIUM AND PHOSPHOROUS LEVELS IN CONTROLS AND CASES
12
10
8
6
4
2
0
10.21
7.33
2.89
4.28
Calcium
Phosphorous
CONTROLS
CASES

DISCUSSION

Rheumatoid arthritis is a major cause of morbidity as it affects the joints, causing stiffness and loss of mobility. The cause of rheumatoid arthritis is mainly joint inflammation initiated by oxidative stress.

Involvement of oxygen free radicals in the pathophysiology of inflammation in a number of organs and tissues has been reported in literature.[73,74] Evidence of OFR generation in patients with RA has been observed by measuring the product of lipid peroxidation malondialdehyde. Antioxidant status was assessed by measuring superoxide dismutase, glutathione reductase, vitaminA, vitamin E and vitamin C . In view of the recent animal studies strongly suggesting anti-inflammatory role of antioxidants like superoxide dismutase[75] and vitamin E[76] in experimentally induced arthritis, antioxidant therapy strategies have been proposed for the prevention and treatment of RA.[77-83] Hypoxic conditions also disrupt an intracellular ionic environment and alter calcium and phosphorous levels. So estimation of calcium and phosphorous levels was done.

MALONDIALDEHYDE

In the present study mean level of MDA was increased significantly in cases compared to controls. Our findings are in accordance with the study of Yousef Shaabani et al,[84] S.D. walwadkar et al,[85] Soobia Ansari et al.[86] MDA is a decomposition product of lipid peroxidation of polyunsaturated fatty acids which is used as an index of oxidative damage. Gambhir et al,[87] also reported markedly increased concentrations of MDA in patients as compared to controls. Enhanced lipid peroxidation may occur as a result of imbalance between scavenging mechanisms and

free radical generation process. Similar reports of elevated MDA levels have been reported in patients with rheumatoid arthritis.[88]

SUPEROXIDE DISMUTASE (SOD)

The mean level of SOD was significantly decreased in cases compared to controls ($P < 0.0001$). The findings of our study are in accordance with study of Sang-Cheol Bae et al.[82]

DiSilvestro et al[89] showed that the administration of anti-inflammatory drugs increases plasma SOD activity, indicating the inflammation process produces free radicals, thereby decreasing SOD activity. Disease itself may inhibit the activity of SOD and reduce the synthesis of SOD .

Our findings are contradictory to the findings of Krishna Mohan Surapneni et al[90] who showed significant increase in SOD levels in RA.

GLUTATHIONE REDUCTASE

The mean level of glutathione reductase was decreased significantly in cases ($P<0.0001$) compared to controls.

The findings of the present study are in accordance with the results of Palanisamy Pasupathi1 et al.[91] According to Palanisamy Pasupathi1 et al , there is enhanced lipid peroxidation in rheumatoid arthritis patients with concomitant failure of both the plasma, and erythrocyte antioxidants defense mechanism. These results are consistent with the underlying hypothesis that there is an imbalance between ROS production and the antioxidant defense system in inflammatory rheumatoid arthritis disease.

VITAMIN-A

The mean level of vitamin A was lowered significantly in cases compared to controls.

The findings of our study are in accordance with the study of M.Mezes et al,[92] who have mentioned in their study that decreased level of vitamin A is because of its inability to combat increased oxidative stress and inflammation.

VITAMIN-E

The mean level of vitamin E was lowered significantly in cases compared to controls.

The findings of our study are in accordance with the study of S.D. Walwadkar et al,[85] Krishna Mohan Surapneni et al.[90]

According to Krishna Mohan Surapneni et al the lowered levels of vitaminE may be due to the increased turnover, for preventing oxidative damage in these patients suggesting an increased defense against oxidant damage in RA. Decreased levels of vitamin E in RA patients, are comparable with previous reports by Fairburn K et al.[93]

In another study, Edmonds et al showed vitamin E supplementation (600mg per day) improved clinical symptoms of RA patients.[94]

VITAMIN C

The mean level of vitamin C was decreased significantly in cases compared to controls.

The findings of our study are in accordance with the study of Krishna Mohan Surapneni et al .

According to Krishna Mohan Surapneni et al, lowered levels of vitamin C is due to its enhanced consumption because of increased oxidant stress caused by free radicals which indicates asynchrony between oxidants and anti oxidants in RA patients.

Calcium

Serum calcium levels were significantly decreased in cases compared to controls.

The findings of our study are in accordance with the study of S.D. Walwadkar et al.

Decrease in calcitriol level may contribute to a negative calcium balance and acceleration of immunomodulatory effects. as observed by studies of Oelenzer.P et al[95] and Kroger.H et al.[96]

There is another study by D. L. Scott et al[97] where the authors have noticed that transient hypercalcaemia and hypocalcaemia occurred occasionally, but for most of the time calcium levels were normal. Changes in calcium levels were not related to changes in clinical, haematological, or immunological parameters of disease activity. Mean serum calcium levels are lower in disease than health; this occurs in RA as well as other diseases.

Our findings are contradictory to the findigs of Kennedy A.C et al[98] who showed that hypercalcemia is a common occurrence in RA.

Phosphorous

Serum calcium levels were significantly increased in cases compared to controls.

The findings of our study are in accordance with the study of S.D. Walwadkar et al

According to S .D. Walwadkar et al, it is postulated that the elevation of phosphorous was related to tissue hypoxia with an increase in ATP degradation

resulting in the release of inorganic phosphorous from cells. Acidosis is another factor that may act to promote shift of phosphate from the intracellular to extracellular pool. The rise in serum inorganic phosphate may parallel increase in blood lactate levels suggesting that a state of parallel anaerobic metabolism may be contributory factor.[99]

As Calcium and phosphorous are important constituents of bone, ultimately bone metabolism is altered in RA, the event observed by many workers.[95,100]

SUMMARY

Our study revealed that there was increased oxidative stress and decreased antioxidant defense in patients of Rheumatoid Arthritis as evidenced by –

- Increased levels of malondialdehyde.
- Decreased levels of enzymatic antioxidants like superoxide dismutase and glutathione reductase.
- Decrease in the concentration of non- enzymatic antioxidants like vitaminA, vitaminE and Vitamin C.

Our study revealed the following information about calcium and phosphorous levels

- Calcium levels were significantly decreased in cases compared to controls.
- Phosphorous levels were significantly increased in cases compared to controls.

CONCLUSION

In RA there is an increased oxidative stress and decreased antioxidant defense (enzymatic and non- enzymatic) which is the root cause of joint inflammation causing arthritis. Also there is altered serum calcium and phosphorous levels in these patients. Supplementation of natural antioxidants and certain minerals like calcium to the individual who are prone for RA may help prevent the morbidity to certain extent.

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ANNEXURE – I

INFORMED CONSENT (MODEL OF THE CONSENT FORM IS ENCLOSED) DESCRIBING THE FOLLOWING.

a) Purpose of Study:

The purpose of the study is to know the status of oxidative stress , antioxidants and calcium, phosphorous levels in Rheumatoid Arthritis.

There will be approximately 40 clinically diagnosed Rheumatoid Arthritis patients and equal number of normal healthy volunteers participating in the study. It will be conducted under supervision of Dr.P.B.Desai, Professor and HOD, Dept. of Biochemistry, JNMC, Belgaum and will be carried out by Dr.Manjunath.S, Postgraduate, Dept. of Biochemistry, JNMC, Belgaum for his MD dissertation to be submitted to KLE University, Belgaum.

b) Procedure and Treatment:

For patients of Rheumatoid Arthritis:-

You will qualify for the study if you are diagnosed as Rheumatoid Arthritis, in the age group of 40-60 years and willing to provide additional background and medical information.

Your venous blood sample (approximately 10ml will be collected for study under the aseptic precautionary measures using sterile disposable syringe. The required parameters will be estimated from the blood sample.

For Normal Individuals (Control Group):-

You will qualify for the study if you are in age group of 40-60 years, free from diseases, in good health and agree to provide additional background

and medical information. Your venous blood sample (approximately 10ml) will be collected for the study under aseptic precautionary measures using sterile disposable syringe.

The following parameters will be estimated from the blood of patients with acute Myocardial Infarction and control subjects.

- Malondialdehyde
- Superoxide dismutase
- Glutathione reductase
- Vitamin A
- Vitamin E
- Vitamin C
- Calcium
- Phosphorous

c) Risks:

Since blood is drawn under aseptic precautionary measures by trained technicians there is no scope for any risk. Further, only small volume of blood is collected which will be spontaneously replenished in the body.

d) Benefits:

You can make use of levels of studied parameters if desired.

e) Financial incentive for participation:

You will not receive any payment for participating in this study.

f) Alternative:

If you decide not to participate in the study, the hospital will provide you usual standard care and treatment.

g) Authorization to publish results:

The study results will be published in the M.D. dissertation of Dr. Manjunath.S to be submitted to KLE University, Belgaum. Also the results may be published for scientific purposes and/or presented to scientific groups. However, you will not be identified.

h) Sponsors Policy:

Department of Biochemistry, JNMC, Belgaum is the sponsor of this study project which you are participating.

i) Institutional Policy:

The J. N. Medical College will provide within the limitations of laws of the state of Karnataka, facilities and medical attention to subjects who suffer any harm as a result of participating in this project. In the event you believe that you have suffered any harm as the result of your participation in this study you may contact the research guide Dr. P.B.Desai or Dr. Manjunath.S.

j) Emergency Provision:

In the event of emergency you should telephone Immunology Department.

k) Voluntary Participation:

Your participation in the study is voluntary. Your decision whether or not to participate will not affect care or treatment in the hospital. You are free to discontinue participation in this study at any time and for any reason. In case you need any further information regarding your rights as a study participant, you may please contact Dr.V.D. Patil, Principal, JNMC, Belgaum and Chairman of the JNMC Institutional Ethics committee of Human Subjects Research.

l) Statement of Consent:

I volunteer and consent to participate in this study, I have read the consent or it has been read to me. The study has been fully explained to me and I may ask question at any time.

_____ _____

Signature Date

Name of Subject: _____

Sign of participants / guardian: Date:_____

Sign of researcher : _____ Date:_____

Sign of witness : _____ Date:_____

Name of witness : _____

ANNEXURE – II

QUESTIONNARE(PROFORMA) USED FOR COLLECTING THE DATA

"OXIDATIVE STRESS,ANTIOXIDANT STATUS AND CALCIUM PHOSPHOROUS LEVELS IN RHEUMATOID ARTHRITIS"-A CASE CONTROL STUDY

Name :

Sex:

Age :

Place:

IP/OP No:

Occupation:

DOA :

DOD:

Address:

PRESENTING COMPLAINTS:

- Onset
- Fever
- Nature
- Presence of swelling
- Joints involved

PAST HISTORY:

- H/o Diabetes mellitus, hypertension
- H/o any joint pain

PERSONAL HISTORY:

- Smoking, alcohol consumption
- Bowel and bladder habits
- Drug intake
- Sleep

FAMILY HISTORY:

- H/o diabetes mellitus, hypertension, bleeding disorders

GENERAL PHYSICAL EXAMINATION:

- Pallor
- Facial oedema
- Blood pressure
- Pedal oedema
- Lymphadenopathy
- Temperature
- Cyanosis
- Pulse rate
- Clubbing
- Respiratory rate

SYSTEMIC EXAMINATION:

- Examination of joints

  Inspection

  Palpation

  Tests to determine the mobility/integrityof joints
- Respiratory system
- Cardiovascular system
- Central nervous system
- Per Abdomen

INVESTIGATIONS:

1. Blood/ Serum

    a. Routine

- Hb
- TC
- DC
- ESR
- Bleeding time
- Clotting time
- Prothrombin time
- HbsAg, HIV
- RF factor titres
- ASLO titres

    b. Biochemical

- Blood urea
- Serum creatinine, uric acid
- Blood sugar – FBS/ PPBS

2. Urine
    - Albumin
    - Sugar
    - Microscopy
3. X-ray

    Chest

    Concerned joints

DIAGNOSIS:

TREATMENT:

ANNEXURE-III: MASTER CHART- CONTROLS

Sl. No.	Names of controls	Age in (Years)	Sex	MDA nmol/ml	SOD Iu/g Hb	G.R. IU/g Hb	Vit A «g/dl	Vit E mg/dl	Vit C mg/dl	Calcium mg/dl	Phosphorous mg/dl
1	Neela Patil	46	F	7.40	854.30	7.90	36.40	0.87	0.76	9.40	2.40
2	Nagamma	50	F	7.36	984.60	10.10	64.20	1.15	0.98	9.60	3.00
3	Sandesh Nagraj	46	M	6.08	823.05	8.90	40.40	0.92	0.85	10.20	2.90
4	Vittal Hegade	62	M	5.38	757.70	7.30	36.30	0.82	0.71	11.10	2.80
5	Ramesh Hegde	60	M	5.06	925.92	8.50	40.77	0.77	0.73	9.90	3.20
6	Rashmi Kamath	45	F	6.12	909.09	9.00	40.20	0.93	0.88	9.70	3.60
7	Shivkumar	60	M	6.79	984.80	9.80	36.00	0.82	0.87	10.90	3.80
8	Mahantshetti	60	F	6.15	952.38	10.40	40.20	0.97	0.88	9.90	2.90
9	Savita Latti	48	F	6.21	1041.66	8.20	38.60	0.84	0.93	9.60	3.80
10	Ramnath Golmal	70	M	5.38	980.39	7.50	36.40	1.01	0.88	10.40	2.50
11	Sri. Lakshmi	46	F	5.89	833.33	7.60	41.24	1.13	0.82	11.20	4.00
12	Gowramma	49	F	6.79	1075.26	8.70	40.30	0.82	0.97	9.80	3.60
13	Ningamma	60	F	7.75	787.08	8.50	42.00	0.93	0.82	9.20	2.80
14	Yallawa	52	F	7.53	1136.36	9.60	42.40	1.02	0.85	11.00	2.40
15	Kamakshi	48	F	7.01	757.70	9.78	36.00	0.77	0.78	10.40	3.60
16	Krishnaveni	52	F	6.15	1000.00	8.70	36.20	0.84	0.84	10.60	3.80
17	Manjula	45	F	5.06	959.08	8.19	34.20	0.91	1.10	9.10	2.60
18	Rajendra	62	M	4.83	956.90	8.54	28.30	0.87	0.85	9.70	2.40
19	Balu Sumane	60	F	6.21	1176.40	7.90	32.40	0.91	0.70	10.80	2.30
20	Jnaneth	58	M	5.41	826.40	8.84	20.60	1.13	0.96	11.00	2.30

Sl. No.	Names of controls	Age in (Years)	Sex	MDA nmol/ml	SOD Iu/g Hb	G.R. IU/g Hb	Vit A «g/dl	Vit E mg/dl	Vit C mg/dl	Calcium mg/dl	Phosphorous mg/dl
21	Maruti	48	M	5.60	1041.60	11.12	26.00	1.00	1.40	9.90	1.90
22	Krishnamma	62	F	6.98	980.39	9.78	34.50	0.98	0.88	9.80	2.20
23	Channabasappa	70	M	7.30	1012.01	7.50	36.40	0.96	0.85	10.80	2.50
24	Roopa	54	F	6.98	888.96	7.19	34.20	0.81	0.95	11.10	3.40
25	Chandrashekar	60	M	5.41	1041.60	8.19	31.30	1.13	0.82	10.90	2.60
26	Devendra	55	M	6.12	925.92	8.84	38.60	0.82	0.83	9.40	2.80
27	Maheshrappa	54	M	7.46	980.39	7.40	28.00	0.97	0.86	9.80	3.10
28	Iftikar	60	M	5.06	956.93	10.37	28.40	0.95	0.88	9.90	2.50
29	Gurupadappa	52	M	7.01	1000.00	9.79	29.00	0.88	0.88	10.50	2.40
30	Rajendra	59	M	7.46	925.93	8.19	36.80	0.98	0.85	11.20	3.00
31	Gajanan	49	M	6.15	757.57	8.76	34.40	0.77	0.98	9.10	2.40
32	Bimappa	58	M	5.38	925.38	9.79	26.80	0.88	0.97	10.20	2.60
33	Kallappa	62	F	6.12	984.00	8.62	36.20	0.79	1.03	10.00	2.10
34	Rangavva	60	F	5.60	956.30	10.88	29.50	0.95	0.91	9.90	3.20
35	Paramesh	49	M	6.98	980.39	10.52	43.50	1.00	0.72	10.40	3.60
36	Suresh	50	M	7.75	925.00	9.10	39.20	0.79	0.84	11.20	4.00
37	Mahima	60	F	5.89	1111.10	10.04	28.00	0.88	0.92	10.80	2.60
38	Rajeshwari	51	F	4.60	980.00	9.60	40.00	1.01	1.00	10.90	2.60
39	Hanamatappa	52	M	5.60	1011.20	8.34	35.70	0.96	0.70	9.60	3.00
40	Rajesh	46	M	3.85	826.00	8.52	35.70	0.88	0.88	9.50	2.90

MASTER CHART- CASES

Sl. No.	Names of cases	Age in (Years)	Sex	MDA nmol/ml	SOD Iu/g Hb	G.R. IU/g Hb	Vit A «g/dl	Vit E mg/dl	Vit C mg/dl	Calcium mg/dl	Phosphorous mg/dl
1	Raj Gopal	60	M	10.83	449.20	1.89	14.40	0.77	0.39	6.50	4.40
2	Anand Rao	50	M	11.22	389.60	2.60	12.60	0.51	0.43	7.60	4.60
3	Kashinath	48	M	10.98	485.50	2.30	10.40	0.43	0.50	6.40	5.10
4	Sumitra	41	F	11.41	502.70	3.10	12.60	0.52	0.39	7.80	5.20
5	Mahalingappa	51	M	11.92	239.80	3.80	14.40	0.49	0.58	6.60	5.40
6	Noor Jahan	50	F	11.40	423.10	2.50	15.80	0.36	0.63	8.80	4.30
7	Mohammad	48	M	12.10	416.50	2.71	12.60	0.37	0.47	8.40	4.60
8	Raghu	42	M	12.25	263.10	3.75	14.40	0.48	0.58	6.50	4.40
9	Radha	50	F	11.00	285.70	3.56	16.20	0.51	0.61	7.70	4.00
10	Ravi	52	M	11.38	472.90	3.67	10.40	0.75	0.38	6.60	5.00
11	Kamalamma	50	F	11.45	416.00	3.37	16.20	0.63	0.43	7.60	5.40
12	Rana	50	F	11.67	555.80	4.94	10.40	0.82	0.50	8.00	5.20
13	Appasaheb	40	M	12.82	285.70	3.75	13.60	0.74	0.67	6.90	5.30
14	Kasturi	50	F	11.35	476.10	3.40	12.40	0.55	0.63	7.50	4.80
15	Basavva	60	F	11.50	401.30	3.67	13.20	0.66	0.71	8.40	4.40
16	Shivaji	58	M	11.53	285.10	3.37	12.00	0.54	0.62	9.60	3.90
17	Jnanesh	60	M	12.20	277.70	2.54	15.60	0.32	0.55	8.80	2.50
18	Mohan	60	M	12.24	333.30	3.40	14.40	0.44	0.38	6.60	3.60
19	Mahalakshmi	50	F	11.50	598.30	3.88	10.60	0.57	0.39	7.40	2.80
20	Rajamma	52	F	10.90	614.40	4.13	12.40	0.36	0.53	6.60	2.40

Sl. No.	Names of cases	Age in (Years)	Sex	MDA nmol/ml	SOD Iu/g Hb	G.R. IU/g Hb	Vit A «g/dl	Vit E mg/dl	Vit C mg/dl	Calcium mg/dl	Phosphorous mg/dl
21	Lakshmesh	46	M	8.90	483.00	2.10	14.00	0.68	0.49	6.60	4.20
22	Siddaramappa	63	M	12.46	503.00	2.43	12.30	0.32	0.51	6.50	4.10
23	Akram	68	M	12.20	478.00	3.10	12.20	0.92	0.41	7.60	5.10
24	Ganesh	50	M	11.82	554.20	2.00	8.80	0.81	0.36	7.40	4.60
25	Meenakshi	49	F	13.17	611.50	1.98	3.80	0.62	0.44	5.90	3.90
26	Gowramma	66	F	11.18	478.30	2.50	10.60	0.45	0.39	8.40	4.50
27	Rakesh	60	M	11.92	572.10	3.26	9.40	0.51	0.43	8.50	3.80
28	Naffeeza	58	F	11.53	534.80	2.80	10.60	0.42	0.50	6.20	4.40
29	Mahesh	52	M	10.43	285.70	4.99	13.20	0.49	0.50	6.30	4.20
30	Roopashri	48	F	9.90	416.60	3.27	12.30	0.50	0.47	7.40	5.00
31	Roopa Bellad	60	F	11.00	357.50	2.18	14.50	0.47	0.44	6.80	4.40
32	Pragna Rao	52	F	10.98	265.55	2.74	3.50	0.35	0.51	6.60	6.00
33	Krishna	49	M	11.53	333.30	3.20	6.20	0.56	0.54	6.30	3.20
34	Uma	48	F	12.46	454.50	1.97	11.10	0.38	0.43	7.20	4.40
35	Rajesh	53	M	11.82	526.30	2.10	10.20	0.43	0.45	9.40	4.00
36	Mahalaxmi	60	F	11.38	504.30	2.00	9.40	0.60	0.41	7.60	3.60
37	Krishnaveni	45	F	11.57	488.60	1.95	10.20	0.58	0.37	8.20	4.10
38	Rajesh	60	M	11.00	592.50	2.45	11.20	0.34	0.45	6.60	3.80
39	Lakshmamma	52	F	10.98	605.40	2.50	10.40	0.62	0.50	6.50	3.20
40	Ningamma	46	F	11.53	526.31	2.70	5.60	0.54	0.48	7.10	3.30