“OXIDATIVE STRESS AND ANTIOXIDANT STATUS IN PRIMARY PULMONARY TUBERCULOSIS - A CASE CONTROL STUDY”

REG. NO. BC0108002

Dissertation

Submitted to the
KLE University, Belgaum, Karnataka

In Partial Fulfillment
of the requirements for the degree of

M. D.
in
BIOCHEMISTRY

DEPARTMENT OF BIOCHEMISTRY,
JAWAHARLAL NEHRU MEDICAL COLLEGE,
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ENDORSEMENT

This is to certify that the dissertation entitled **"OXIDATIVE STRESS AND ANTIOXIDANT STATUS IN PRIMARY PULMONARY TUBERCULOSIS - A CASE CONTROL STUDY"** is a bonafide research work done by **CANDIDATE, REG. NO. BC0108002** from the Department of Biochemistry, Jawaharlal Nehru Medical College, Nehru Nagar, Belgaum-590010.

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LIST OF ABBREVIATIONS USED

ΔOD	-	Change in optical density per minute
$^{\bullet}CCl_3$	-	Trichloromethyl radical
^{0}C	-	Degree centigrade
$A^{\bullet}$	-	Armstrong
AFB	-	Acid fast bacilli
BC	-	Before Christ
CCl_4	-	Carbon tetrachloride
CH_2	-	Methylene
COPD	-	Chronic obstructive pulmonary disease
Cu / Zn SOD	-	Copper / Zinc containing superoxide dismutase
Cu^{+}	-	Cuprous ion
Cu^{+2}	-	Cupric ion
D/W	-	Distilled water
DNA	-	Deoxyribonucleic acid
DOTS	-	Directly Observed Treatment – Short Course
DTH	-	Delayed type of hypersensitivity
EDTA	-	Ethylenediamine tetraacetic acid
ESR	-	Erythrocyte sedimentation rate
FAD	-	Flavin adenine dinucleotide
Fe^{+2}	-	Ferrous ion
Fe^{+3}	-	Ferric ion
$FeCl_3$	-	Ferric chloride
g%	-	Gram percent
gm	-	Gram

GSH	-	Reduced glutathione
GSH-Px	-	Glutathione peroxidase
GSSG	-	Oxidized glutathione
H^+	-	Hydrogen ion proton
H_2O	-	Water
H_2O_2	-	Hydrogen peroxide
H_2SO_4	-	Sulphuric acid
HCl	-	Hydrochloric acid
HiCN	-	Cyanmethemoglobin
HIV	-	Human immunodeficiency virus
HOCl	-	Hypochlorous acid
HPLC	-	High pressure liquid chromatography
INFγ	-	Interferon γ
KD	-	Kilo Daltons
L	-	Litre
MDA	-	Malondialdehyde
MDR-TB	-	Multidrug resistant tuberculosis
mg	-	Milli gram
ml	-	Milli litre
Mn SOD	-	Manganese containing superoxide dismutase
MPO	-	Myeloperoxidase
Na_2CO_3	-	Sodium Carbonate
$Na_2H_2PO_4$	-	Sodium dihydrogen phosphate
NAD	-	Nicotinamide adenine dinucleotide
NADP	-	Nicotinamide adenine dinucleotide phosphate

$NaHCO_3$	-	Sodium bicarbonate
nM	-	Nanometer
nmol	-	Nano moles
$NO^{\bullet}$	-	Nitric oxide
O_2	-	Oxygen
$O_2^{\bullet -}$	-	Super oxide anion radical
ODc	-	Optical density of control
ODs	-	Optical density of standard
$OH^{\bullet -}$	-	Hydroxyl radical
$ONOO^-$	-	Peroxynitrite
PCR	-	Polymerase chain reaction
PPD	-	Purified protein derivative
PUFA	-	Poly unsaturated fatty acids
$R^{\bullet}$	-	Free radical
RBC	-	Red blood cell
$ROO^{\bullet}$	-	Lipid peroxyl radical
$ROO^{\bullet}$	-	Peroxyl radical
ROOH	-	Lipid hydroperoxides
ROS	-	Reactive oxygen species
rpm	-	Revolutions per minute
$RS^{\bullet}$	-	Thiyl radicals
RSH	-	Thiol compounds
Se	-	Selenium
SOD	-	Superoxide dismutase
TBA	-	Thiobarbituric acid

t-BHP	-	t butyl hydrogen phosphate
UV	-	Ultravoilet
Vc	-	Volume of cuvette
W/W	-	Weight / Weight
WHO	-	World Health Organization
XDR-TB	-	Extensively drug resistant tuberculosis
µL	-	Micro Litre
ω3	-	Omega 3
ω6	-	Omega 6

ABSTRACT

Background and Objectives

The objectives of present study were to find out oxidative stress in genesis of pulmonary tuberculosis, assess role of antioxidant defense in pathophysiology of disease process and to correlate oxidative stress and antioxidant defense mechanism in pulmonary tuberculosis.

Methods

Present case control study was conducted at Department of Biochemistry, KLES Dr. Prabhakar Kore Hospital and Medical Research Centre, Belgaum between February 2009 to January 2010 on 50 cases of primary pulmonary tuberculosis and 50 healthy controls in the age of group of 25 to 50 years of both sexes. Ten ml of blood was collected from the patients immediately after admission. It was used for estimation of malondialdehyde, oxidative stress parameter and antioxidants like SOD, GSH Px, catalase, vitamin C and E.

Results

Mean age of controls was 33.7±5.61 years and in cases it was 34.8±6.39 years. Mean BMI in control group was 24.3±3.06 kg/m^2 and 21.1±2.53 Kg/m^2 in patients with pulmonary tuberculosis. Mean MDA levels in control were 5.2±1.42 nmol/ml and in cases it was 14.2±2.21 nmol/ml. Enzymatic antioxidants SOD, GSH Px and catalase levels in controls was 882.1±116.81 IU/gmHb, 19.2±1.67 IU/gmHb and 5.9±1.40 IU/gmHb and in cases it was 504.1±116.24 IU/gmHb, 8.8±2.22 IU/gmHb and 3.7±1.02 IU/gmHb respectively. Non enzymatic antioxidant vitamin E and C levels in the control group were

0.92±0.16 mg/dL and 1.01±0.18 mg/dL, and in cases it was 0.31±0.16 mg/dL and 0.43±0.16 mg/dL respectively. There was a significant increase in the MDA levels and decrease in enzymatic antioxidants in cases compared to controls ($p<0.001$). Non enzymatic antioxidants in the cases were significantly decreased when compared to controls ($p<0.001$).

Conclusion and interpretation

The findings of this study suggest a significant association between oxidative stress and antioxidant defense mechanism among patients tuberculosis.

Keywords

Catalase; Glutathione peroxidase; Malondialdehyde; Superoxide dismutase; Oxidative stress; Tuberculosis; Vitamin C; Vitamin E;

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INTRODUCTION

Tuberculosis is a chronic granulomatous infectious disease caused by mycobacterium tuberculosis. It affects almost all the organs in the body, lungs being the primary. It spreads primarily through air borne transportation by aerosolized droplet inhalation during coughing, sneezing, talking especially in poorly ventilated areas.[1]

Tuberculosis is a major global health problem. It remains one of the top three killer among the infectious diseases. It is a major cause of morbidity and mortality in densely populated countries like India, China, Africa and Latin America.[2] It is an ancient disease but has stayed in modern times.[3] It is a curse to the mankind since time immemorial and is a ubiquitous disease. The earliest known Babylonian scriptures described tuberculosis. The first evidence of the infection in humans was found in the neolithic bone remains that show evidence of spinal tuberculosis. It was called consumption in Latin and Yakshma in India, at about 2000 BC. Schoenlein named it as tuberculosis in the year 1839. Since then man has continued to live with tuberculosis infection in a state of unsteady equilibrium which can be readily disturbed in favor of the disease.[4]

There is resurgence in the incidence of tuberculosis in developing countries the recent years due to co-infection in human immunodeficiency virus (HIV). Emergence of multidrug resistant tuberculosis, inadequate treatment, poverty, malnutrition, over-crowding armed conflict and increasing number of displaced persons.[1]

World Health Organization (WHO) has declared tuberculosis as a global emergency in the year 1993.[5] In the world two billion patients are diagnosed as tuberculosis every year, of this eight to nine million succumb to death. According to Global Tuberculosis Control Program 2009 the incidence of tuberculosis was 139 per 100000 population in 2007.[6] In Belgaum district 5000 cases were diagnosed as tuberculosis positive out of 45 lakh population (According to survey of District Tuberculosis Health Centre, Belgaum).[7] Tuberculosis, a major barrier to economic development of the country costing India about Rs. 12,000 crore a year.[6]

Recent literatures implicate free radicals in physiologic and pathophysiologic process and in wide spectrum of diseases. Mycobacteria are intracellular pathogens that grow and replicate in the host macrophages. In an attempt to kill mycobacteria, host cells namely macrophages, neutrophils and monocytes generate huge amounts of reactive oxygen species, which contribute to inflammatory injury to host tissues.[3]

In pulmonary tuberculosis increased formation of reactive oxygen species (ROS) can produce injury to cell membrane by initiation of lipid peroxidation, inactivation of membrane enzyme receptors, protein cross linking and fragmentation.[8] The resulting tissue damage can be assessed by measuring malondialdehyde (MDA) a product of lipid peroxidation.[8,9]

To defend themselves against deleterious effects of ROS, cells have endogenous enzymatic antioxidants like superoxide dismutase (SOD), catalase,[10]

glutathione peroxidase (GSH-Px),[11] and exogenous antioxidants like vitamin C and vitamin E.[8,12]

OBJECTIVES

Objectives of the present study were;

1. To find out the oxidative stress in genesis of pulmonary tuberculosis.
2. To assess the role of antioxidant defense in the pathophysiology of the disease process.
3. To correlate the oxidative stress and antioxidant defense mechanism in pulmonary tuberculosis.

REVIEW OF LITERATURE

HISTORY[4]

Consumption, Phthisis, Scrofula, Pott's disease, and the White Plague are all terms used to refer to tuberculosis throughout history. Over time, the various cultures of the world gave the illness different names: Yaksma (India), Phthisis (Greek), Consumption (Latin) and Chaky Oncay (Incan), each of which make reference to the "drying" or "consuming" affect of the illness, cachexia. Its high mortality rate among middle-aged adults and the surge of romanticism, which stressed feeling over reason, caused many to refer to the disease as the "romantic disease."

Tuberculosis in ancient times[4]

The first evidence of the infection in humans was found in the neolithic bone remains that show evidence of spinal tuberculosis. Signs of the disease have also been found in Egyptian mummies dated between 3000 and 2400 BC. The term phthisis first appeared in Greek literature around 460 BC. Hippocrates identified the illness as the most common cause of illness in his time, stated that it typically affected individuals between 18 and 35 and was nearly always fatal, Galen, the most eminent Greek physician after Hippocrates, defined phthisis as the "ulceration of the lungs, thorax or throat, accompanied by a cough, fever, and consumption of the body by pus." The first references to tuberculosis in Asian civilization is found in the Vedas. The oldest of them (Rigveda, 1500 BC) calls the disease yaksma. The Sushruta Samhita, written around 600 BC, recommends

that the disease be treated with breast milk, various meats, alcohol and rest. The first mention of tuberculosis in Chinese literature appears in a medical text written by Emperor Shennong of China (2700 BC) in which he describes xulao bing (weak consumptive disease).

Renaissance and after (1400-1800)[4]

With the spread of Christianity, the touch of the sovereign of England or France, could cure diseases due to the divine right of sovereigns. King Henry IV of France usually performed the rite once a week, after taking communion.

Seventeenth and eighteenth centuries[4]

In the book ‘Systematik de speziellen Pathologie und Therapie’, J. L. Schönlein, Professor of Medicine in Zurich, proposed that the word "tuberculosis" be used to describe the affliction of tubercles.

Nineteenth century[4]

It was during this century that tuberculosis was dubbed the White Plague, mal de vivir, and mal du siècle. It was seen as a "romantic disease." Suffering from tuberculosis was thought to bestow upon the sufferer heightened sensitivity.

Scientific advances[4]

One of the most important physicians dedicated to the study of pthisisology was René Laennec, who died from the disease at the age of 45, after contracting tuberculosis while studying contagious patients and infected bodies. Laennec invented the stethoscope which he used to corroborate his auscultatory

findings. In 1869, Jean Antoine Villemin demonstrated that the disease was indeed contagious, conducting an experiment in which tuberculous matter from human cadavers was injected into laboratory rabbits, who then became infected.

On 24 March 1882, Robert Koch revealed the disease was caused by an infectious agent. In 1895, Wilhelm Roentgen discovered the X-ray, which allowed physicians to diagnose and track the progression of the disease, in 1890 Koch developed tuberculin, a purified protein derivative (PPD) of the bacteria. It proved to be an ineffective means of immunization but in 1908, Charles Mantoux found it was an effective intradermic test for diagnosing tuberculosis.

Twentieth century[4]

In 1902, the International Conference on Tuberculosis convened in Berlin. National campaigns spread across Europe and the United States to tamp down on the continued prevalence of tuberculosis.

After the establishment in the 1880s that the disease was contagious, tuberculosis was made a notifiable disease in Britain; there were campaigns to stop spitting in public places, and the infected poor were pressured to enter sanatoria that resembled prisons; the sanatoria for the middle and upper classes offered excellent care and constant medical attention.

Vaccines[4]

The first genuine success in immunizing against tuberculosis was developed from attenuated bovine-strain tuberculosis by Albert Calmette and Camille Guérin in 1906. It was called Bacille Calmette-Guérin (BCG). The BCG

vaccine was first used on humans in 1921 in France, but it was not until after World War II that BCG received widespread acceptance in the United States, Great Britain, and Germany.

Treatments[4]

As the century progressed, some surgical interventions, including the pneumothorax or plombage technique - collapsing an infected lung to "rest" were used to treat tuberculosis. In 1696, Giorgio Baglivi reported a general improvement in tuberculosis sufferers after they received sword wounds to the chest. F. H. Ramage induced the first successful therapeutic pneumothorax in 1834, and reported subsequently the patient was cured. The search for a medicinal cure, however, continued in earnest.

In 1944 Albert Schatz, Elizabeth Bugie, and Selman Waksman isolated Streptomyces griseus or streptomycin, the first antibiotic and first antibacterial agent effective against M. tuberculosis. Isoniazid was the first oral mycobactericidal drug. The advent of Rifampin in the 1970s hastened recovery times, and significantly reduced the number of tuberculosis cases until the 1980s.

Tuberculosis resurgence[4]

Hopes that the disease could be completely eliminated were dashed in the 1980s with the rise of drug-resistant strains. Due to the elimination of public health facilities in New York and the emergence of HIV, there was a resurgence of tuberculosis in the late 1980s. The number of patients failing to complete their course of drugs is high.

In response to the resurgence of tuberculosis, the WHO issued a declaration of a global health emergency in 1993. Every year, nearly half a million new cases of multidrug-resistant tuberculosis (MDR-TB) are estimated to occur worldwide.

EPIDEMIOLOGY

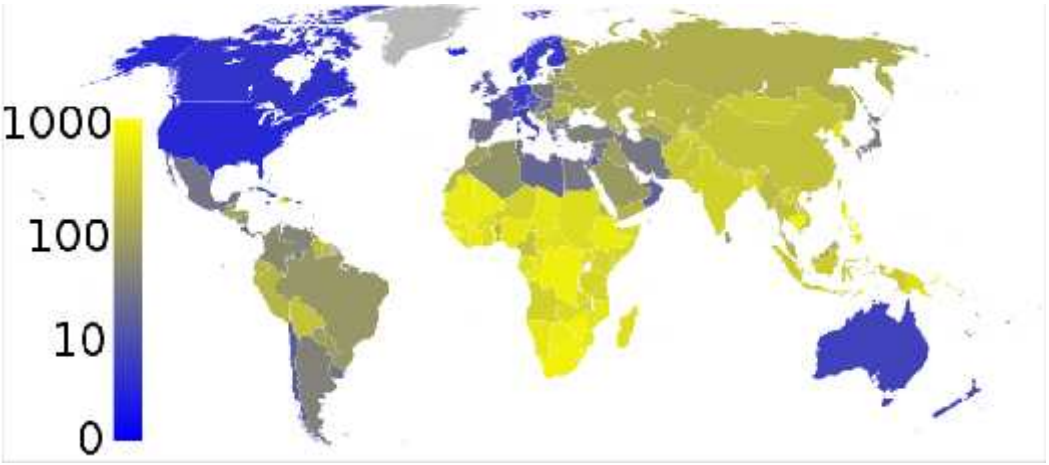


Figure 1: In 2007, the prevalence of TB per 100,000 people was highest in sub-Saharan Africa, and was also relatively high in Asia.[13]

Incidence[6]

- Globally, an estimated 9.27 million new cases (15% amongst HIV +ve) occurred in 2007; mostly in Asia (55%) and Africa (31%).
- The maximum number of cases occurred in India (2.0 million), China (1.3 million), Indonesia (0.53 million), Nigeria (0.46 million) and South Africa (0.46 million).
- There were about 1.75 million TB deaths; over 25% occurred in HIV-positive persons.

- The incidence decreased marginally from 142 per 100 000 in 2004 to 139 per 100,000 population in 2007; prevalence and mortality rates also falling globally in all six WHO regions.

- Tuberculosis was the most common cause of death among people living with HIV/AIDS in 2007. HIV-positive people are about 20 to 37 times more likely to develop tuberculosis.

- There were an estimated 0.5 million cases of MDR -TB in 2007 with maximum number from India (131,000). Extensively drug resistant tuberculosis (XDR -TB) has been reported from 55 countries.

- In 2007, 5.5 million tuberculosis cases (2.6 million smear-positive) were notified by Directly Observed Treatment – Short Course (DOTS) Programs (99% of total case notifications). The case detection rate of new smear-positive cases under DOTS was 63%; seven percent short of the target of 70% or more for 2005.

- Globally, the rate of treatment success for new smear-positive cases treated in DOTS programs in 2006 reached the target of 85%.

- In 2009, a total of US$ 3.0 billion was available for tuberculosis control in 94 countries which account for 93% of the world's tuberculosis cases as against the requirement of US$ 4.2 billion. Most of the extra funding required was for MDR -TB diagnosis and treatment in India and China, and for DOTS and collaborative tuberculosis and/or HIV activities in Africa.

PATHOPHYSIOLOGY[14]

Tuberculosis is a constitutional disease caused by infection with Mycobacterium tuberculosis, characterized by the production of tubercles in the internal organs, especially in the lungs, where it constitutes the most common variety of pulmonary phthisis.

Etiology[14]

Tuberculosis is one of the oldest known diseases known to affect humans caused by bacteria mycobacterium tuberculosis (M. tuberculosis)**,** characterized by the production of tubercles in the internal organs, especially in the lungs, where it constitutes the most common variety of pulmonary phthisis (consumption). If properly treated it is curable in virtually all the cases, if untreated the disease may be fatal within five years in more than half the cases. Pulmonary tuberculosis is the one involving the lung with progressive wasting of the body.

Mycobacterium belongs to the family Mycobacteriaceae and order Actinomycetales. They are rod shaped, non-spore forming thin aerobic bacteria measuring 0.5 µm by 3 µm. They do not stain by gram method of stain, faintly gram positive, but once stained cannot be decolorized by acid alcohol hence classified as acid fast bacilli-acid fastness is due to the organisms' high content of mycolic acids, long chain cross linked fatty acids and other cell wall lipids.

The interaction of M. tuberculosis with human host begins when droplet nuclei (less than 10 µm in diameter) which are aerosolized by coughing, sneezing

or speaking from an infectious patient are inhaled. While the majority of the inhaled bacilli are trapped in the upper airways and expelled by ciliated mucosal cells, a fraction reaches the alveoli.

Risk Factors for active tuberculosis among persons who have been infected with tubercle bacilli[14]

Risk Factor	Relative risk / Odds
Recent Infection (less than one year)	12.9
Fibrotic lesion spontaneously healed	2 – 30
Comorbidity	
HIV Infection	100
Silicosis	30
Chronic renal failure / Hemodialysis	10 – 25
Diabetes	2 – 4
Intravenous drug use	2 – 4
Immunosuppressant treatment	10
Gastrectomy	2 – 5
Jejunoleal bypass	30 – 60
Post transplantation period	20 – 70
Malnutrition and underweight	2

The risk of developing the disease after being infected depends on factors like individual's innate susceptibility to the disease and level of cell mediated

immunity. Clinical illness directly following infection is classified as primary tuberculosis. Among the infected individuals the incidence is highest during late adolescence and early adulthood peaks between 25 to 34 years of age. In children and in persons with impaired immunity (for example, malnutrition or HIV infection) the disease may progress rapidly to clinical illness.

In the initial stage of host bacterium interaction, either the host macrophages contain bacillary multiplication by producing proteolytic enzymes and cytokines or the bacilli begin to multiply. If the bacilli multiply, their growth quickly kills the macrophages which lyse. The balance between the bactericidal activity of the macrophage and the number and virulence of the bacilli determines the events following phagocytosis.

After about two to four weeks after infection, two additional host responses to M. tuberculosis develop a tissue damaging response and a macrophage activating response.

The tissue damaging response is result of delayed type hypersensitivity (DTH) reaction to various bacillary antigens; it destroys non activated macrophages. The macrophage activating response is a well mediated phenomenon resulting in activation of macrophages that ingest and kill the bacilli.

With the development of specific immunity the accumulation of large number of activated macrophages at the primary sites, granulomatous lesions are formed. These lesions are comprised of lymphocytes, epitheloid cells and giant cells. Initially the newly developed tissue damaging response is the only event

capable of limiting mycobacterial activity. This not only destroys the macrophages but also produces early solid necrosis in the centre of the tubercle of these lesions, some may heal by fibrous and calcification while others may undergo further evolution cell mediated immunity is critical at this early stage.

In the majority of infected individuals, local macrophages are activated when bacillary antigens processed by macrophages stimulate T lymphocytes to release a variety of lymphokines.

In a minority of cases the macrophage activating response is weak and mycobacterial growth can be inhibited only by intensified DTH reaction which leads to tissue destruction. Bronchial walls and blood vessels may be invaded and destroyed leading to spread into the airways and discrimination through blood vessels, hematogenous dissemination may results leading to military tuberculosis or tuberculous meningitis.

Classification of tuberculosis[14]

1. Pulmonary tuberculosis
    a. Primary disease
    b. Post primary (Secondary)
2. Extrapulmonary tuberculosis
3. HIV associated tuberculosis

Pulmonary tuberculosis

Primary disease

Results from an initial infection with tubercle bacilli. Common in persons with impaired immunity (for example, malnutrition and HIV infection) and may progress rapidly to clinical illness. The lesion forming after the infection is usually peripheral accompanied by hilar or paratracheal lymphadenopathy called Ghon's lesion.

Post Primary (secondary)

Results from endogenous reactivation of latent infection, localized to apical and posterior segments of lung. May lead to cavitation or become fibrotic and undergo calcification and may undergo spontaneous remission or proceed along a chronic debilitating course.

Extra-pulmonary tuberculosis

Commonly involve the lymphnodes, pleura, genitourinary tract, bones and joints, meninges, peritoneum, pericardium. It occurs due to hematogenous spread of infection.

Human immunodeficiency virus associated tuberculosis

Tuberculosis is an important opportunistic disease among HIV infected persons, worldwide. A person with skin test documented M. tuberculosis infection who acquires HIV infection has three to fifteen percent annual risk of developing active tuberculosis.

Tuberculosis can appear at any stage of HIV infection and its presentation varies with the stage. When cell mediated immunity is only partially compromised, pulmonary tuberculosis present as a typical pattern of upper lobe infiltrates and cavitation, without significant lymphadenopathy or pleural effusion.

In late stages of HIV infection a primary tuberculosis like pattern with diffuse interstitial or military infiltrates, little or no cavitation and intrathoracic lymphadenopathy is more common.

Overall, sputum smears may be positive less frequently among tuberculosis patients with HIV infection than among those without; thus the diagnosis of tuberculosis may be unusually difficult, especially in view of the variety of HIV related pulmonary conditions mimicking tuberculosis.

Extrapulmonary tuberculosis is common among HIV infected patients. The most common forms are lymphatic disseminated pleural and pericardial.

The diagnosis may be difficult not only because of increased frequency of sputum negativity but because of atypical radiographic findings, a lack of classic granuloma formation and negative results in PPD skin tests.

Symptoms and signs of pulmonary tuberculosis[14]

In the early course of disease symptoms and signs are non specific and insidious, consisting of night sweats and fevers, weight loss, anorexia, general malaise and weakness.

Cough eventually develops initially, non productive but subsequently accompanied by production of purulent sputum. Blood streaking of sputum is frequently documented.

Massive hemoptysis may ensue. Pleuritic chest pain sometimes develops in patients with subpleural parenchymal lesion. It may also be result of muscle strain due to persistent coughing. Extensive disease may produce dyspnoea and acute respiratory distress syndrome.

Physical findings[14]

May have no abnormalities detectable by chest examination while other have detectable rales in the involved areas. Occasionally rhonchi due to partial bronchial obstruction and classic amphoric breath sounds in areas of large cavities are heard.

Systemic features include fever (low grade intermittent) and wasting some cases pallor and finger clubbing develop.

Diagnosis[14]

1. Hematology: Mild anemia and leucocytosis
2. Chest radiography: Shows classic upper lobe disease with infiltrates and cavities, virtually any radiographic pattern from a normal film or a solitary pulmonary nodule to diffuse alveolar infiltrates like in patients with acute respiratory distress syndrome may be seen.
3. Microbiological analysis:

a. Acid fast bacilli (AFB) Microscopy: Finding AFB on microscopic examination of a smear of expectorates sputum. Staining is done with Ziel Neilson basic fuschsin dye.

b. Mycobacterial culture: Definitive diagnosis – Specimens may be inoculated onto egg or agar based medium (Lowenstein – Jenson) and incubate at 37^0 C under five percent CO_2. Growth bacilli show four to eight weeks.

Sputum collected early morning to be submitted for microbiology assays.

4. Tuberculin test (Mantoux test): Widely used for screening. The test is of limited value in diagnosis because of low sensitivity and specificity. False negative reactions are common in immuno-compromised patients.
5. Nucleic acid amplification: Polymerase chain reaction (PCR) and deoxyribo nucleic acid (DNA) Probes Their applicability is limited by low sensitivity and high cost.
6. Cytokine release assay: QuantiFERON tuberculosis Test: This test requires over night incubation of peripheral blood sample with PPD and control antigens followed by measurement of interferon γ (IFN-γ) released by sensitized lymphocytes in an enzyme linked immunosorbant assay (ELISA)
7. High pressure liquid chromatography (HPLC) of mycolic acids: Non specific and less sensitive.

If sputum is difficult to obtain, fiberoptic bronchoscopy with bronchial brushing or transbronchial biopsy may be performed and specimens may be submitted for AFB smear and mycobacterial culture.

FREE RADICALS ($R^{\bullet}$)

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.[15] 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.[16] A free radical is conventionally represented by a superscript dot ($R^{\bullet}$).

A compound becomes a $R^{\bullet}$ by gaining an additional electron, as in the case of reduction of molecular oxygen (O_2) 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,[17]

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

Generation

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

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.

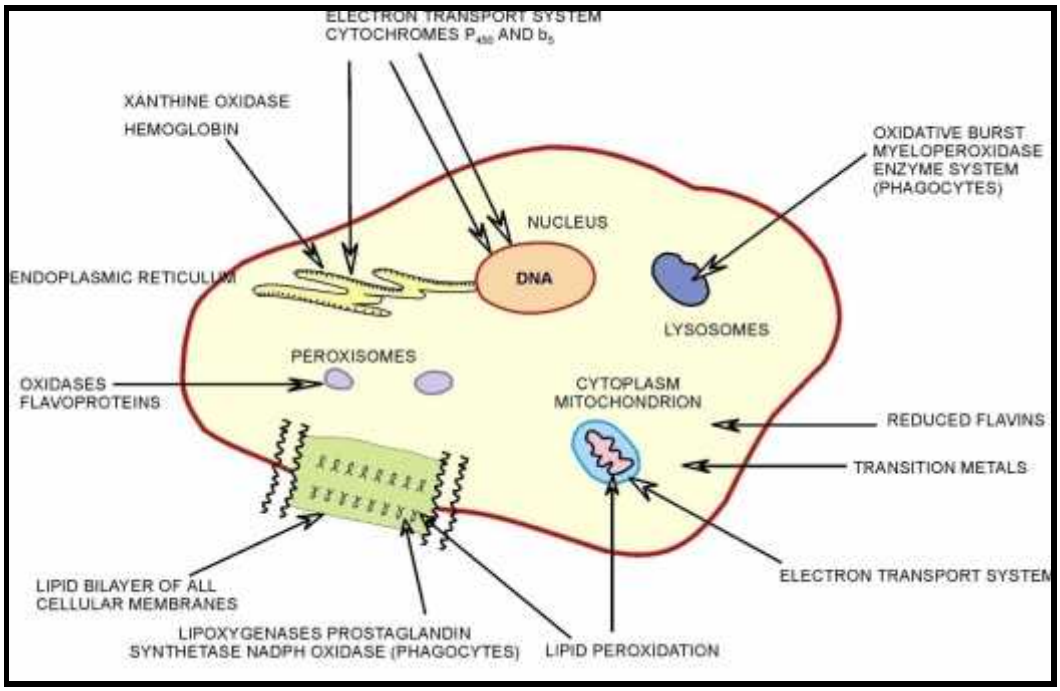


Figure 2. Sub-cellular sources of free radicals

Those secondary to xenobiotics, either because the xenobiotics includes oxidants (e.g. inhaled oxidant gases) or because these xenobiotics induce oxidant generation within cells, for example drugs that injure tissues through oxidant mechanisms.[18]

Endogenous

a. Endogenous Sources of free radicals include those that are generated and act intracellular as well as those that are formed within the cells and are released into the surrounding area[19] (Figure 2). 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.[20] Oxidases and electron transport systems are prime, continuous sources of intracellular reactive O_2 free metals. Electron transfer from transition metals such as iron (Fe) to oxygen can initiate free radical reactions.[19] An important source of $O_2^{\bullet-}$ is the "univalent leak" of $O_2^{\bullet-}$ 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, cyclooxygenases, lipoxygenases, dehydrogenases and peroxidases[19] (Figure 2). A variety of enzyme systems catalyze the univalent reduction of molecular oxygen to $O_2^{\bullet-}$. Such univalent reduction of molecular O_2 also occurs in vivo in non-enzymatic electron-transfer oxidation-reduction reactions. (For example hydroquinone + O_2 $\longrightarrow$ semi Quinone + $O_2^{\bullet-}$ + H^+) and during auto-oxidation reactions including those that involve catechol amines, flavins and reduced ferridoxins.[22]

c. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in the inflammatory cells (neutrophils, eosinophil, monocytes and macrophages)

produces $O_2^{\bullet-}$ by the process of respiratory burst during phagocytosis. The $O_2^{\bullet-}$ is converted to hydrogen peroxide (H_2O_2) and then to hypochlorous acid (HOCI) with the help of SOD and myeloperoxidase (MPO). The $O_2^{\bullet-}$ and HOCl ions are the final effectors of bactericidal action. The gene for MPO is on chromosome 17. The enzyme MPO 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 O_2 uptake by macrophage is used for free radical generation. Along with the activation of macrophages, the consumption of O_2 by the cell is increased drastically; this is called respiratory burst[23] (Figure 3).

d. Hydroxyl radicals are formed in the "Fenton reaction" whenever H_2O_2 comes into contact with ferrous (Fe^{+2}) or cupric ions (Cu^{+2}). An iron catalyzed Haber-weiss type of reaction may also form this radical. The net effect of which is an interaction between H_2O_2 and $O_2^{\bullet-}$ in the presence of traces of transition metal ions to form $OH^{\bullet}$. Finally, the $OH^{\bullet}$ radical is also a product of ionizing radiation.[22, 24]

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

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

(Haber Weiss reaction)

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

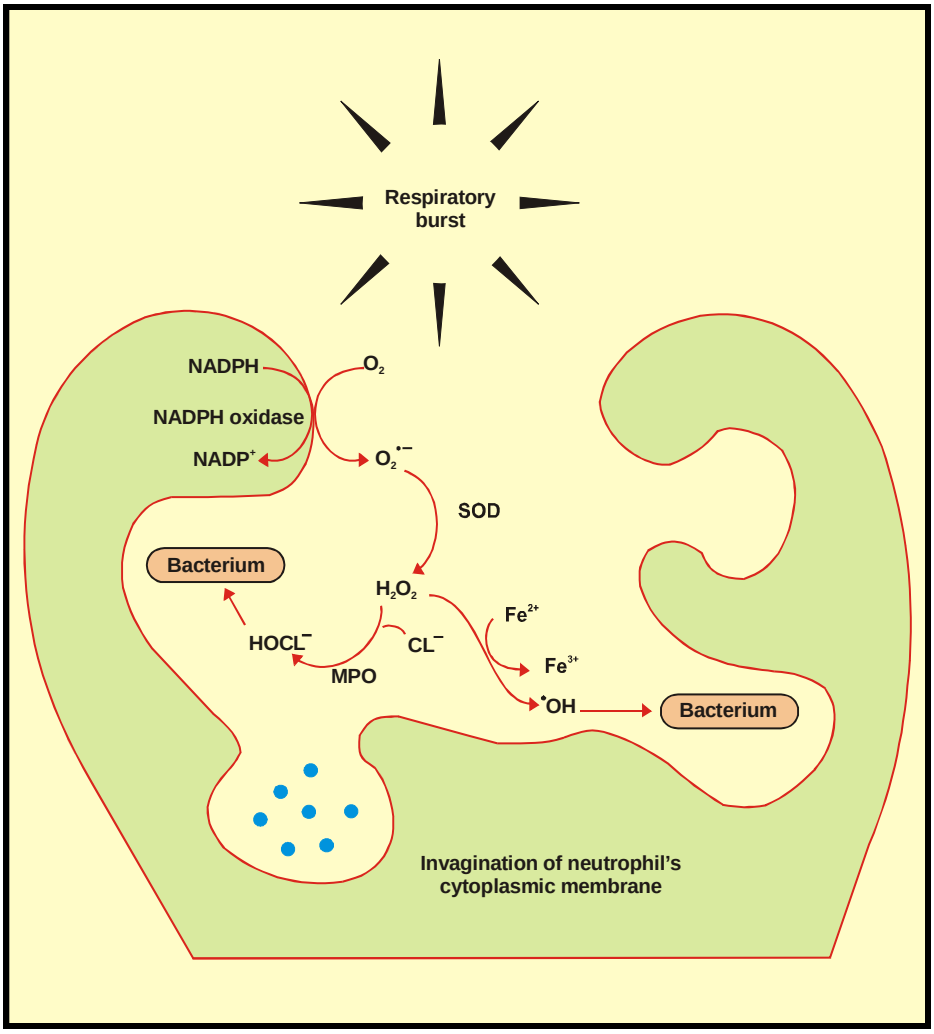


Figure 3. Generation of Oxygen Free Radicals in Respiratory Burst

Exogenous

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.[19]

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

$$H_2O \xrightarrow{\text{Gamma, UV radiation}} H^{\bullet}+OH^{\bullet}$$

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

Sites of generation

Main sites of free radical generation are mitochondria, lysosomes, peroxisomes, nuclei, endoplasmic reticulum, plasma membranes and the cytosol.[19]

Endoplasmic Reticulum, Nuclear Membrane and Electron Transport Systems

Free radicals produced by the endoplasmic reticulum and nuclear membrane can undergo both intra organelle and cytosolic reactions. In case of nuclear membrane generated radicals DNA would be particularly susceptible to free radical damage.[25]

Plasma Membrane

Plasma membrane is a site of action of extracellular 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 amino acids 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.[25]

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.[25]

Peroxisomes

Peroxisomes are potent sources of cellular H_2O_2 because of high concentrations of oxidases.[25]

REACTIVE OXYGEN SPECIES (ROS)

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

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

This ROS is formed, when O_2 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).[26] This species is reduced and forms H_2O_2. The production of $O_2^{\bullet -}$ at the membrane level (NADPH oxidase) is initiated in specialized cells with phagocytic functions (macrophages) and contributes to their bactericidal action (oxidative burst).[27] The flavin cytosolic enzyme xanthine oxidase found in quite all tissues and in milk fat globules generates $O_2^{\bullet -}$ from hypoxanthine and O_2 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 H_2O_2 production is relatively important and leads to a constant cellular concentration between 10^{-9} and 10^{-7} M. In plant and animal cells, SOD 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 SOD and catalase contributes to remove H_2O_2 and thus has a true cellular antioxidant activity. Hydrogen peroxide is also able to diffuse easily through membranes.[28]

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.[27]

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[29] and endothelial cells[30] 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 ($O^{\bullet}$)

This chemical form of O_2 is not a true radical but is reported to be an important ROS in reactions related to ultraviolet exposition [(UV) A 320-400 nm]. Its toxicity is reinforced when appropriate photo excitable compounds (sensitizers) are present with molecular O_2.[31] 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 $O_2^{\bullet-}$, as well as $O^{\bullet}$, and thus accelerates the oxidation of unsaturated lipids generating hydro peroxides. 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 $O^{\bullet}$ formation may account for the chemiluminescence observed during lipid peroxidation.[32]

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 **$RS^{\bullet}$** have strong reactivity in combining with O_2.[33, 34]

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

Furthermore, they are able to oxidize NADH into NAD, ascorbic acid and to generate various free radicals ($OH^{\bullet}$ and $O2^{\bullet-}$). These $RS^{\bullet}$ 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 (that is $^{\bullet}O_2\,CCl_3$).[35]

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 for example;

a) Peroxidation of PUFA in plasma membranes,
b) Oxidative inactivation of sulfhydryl 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.[36]

Proteins

Protein molecules undergo substantial modifications through reactive reactions with free radicals. Proteins containing tryptophan, tyrosine, phenylalanine histidine, methionine and cysteine can undergo free radical mediated amino acid modification. Free radicals 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.[37] Enzymes undergo cross-linking with resulting increase in molecular weight, such enzymes cross-link with their neighbors 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.[38]

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 monosaccharaides undergo oxidation but conditions are appropriate.[37] 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.[39]

Lipids

All of the major classes of biomolecule may be attacked by free radicals but lipids are probably the most susceptible.[40] Cell membranes are rich sources of PUFA. 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.[25] The membrane fluidity is due to the presence of PUFA 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 O_2 and

iii) The presence of transitional metal catalysts.[41]

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.[42]

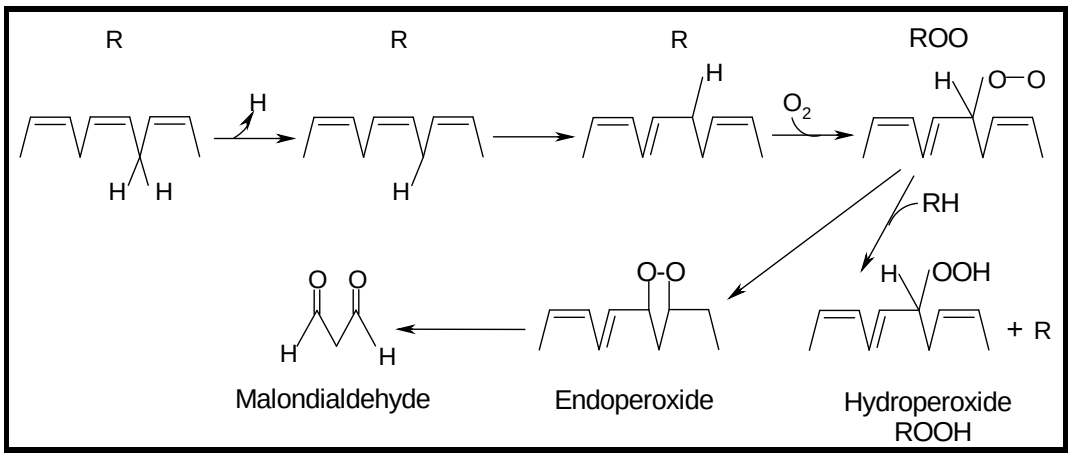


Figure 4. The lipid peroxidation process

The reaction is initiated by an existing free radical, 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.[43]

Peroxidation of PUFA usually involve three operationally defined processes.[25]

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[37] 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 peroxyl radical, R-OO$^{\bullet}$. The presence of the redox active metals such as iron or copper can facilitate the initiation process.[25]

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

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

Propagation Phase

During this phase lipid peroxidation relies on the interaction of molecular O_2 with carbon-centered free radicals to form lipid hydroperoxides.[37] The peroxyl radical abstract a hydrogen atom from another lipid molecule and once the process begins it tends to continue. The peroxyl radical combines with the hydrogen atom that it abstracts to give a lipid hydroperoxides R-OOH. A probable alternative fate of peroxyl radicals is to form cyclic peroxides. With the help of metal catalysts, the decomposition of hydroperoxides results in the formation of alkoxyl or peroxyl radicals. These radicals are capable of further reactions and thus the propagation of lipid peroxidation continues.[25,37]

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

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

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 for example MDA. Any kind of lipid free radical can react with a lipid peroxyl radical ($ROO^{\bullet}$) 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.[44] 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 MDA is diffusible, it will also react with nitrogenous bases of DNA[45] lipid hydroperoxides can directly inhibit enzymes.[44]

ANTIOXIDANT DEFENSE SYSTEMS

Detoxification of reactive oxygen species (ROS) 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 (Figure 5).[46]

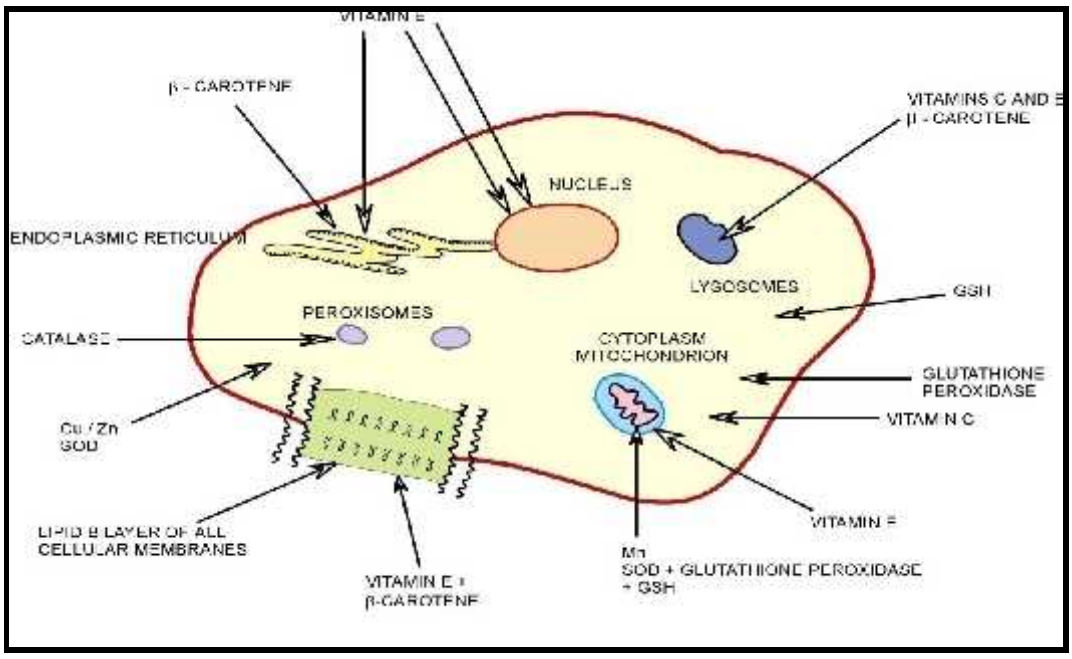


Figure 5. Antioxidant protection within the cell

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 H_2O_2 and also the lipid hydroperoxides, which are produced during lipid peroxidation. Catalase and GSH Pxs are the enzymes, their role is to safely decompose peroxides.[25] 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.[47,48] Ceruloplasmin an important extracellular antioxidant[49] which oxidizes Fe^{2+} to Fe^{3+} that swiftly binds to transferrin and any iron mobilized from serum ferritin.[50]

Interception

The free radical scavenging enzyme is SOD 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 (GSH), uric acid etc.[20]

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.[51] 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”.[52] 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 SOD, catalase and GSH Px. In the second category is the chain breaking agents such as α tocopherol, ubiquinone, β 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 PUFA. Peroxidation of PUFA will result in a disruption of membrane. So, the antioxidants lie functionally at the heart of this protective mechanism.[51]

Intracellular defense system is largely dependent on the antioxidant enzymes such as GSH-Px, SOD, and catalase requiring the antioxidant micronutrients like selenium (Se), copper (Cu), zinc (Zn) and iron. Riboflavin functions as an antioxidant cofactor (FAD) needed for the glutathione reductase.[51]

ENZYMATIC ANTIOXIDANTS

Superoxide Dismutase

McCord and Fridorich described the metalloenzyme superoxide dismutase.[53] Superoxide dismutase is the major intracellular antioxidant enzyme, which is essential for the survival of aerobic cells. It catalytically scavenges the $O_2^{\bullet-}$, which appears to be important agent for toxicity of O_2 and thus provides a defense against O_2 toxicity.

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

Superoxide dismutase catalyzes the dismutation of the $O_2^{\bullet-}$ to $H_2O_2$and molecular O_2 at a rate 104 times faster than spontaneous dismutation at physiological pH[54] resulting in no $O_2^{\bullet-}$ available to react with H_2O_2 to from $OH^{\bullet}$ 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. Copper and Zn containing SOD found in cytosol.
2. Manganese (Mn) containing SOD found in mitochondria.

Copper / Zinc containing SOD: 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.[55]

Manganese containing SOD: Manganese SOD has been isolated from human liver mitochondria.[55] It contains four subunits and has a molecular weight of 80,000.

Glutathione peroxidase

Mills established the presence of GSH Px in mammalian erythrocytes.[56] It has a molecular weight of about 85,000 and consisting of four apparently identical subunits and contains four atoms of Se/mol. The enzyme bound

selenium can undergo a substrate induced redox change, and it is also essential for the activity. Selenium deficiency may lead to depleted activity of selenium containing GSH Px, which controls the concentration of H_2O_2 and catalyzes the reduction of lipid peroxides with the formation of safely disposable hydroxy acids.

Glutathione peroxidase apparently reduces the Se and reduced form of enzyme then reacts with H_2O_2.[25] Both types of GSH Px enzymes Se dependent and Se independent have been shown to protect against radical damage by reducing peroxides. Glutathione peroxidase enzyme has been shown to catalyze with high specificity the invitro detoxification of H_2O_2 by the oxidation of reduced GSH.[57]

$$2GSH + H_2O_2 \xrightarrow{GSH\ Px} GSSG + 2H_2O$$

This enzyme is found in liver, kidney, erythrocytes, the endothelial lining of vessels, lens of the eye etc.

Mills showed that GSH Px is one of the important components in the metabolic pathway of the GSH system.[56]

In biological systems, high concentrations of H_2O_2 are disposed off by catalase and lower concentration by GSH Px. Glutathione peroxidase essentially utilizes glutathione as a reductant in disposing off H_2O_2. The enzyme is a metalloenzyme and remains associated with Se as the metal prosthetic group.[58]

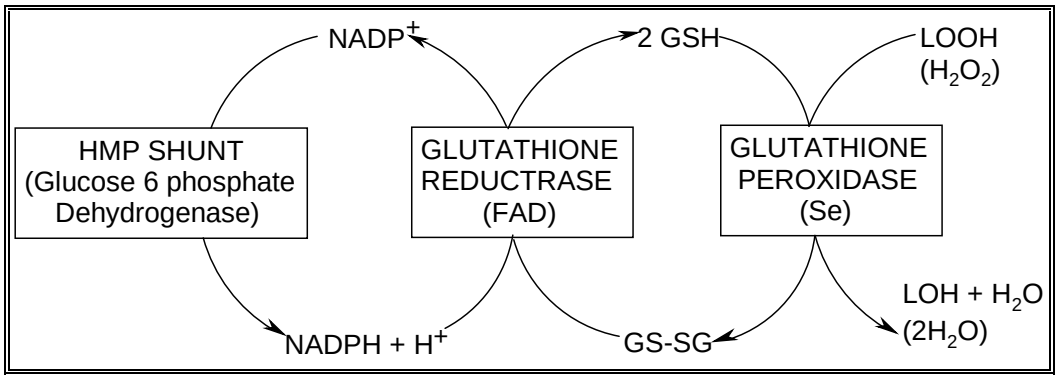


Figure 6. Glutathione system and its components

The glutathione system and its components

Glutathione peroxidase is one of the primary antioxidants present in tissues that limit the amount of lipid peroxides.[59,60]

Reduction of lipid peroxides to non-toxic hydroxyl fatty acids by GSH Px, protects the cellular components from deleterious effects of peroxides and also prevents the decomposition of these peroxides into free radicals that can reinitiate peroxidation.

Glutathione peroxidase uses GSH as its cofactor to convert lipid peroxides into relatively harmless hydroxylated fatty acids, water and glutathione disulfide. Therefore, if GSH activity were deficient, lipid peroxides could increase in the tissue.

Mechanisms of action of glutathione peroxidase

a. Lack of specificity with respect to the hydroperoxide.
b. High specificity for GSH.
c. Selective inhibition by iodoacetate of the substrate reduced enzyme.

d. The identification of selenol as a functional group.

e. The reactivity of the enzyme bound selenium with the physiological substrates.

Glutathione peroxidase actively reduces the primary products of PUFA peroxidation ROOH, but not secondary products. ROOR, which are minor products in natural system.

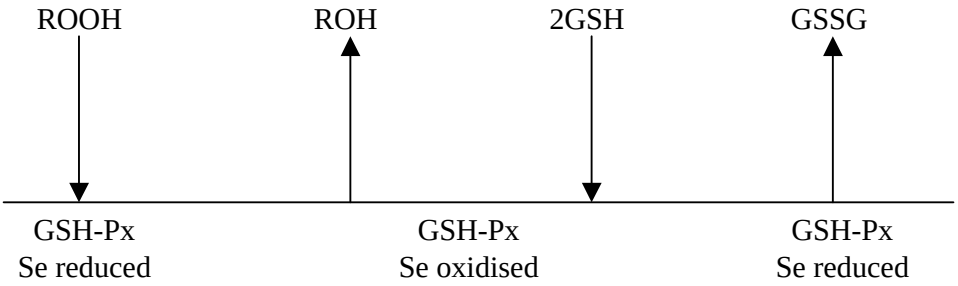


Figure 7. Glutathione peroxidase and its reactions

Glutathione Peroxidase Reactions

An important feature of the mechanism is that with the physiological concentrations of GSH the enzyme is activated immediately to reduce any hydroperoxides with which it comes in contact.[58]

Catalase

Catalase is major antioxidant defense component present in all mammalian cell types, reacts rapidly with H_2O_2 inside of cells and converts it into water and O_2.[61] Selenium dependent GSH Px also inactivates H_2O_2.[37] Catalase the breakdown of toxic H_2O_2 directly to water and preventing the secondary generation of toxic intermediates such as $OH^{\bullet}$.[62]

$$2\ H_2O_2 \rightarrow O_2 + H_2O$$

Catalase activity is present in all aerobic cells. The level of activity varies not only between tissues but also within the cell itself. High level of catalase activities are found in lives. Kidney red blood cells, microsomes and also in cytosol.[63] Purified catalase have been shown to consist of four protein subunits, each of which contains Fe^{+3}, protoporphyrin group which is bound to its active site.[25]

$$\text{Catalase } Fe^{3+} + H_2O_2 \longrightarrow \text{Compound 1}$$

$$\text{Compound 1} + H_2O_2 \rightarrow \text{catalase} \rightarrow Fe^{3+} + 2H_2O_2 + O_2$$

Vitamin – E (α–Tocopherol)

Vitamin E or α 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 PUFA from peroxidative damage by donating hydrogen to the $ROO^{\bullet}$. 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 (for example 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 $ROO^{\bullet}$ and alkoxyl radicals.[37]

Alpha tocopherol is known as a chain breaking antioxidant because it functions to intercept $ROO^{\bullet}$ and so terminates lipid peroxidation chain reactions. Peroxyl and alkoxyl radicals generated during lipid peroxidation preferentially combine with the antioxidant.[64]

$$ROO^{\bullet} + \alpha - \text{tocopherol OH} \longrightarrow ROOH + \alpha - \text{tocopherol} + O^{\bullet}$$

Reaction is faster, then these radicals can react with membrane proteins or with adjacent fatty acid side chains. The resultant tocopheryl radical is relatively stable and in normal circumstances insufficiently reactive to initiate lipid peroxidation itself. Evidence exists that this tocopherol radical can migrate to the membrane surface and reconvert to α-tocopherol by reaction with ascorbic acid.[65,66] Some thiol compounds, such as GSH might also be involved in regenerating α-tocopherol from its radical.[65,67] α-tocopherol quenches and reacts with $O^{\bullet}$ and could therefore protect the membrane against this species. It also reacts with the $O_2^{\bullet -}$.[68]

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.[69] 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.[70] 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.[37]

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 $O_2^{\bullet -}$ and $OH^{\bullet}$ 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. Ferrous ion is known to be a potent free radical inducer.[37]

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 ROS (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).[43]

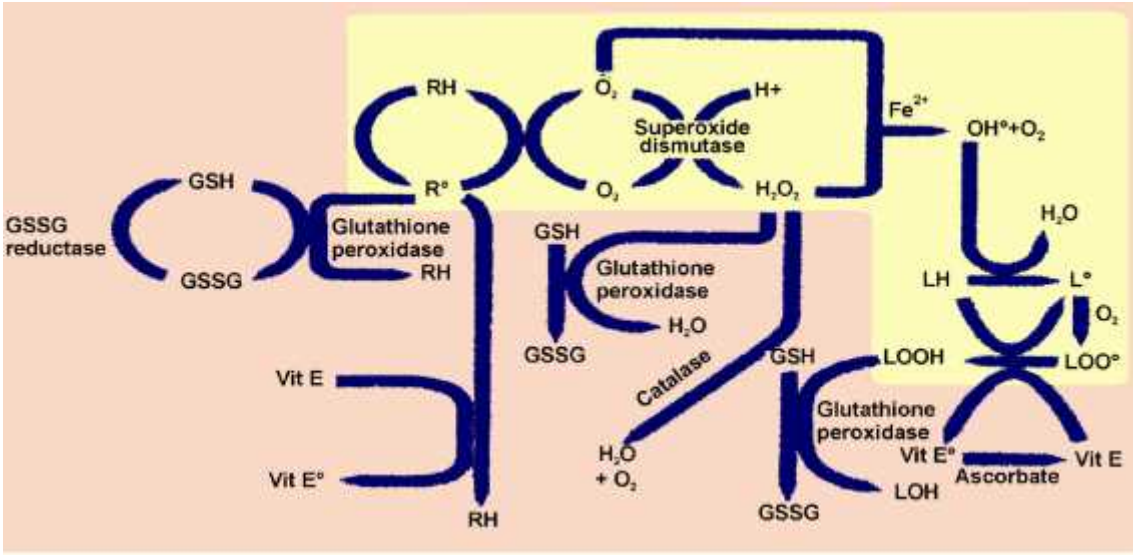


Figure 8. Effects of free radicals in cell injury and cellular defense mechanisms

METHODOLOGY

The present study was conducted in Department of Biochemistry, KLES Dr. Prabhakar Kore Hospital and Medical Research Centre, Belgaum between February 2009 to January 2010.

Study design

One year case control study.

Source of data

The present study comprises of clinically, diagnostically confirmed and untreated cases of pulmonary tuberculosis admitted and attending the respiratory medicine unit of KLES Dr. Prabhakar Kore Hospital and Medical Research Centre, Belgaum. Study was undertaken over a period of one year from February 2009 to January 2010.

Sample size

Fifty cases of primary pulmonary tuberculosis and 50 healthy controls in the age of group of 25 to 50 years of both sex were included in the study based on 80% average of last three year hospital records.

Selection criteria

Inclusion criteria

- Clinically, diagnostically confirmed and untreated cases of primary pulmonary tuberculosis.

- Following positive parameters were considered for inclusion of cases in the present study.
    1. Chest X-ray.
    2. Sputum – AFB.
    3. Blood examination – Erythrocyte sedimentation rate (ESR).
    4. Clinical history and findings

Exclusion criteria

- Extrapulmonary tuberculosis.
- Pulmonary diseases like pneumonia, asthma, chronic obstructive pulmonary disease.
- Hepatocellular disorders, renal disorders.
- Diabetes mellitus.
- Rheumatoid arthritis
- Smoking and alcoholic.
- Cerebrovascular diseases
- Neoplasias

Procedure

The study was approved by the Ethical and Research Committee of Jawaharlal Nehru Medical College, Belgaum. All the cases were evaluated and selected by simple random technique after fulfilling selection criteria. The cases of primary tuberculosis reported to the Department of Respiratory Medicine, KLES Dr. Prabhakar Kore Hospital and Medical Research Center were screened. After finding the suitability as per selection criteria they were requested to

participate in the study and briefed about the nature of the study and interventions used. A written informed consent was obtained (Annexure I). The consented patients were enrolled in the study, further descriptive data of the participants like name, age, sex, detailed history were obtained by interviewing the participants and were recorded on a predesigned and pretested proforma (Annexure II).

Collection and storage of blood sample

Ten ml 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 and transferred to heparin containing vials. Out of this 1ml of whole blood was used for estimation of MDA and one ml was used for the preparation of hemolysate. Enzymes like SOD, GSH Px and catalase were analyzed from the hemolysate within one hour and the remaining sample of blood was centrifuged immediately and plasma was obtained for estimating vitamin E and vitamin C.

Methods of assay

Whole blood

- Malondialdehyde (Thiobarbituric acid method)

Hemolysate

- Hemoglobin (Drabkin's method)
- Enzymatic antioxidants
    - Superoxide dismutase (Misra and Fridorich method)
    - Glutathione peroxide (Beutler's method)
    - Catalase (Beutler's method)

Plasma

- Vitamin E (Quafie, Baker and Frank Method)
- Vitamin C (Evelyn and Melloy method)

STATISTICAL ANALYSIS:

The data was tabulated and statistical analysis was carried out by using unpaired student's t – test and comparison within groups was done using analysis of variance.

ESTIMATION OF MALONDIALDEHYDE IN WHOLE BLOOD[71]

Principle

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

Thiobarbituric acid reagent

- 75 mg TBA
- 15 ml trichloroacetic acid (TCA)
- 2.08 ml – 0.2 N hydrochloric acid (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	-
TBA reagent	3	3

- Keep in boiling water bath for 15 minutes
- Cool, centrifuge for 10 minutes at 10,000 revolutions per minute (rpm).
- Read absorbance of supernatants of blank and test immediately at 535 nm.

Calculation

Malondialdehyde (Nano moles / ml)

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

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

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

PREPARATION OF (RBC) HEMOLYSATE

Isolation of Red Blood Cells[72]

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 white blood cells (WBC). In order to isolate red blood cells (RBC) the whole blood was filtered through a column of α-cellulose and microcrystalline cellulose mixture.

α – Cellulose and microcrystalline cellulose in 1:1 weight/weight (W/W) was mixed with isotonic (9.0 gm/L) sodium chloride (NaCl) solution. Five ml 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 two ml mark. The bed was washed with five ml isotonic sodium chloride and one 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 NaCl. After washing, the packed cells were resuspended in isotonic NaCl to give an approximately 50% suspension (1:1) dilution). This suspension was subjected to hemolysis.

LYSING OF THE RBCS[73]

Reagents

Stabilizing solution: 2.7 mM Ethylene diamine tetra acetic acid (EDTA) (pH 7.0) and 0.7mM β- mercaptoethanol: This solution was prepared by dissolving 100 mg of disodium salt of EDTA in distilled water (D/W) and five µl 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, one volume of the RBC suspension was mixed with nine 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

b) Glutathione peroxidase

c) Catalase

DETERMINATION OF HEMOGLOBIN[74]

Principle

Drabkins reagent contains potassium cyanide and potassium ferricyanide Hemoglobin (Hb) 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 HiCN 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:

	Blank (ml)	**Standard (ml)**	**Test (ml)**
Drabkin's reagent	5.0	---	5.0
HiCN standard	----	5.0	----
Unknown	----	----	0.02

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

Calculation

$$\text{Hemoglobin g\%} = \frac{\text{OD test}}{\text{OD std}} \times 15$$

PROCEDURE FOR ENZYME ASSAYS IN 1:20 HEMOLYSATE

SUPEROXIDE DISMUTASE[75]

Principle

Epinephrine can be auto-oxidized to adrenochrome by $O_2^{\bullet -}$. Maximum auto-oxidation of epinephrine takes place at pH-10.2. The ability of SOD 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 $O_2^{\bullet -}$ 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 100 ml 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) 33 ml was mixed with solution (b) 17 ml 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 three ml of D/W. To dissolve epinephrine completely minimum amount of 1N HCl was added and volume was made to 10 ml 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 10 ml with D/W.

Procedure: Preparation of hemoglobin free filtrates: Chloroform, ethanol and water were chilled before use. In a test tube, chloroform (0.125 ml), ethanol (0.25 ml) and D/W (0.8 ml) were added. To this mixture, 0.2 ml of cold hemolysate was transferred. The suspension was subjected to vortex agitation for two 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 (3 x 10^{-4} M), EDTA (1 x 10^{-4} M) and carbonate buffer (0.05 M). 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

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 0.03 ml of epinephrine (3 x 10^{-2} M) was added and after 90 seconds (the lag phase) the auto-oxidation of epinephrine was assayed by measuring the optical densities at zero minute and after three minutes, at 480 nm.

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

Calculations: Superoxide dismutase 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.

$$\text{Percent inhibition} = 100 - \frac{\Delta OD\ s}{\Delta OD\ c} \times 100$$

Where, ΔOD s: Difference in optical density of sample in three minutes.

ΔOD c: Difference in optical density of control in three minutes.

A graph percent inhibition against amount of red cell extract was plotted as shown in the figure 7, to determine 50% inhibition of epinephrine oxidation. The amount of extract, which gave 50% inhibition, as determined from the graph corresponds to one 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

$$= \frac{100 \times 1000 \text{ units}}{V\ \mu l}$$

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

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

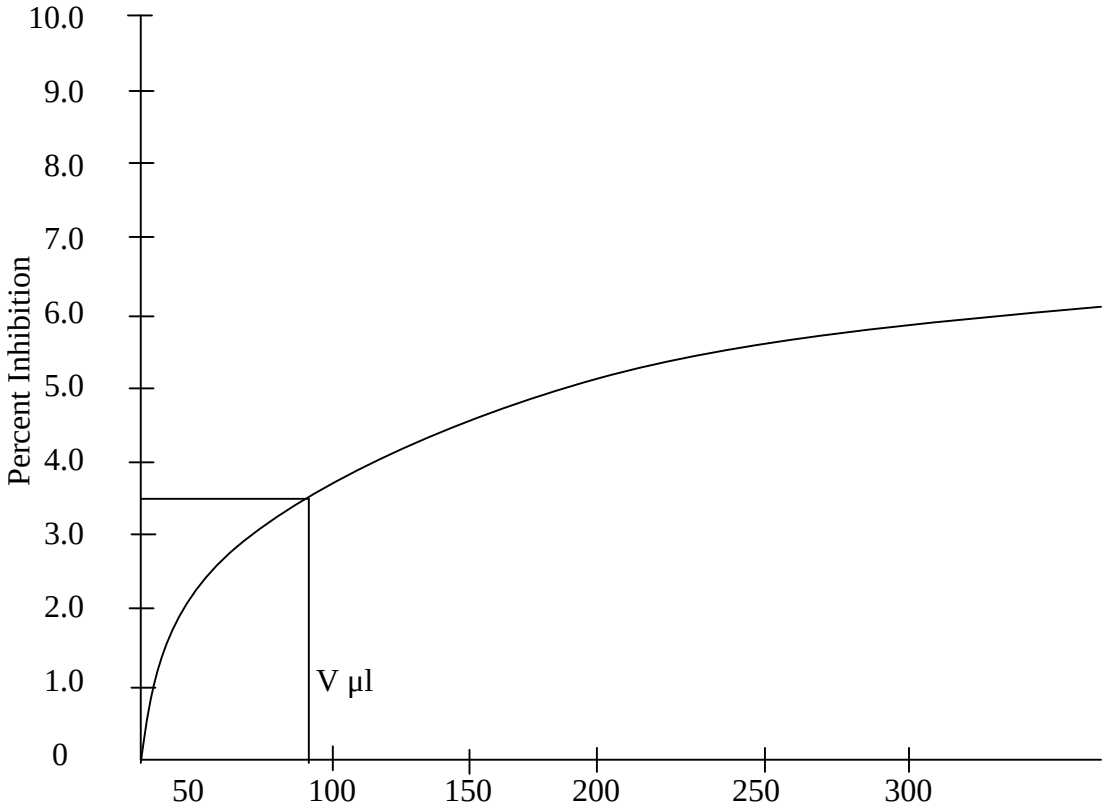


Fig. No 9: Determination of SOD Activity Red cell extract (rl)

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 PEROXIDASE[76]

Principle

Glutathione peroxidase catalyzes the oxidation of reduced GSH to oxidized glutathione (GSSG).

$$2GSH + ROOH \xrightarrow{GSH\ Px} GSSG + H_2O + R\text{-}OH$$

Where ROOH is peroxide. t-butylhydroperoxide (t-BHP) is the most suitable substance for assay of the enzyme. The rate of reduction of GSSG by glutathione reductase (GR) is measured.

$$GSSG + NADPH + H \xrightarrow{GR} 2GSH + NADP$$

The oxidation of NADPH is followed at 340 nm. To ascertain that the auxillary enzyme GR is not contaminated with the enzyme being assayed GSH-Px, blank 2 is also prepared. β - Mercaptoethanol EDTA stabilizing solution is substituted for the hemolysate in the reaction mixture.

Reagents

1. Tris-HCl, 1M;EDTA, 5mM; pH 8.0: 12.1 gm of Tris and 168 mg of disodium salt of EDTA were dissolved in 80 ml of D/W and pH was adjusted to 8.2 with concentrated HCl and further till 8.0 with 1N HCl and final volume was made to 100 ml of D/W.

2. Glutathione reductase: 10 U/ml: 40 µl of Glutathione reductase (EC 1.6.4.2; Type III from Baker's yeast. Sigma) of strength 500 U/2 ml were diluted to one ml with EDTA stabilizing solution.

3. Nicotinamide adenine dinucleotide phosphate reduced (NADPH) 2mM: 2.3 mg tetrasodium salt of NADPH (SRL. Mwt 833.36) was dissolved in one ml of D/W. To 0.85 ml of D/W, 0.1 ml of Tris-HCl, 1M EDTA; 5mM pH 8.0 buffer was added and its optical density was measured at 340nm against D/W blank as Ao. To the above solution 0.05 ml of 2.3 mg/ml

4. NADPH was added and the optical density was measured at 340 nm against D/W blank as A1. The concentration of NADPH in test solution was C = A1-Ao/0.311, where C is the concentration in mM. To dilute the solution to give two mM concentration, two ml of NADPH (2.3 mg/ml) were diluted to C.

5. Glutathione reduced, (GSH), 0.1M: 30.7mg of GSH (SRL Mwt 307.32) were dissolved in 1ml of D/W. (It was prepared prior to use).

6. t-butylhydroperoxide: 7 mM: 0.01 ml of t-BHP (Sigma 7M) was diluted to 10 ml.

Procedure

In 1.0 ml system following additions was carried out in the order given below.

	Blank1 (µl)	Blank2 (µl)	Test (µl)
Tris HCl; 1M EDTA, 5 mM, pH 8.0	100	100	100
Reduced GSH, 0.1 M	20	20	20
Glutathione reductase, 10 U/ml	100	100	100
NADPH, 2 mM	100	100	100
1:20 hemolysate	10	---	10
EDTA – stabilizing solution	---	10	---
D/W	670	660	660
Pre-incubated at 37^0 C for 10 minutes			
t-BHP, 7 mM	--	10	10

The decrease in the optical density of the test at 340nm was measured against Blank at zero minute and after 10 minutes.

Calculations: The number of enzyme units per ml:

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

Where

Δ OD : Change in optical density per minute.

$$\Delta \text{ OD} : \frac{\text{Test 0 min – Test 10 min}}{\text{10 minutes}}$$

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 = 0.01 ml

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 100 ml of the Hemolysate.

CATALASE[76]

Principle

Catalase catalyses the breakdown of H_2O_2 according to the following reaction.

$$2H_2O_2 \xrightarrow{\text{Catalase}} 2H_2O + O_2$$

The rate of decomposition of H_2O_2 by catalase is measured spectrophotometrically at 230 nm. Since H_2O_2 absorbs light at this wave length.

Ethanol is added to stabilize the hemolysate by breaking down the "Complex II" of catalase and H_2O_2.

Reagents

1) Tris-HCl, 1M: EDTA, 5 mM; pH 8.0; 12.1g of Tris and 168 mg of disodium salt of EDTA were dissolved in 80ml of D/W and pH was adjusted to 8.2 with concentrated HCl and further till 8.0 with 1N HCl and final volume was made to 100 ml with D/W.

2) Phosphate buffer, 1 M, pH 7.0: 100 ml of 1M Na_2HPO_4, H_2O_2 (17.79 g%) solution was adjusted to pH 7.0 with 1M NaH_2PO_4.2H2O (15.6g%) solution.

3) Hydrogen peroxide; 10 mM: Optical density of 2.7ml of 1; 10 diluted 1M-phosphate buffer, pH 7.0 was measured at 230 nm as OD1. Three µL of 1:100 diluted 30% H_2O_2 solution were added and optical density was measured as OD2. Since the millimolar extinction coefficient of H_2O_2 at 230 nm is 0.071, the H_2O_2 concentration (C) of the 1:100 diluted peroxide solution is 141

(OD2 – OD1) mM. To obtain H_2O_2 10mM for the assay, 1 ml of 1:100 diluted H_2O_2 was further diluted to C/10ml with D/W.

4) 1:2000 hemolysate with ethanol: 1:20 hemolysate prepared in β-mercaptoethanol EDTA stabilizing solution. Solution was further diluted to 1:100 with EDTA stabilizing solution. Twenty µL absolute ethanol was added per mL of dilute hemolysate (to breakdown "Complex II" which may be present).

Procedure

A set of test tubes was prepared as follows:-

	Blank (ml)	**Test (ml)**
Tris HCl; 1M EDTA, 5mM, pH 8.0	0.15	0.15
H_2O_2 10mM	--	2.70
D/W	2.79	0.09
Tubes were incubated at 37^0C for 10 minutes		
1:2000 hemolysate	0.06	0.06

Calculations: The number of enzyme units per ml is

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

Where

Δ OD : Change in optical density per minute

V_C : Volume of the cuvette in ml

ε : The millimolar extinction coefficient of the hydrogen peroxide = 0.071

N : The number of molecule (1) of hydrogen peroxide converted per

molecule of substrate (H_2O_2) consumed

V_H : The volume of hemolysate in ml = 6×10^{-4}

The enzyme activity in international units/g Hb is

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

A : Number of enzyme units per ml

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

ESTIMATIONS OF α TOCOPHEROL IN PLASMA[77]

Principle

This method is based on the Emmerie Engel reaction. Xylene extract of plasma containing α-tocopherol when reacts with ferric chloride ($FeCl_3$), reduces Fe^{+3} to Fe^{+2}. The Fe^{+2} 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 two 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' – dipyridyl 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 $FeCl_3$ reagent was added and contents were mixed for 30 seconds. The absorbance was read exactly after 90 seconds of the addition of $FeCl_3$ 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 %

ESTIMATION OF ASCORBIC ACID IN PLASMA[78]

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 ⟶

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 (H_2SO_4). Take 18.7ml concentrated H_2SO_4. Mix with D/W make the volume up to one liter with D/W.
3) Metaphosphoric acid solution; 5%: 5g of metaphosphoric acid (Robert Johnson) is dissolved in 100 ml of D/W without heating. Solution should be prepared weekly. Store in refrigerator.
4) 2, 6 dichlorophenol indophenol solution: 13 mg of 2, 6 dichlorophenol indophenol (Loba GR) and three g of anhydrous sodium acetate trihydrate (Qualigens SQ), were dissolved in one liter of D/W. Nine ml of this reagent and one ml of metaphosphoric acid (reagent 1) were mixed to

check the pH which should be around 3.5 to 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 100 ml of metaphosphoric acid.

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

Procedure:

In a clean dry test tube, one ml of plasma, three ml of five percent metaphosphoric acid, 0.5 ml of sodium tungstate and 0.5 ml of 2/3N H_2SO_4 were taken. The contents were mixed and filtered after five 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 520 nm against D/W using a double beam Spectrophotometer.

Calculations:

Amount of ascorbic acid in mg% =

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

RESULTS

The present study was conducted in the Department of Biochemistry, Jawaharlal Nehru Medical College, Belgaum during the period of January 2009 to December 2009. The study comprised of 50 normal healthy controls and 50 primary pulmonary tuberculosis cases in the age raging from 25 to 50 years.

The cases were further grouped into Group I consisting of pulmonary tuberculosis without HIV and Group II pulmonary tuberculosis with HIV infection. The data obtained was tabulated and statistical analysis was done using student unpaired 't' test and comparison between the groups was done by analysis of variance.

Table 1. Gender distribution

Gender	Control		Cases	
	Number	Percentage	Number	Percentage
Male	25	50	33	66
Female	25	50	17	34
Total	50	100	50	100

In the present study 50% of each were males and females in control group with sex ratio 1:1. In cases group 66% were males and 34% were females with male to female ratio of 2.2:1.

Table 2. Mean age

Age (Years)	Control		Cases	
	Mean age	**SD**	**Mean age**	**SD**
Mean age	33.7	5.61	34.8	6.39

The mean age of controls was 33.7 ± 5.61 years and in cases it was 34.8 ± 6.39 years. There was no significant difference in the age of the cases and control group.

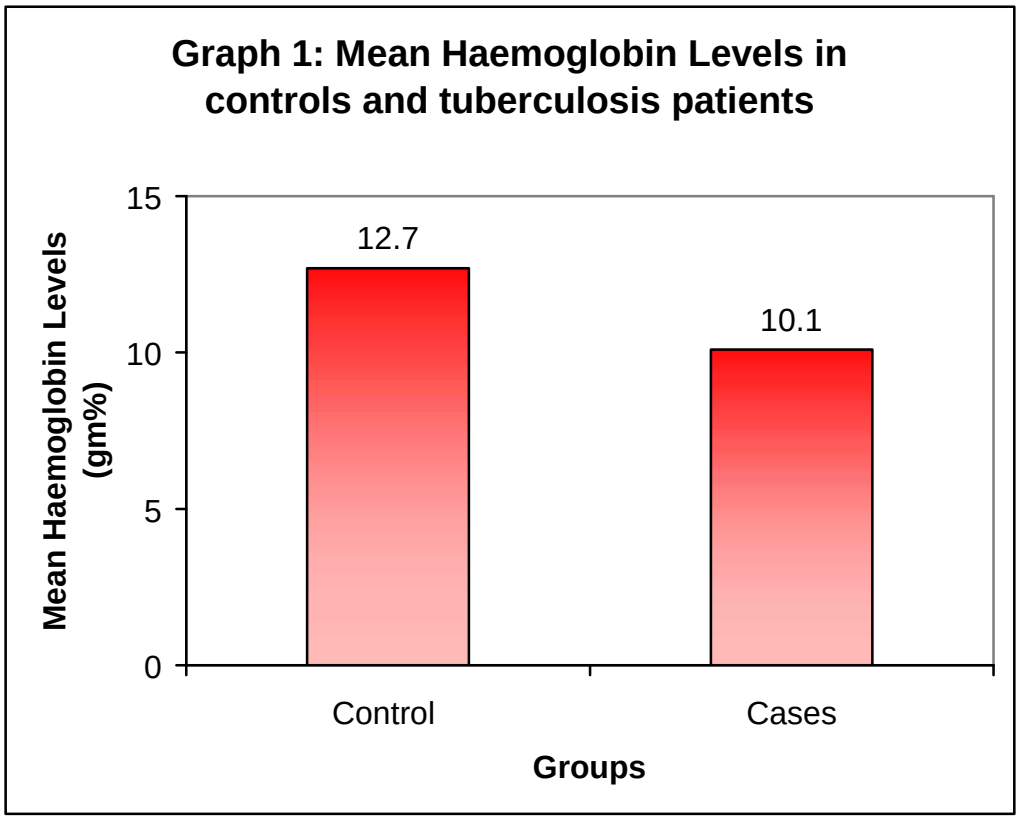
Table 3. Body Mass Index

	Control group		Cases group		t	df	p value
	Mean	**SD**	**Mean**	**SD**			
BMI (kg/m^2)	24.3	3.06	21.1	2.53	5.687	98	<0.001

The mean BMI in control group was 24.3 ± 3.06 kg/m^2 and the mean BMI in patients with pulmonary tuberculosis it was 21.1 ± 2.53 Kg/m^2. There was a significant difference in the BMI of cases and control group.

Table 4. Haemoglobin

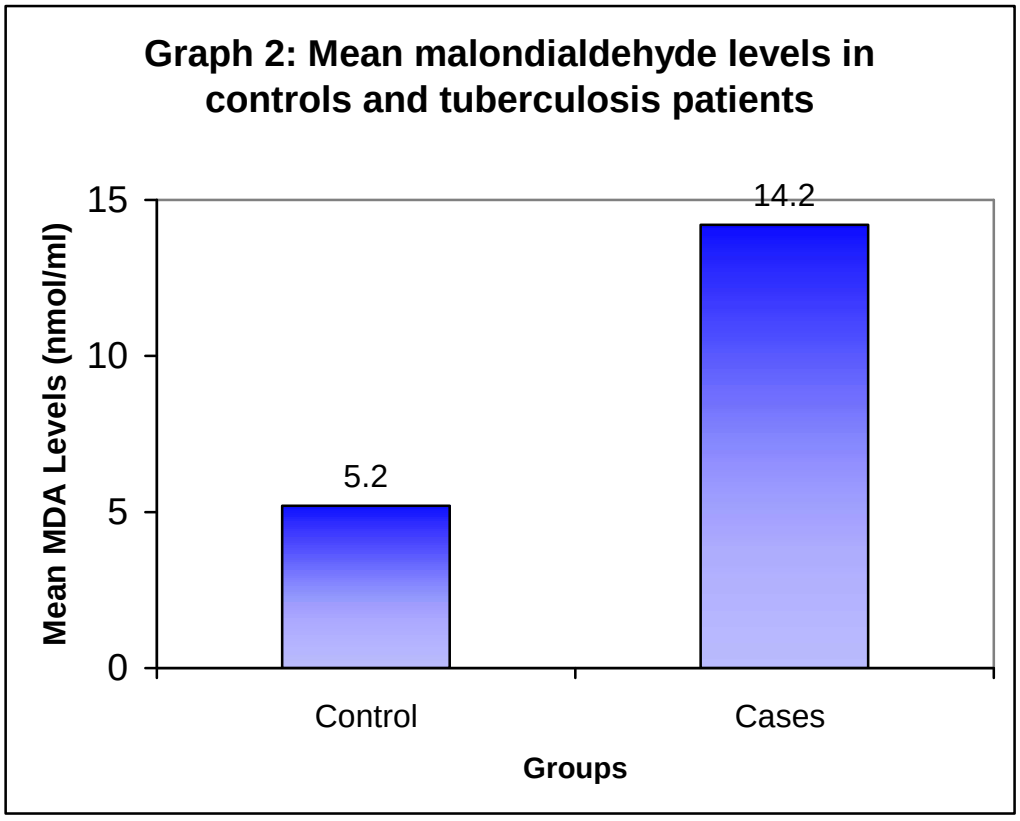
	Control group		Cases group		t	df	p value
	Mean	**SD**	**Mean**	**SD**			
Hb (gm%)	12.7	1.15	10.1	1.26	10.584	98	<0.001



The mean and standard deviation for Hb% in control group was 12.7±1.15 gm% and in cases it was 10.1±1.26 gm% showing a significant decrease ($p<0.001$) in the Hb% in the cases compared to controls.

Table 5. Concentration of malondialdehyde

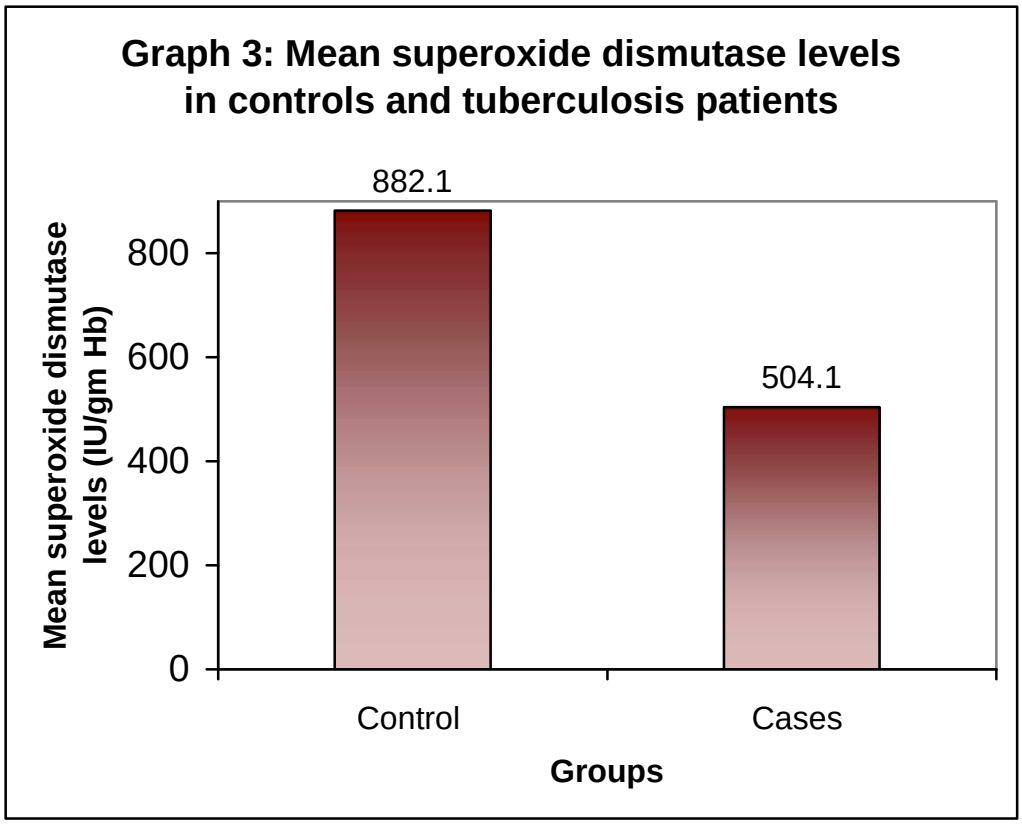
Parameters	Control group		Cases group		t	df	p value
	Mean	SD	Mean	SD			
MDA (nmol/ml)	5.2	1.42	14.2	2.21	24.046	98	<0.001



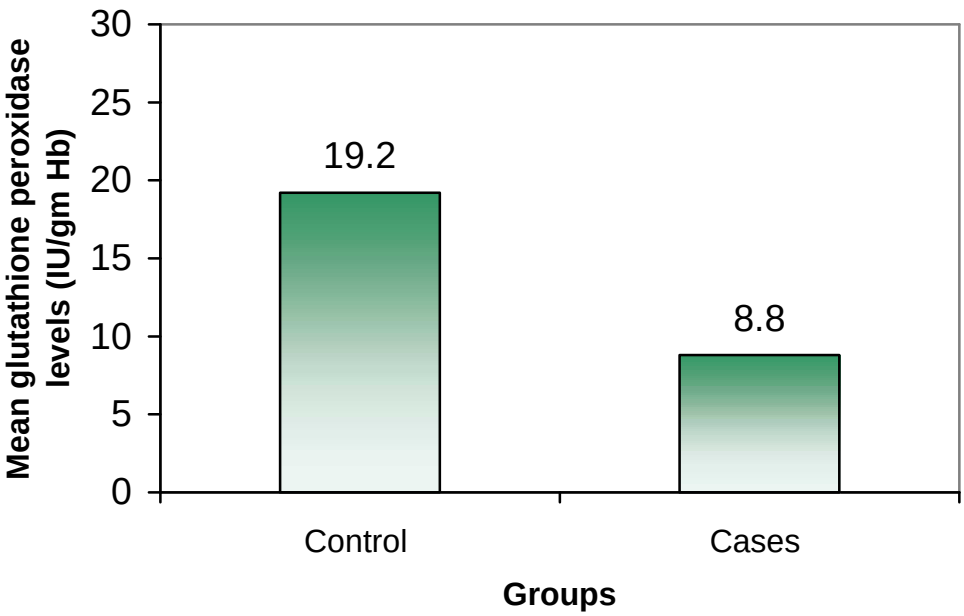
The mean MDA levels in control was 5.2 ± 1.42 nmol/ml and in cases it was 14.2 ± 2.21 nmol/ml. There was a significant increase (p <0.001) in the MDA levels in the cases compared to the controls.

Table 6. Concentration of enzymatic antioxidants (SOD, GSH Px, Catalase)

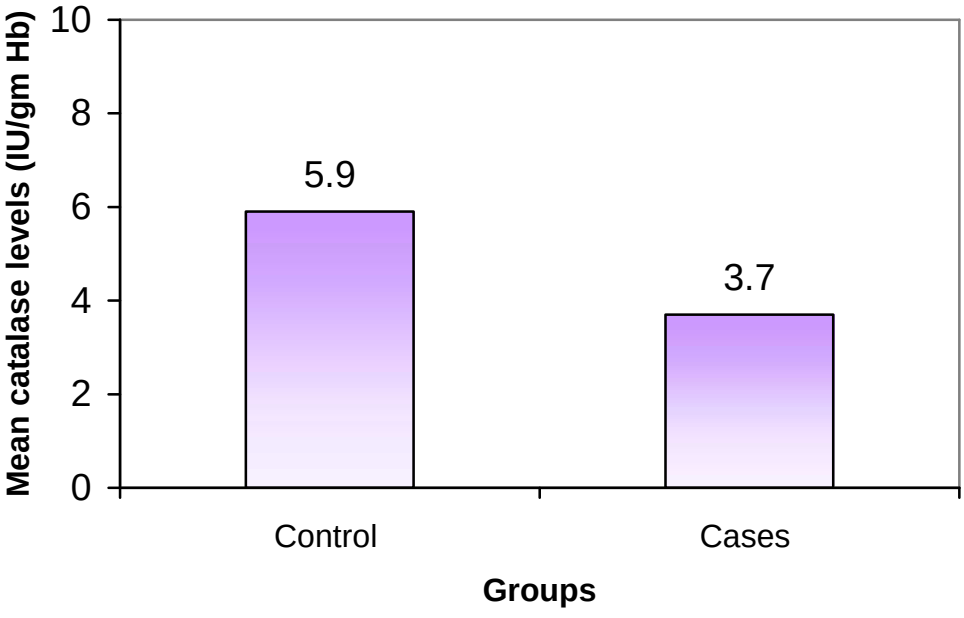
Parameters	Control group		Cases group		t	df	p value
	Mean	SD	Mean	SD			
SOD (IU/gmHb)	882.1	116.81	504.1	116.24	16.22	98	<0.001
GSH Px (IU/gmHb)	19.2	1.67	8.8	2.22	9.16	98	<0.001
Catalase (IU/gmHb)	5.9	1.4	3.7	1.02	26.31	98	<0.001



Graph 4: Mean glutathione peroxidase levels in controls and tuberculosis patients



Graph 5: Mean catalase levels in controls and tuberculosis patients



The mean SOD levels in control group was 882.1 ± 116.81 IU/gmHb and in cases of pulmonary tuberculosis it was 504.1 ± 116.24 IU/gmHb. There was a significant decrease in the SOD levels in the cases as compared to controls.

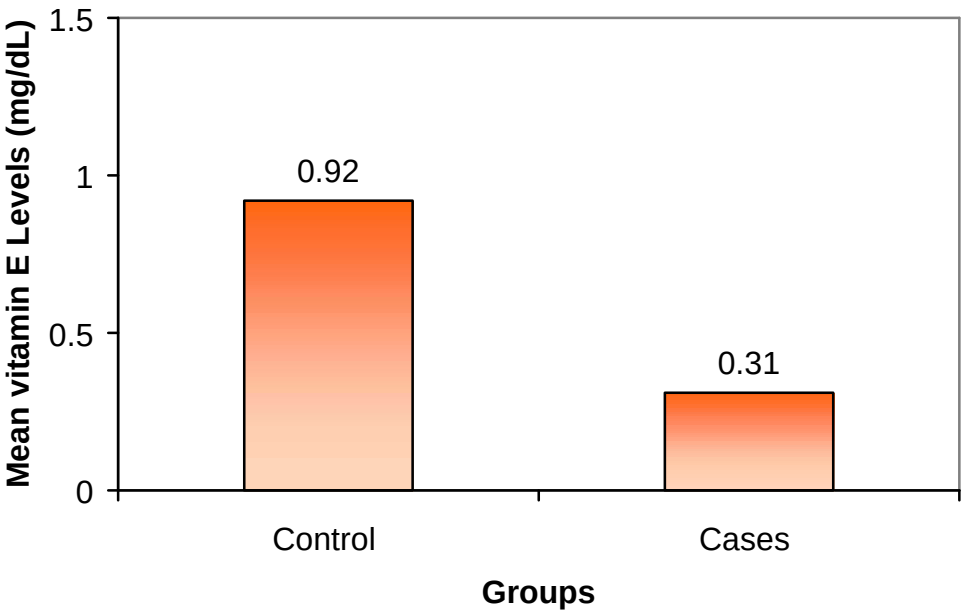
The mean GSH Px levels the controls was 19.2 ± 1.67 IU/gmHb and in study group it was 8.8 ± 2.22 IU/gmHb. There was a significant decrease in the GSH Px levels in cases as compared to control group.

The mean catalase level in the controls was 5.9 ± 1.40 IU/gmHb and in cases of pulmonary tuberculosis it was 3.7 ± 1.02 IU/gmHb. There was significant decrease in catalase levels in cases as compared to controls.

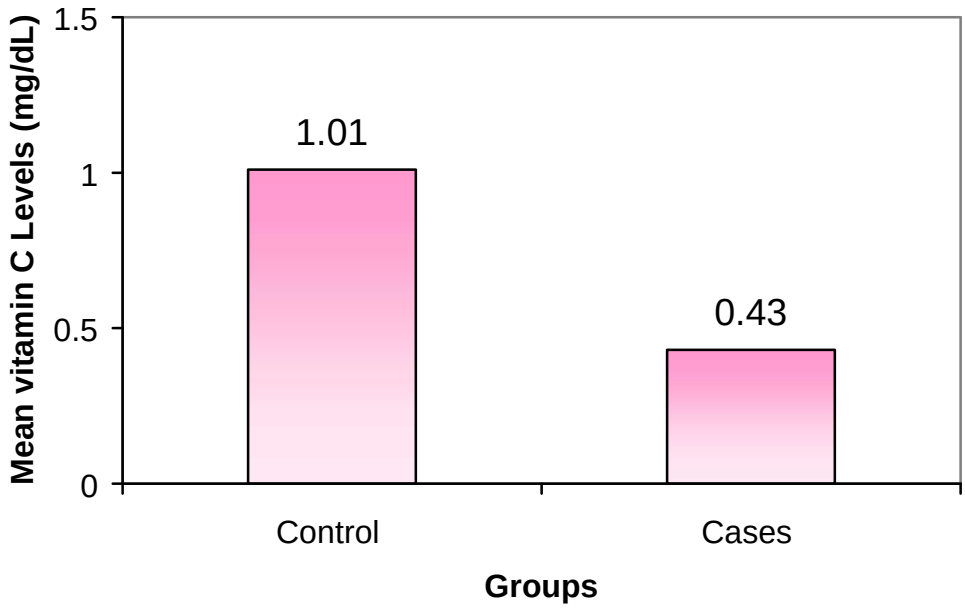
Table 7. Concentration of plasma vitamin E and C levels in controls and cases

Parameters	Control group		Cases group		t	df	p value
	Mean	SD	Mean	SD			
Vitamin E (mg/dL)	0.92	0.16	0.31	0.16	18.679	98	<0.001
Vitamin C (mg/dL)	1.01	0.18	0.43	0.16	17.014	98	<0.001

Graph 6: Mean vitamin E levels in controls and tuberculosis patients



Graph 7: Mean vitamin C levels in controls and tuberculosis patients



The mean vitamin E levels in the control group was 0.92 ± 0.16 mg/dL and in cases it was 0.31 ± 0.16 mg/dL. There was a significant decrease in the vitamin E levels in cases as compared to control group.

The mean vitamin C levels in the control group was 1.01 ± 0.18 mg/dL and in cases it was 0.43 ± 0.16 mg/dL. There was a significant decrease in the vitamin C levels in cases as compared to controls.

The cases of primary pulmonary tuberculosis were further grouped into group I consisting of primary pulmonary tuberculosis without HIV infection and Group II primary pulmonary tuberculosis with HIV infection. Comparison of the parameters among the control group I and group II was done by analysis of variance.

Table 8. Concentration of malondialdehyde, superoxide dismutase, glutathione peroxidase, catalase, vitamin E and C levels in controls and cases with pulmonary tuberculosis without HIV (n=25)

Parameters	Control		Cases (Without HIV)		'p' value
	Mean	SD	Mean	SD	
MDA (nmol/ml)	5.2	1.42	13.2	2.23	<0.001
SOD (IU/gmHb)	882.1	116.81	578.9	110.63	<0.001
GSH Px (IU/gmHb)	19.2	1.67	10.2	1.68	<0.001
Catalase (IU/gmHb)	5.9	1.4	3.7	0.83	<0.001
Vitamin E (mg/dL)	0.92	0.16	0.39	0.18	<0.001
Vitamin C (mg/dL)	1.01	0.18	0.44	0.14	<0.001

The mean MDA, SOD, GSH Px, catalase, vitamin C and E in controls was 5.2 ± 1.42 nmol/ml, 882.1 ± 116.81 IU/gmHb, 19.2 ± 1.67 IU/gmHb, 5.9 ± 1.4 IU/gm Hb and 1.01 ± 0.18 IU/gmHb and 1.01 ± 0.18 mg/dL and 0.92 ± 0.16 mg/dL and in group I it was 13.2 ± 2.23 nmol/ml, 578.9 ± 110.63 IU/gmHb 10.2 ± 1.62 IU/gmHb, 3.7 ± 0.83 IU/gmHb, 0.44 ± 0.14 mg/dL and 0.39 ± 1.00 mg/dL respectively. There was significant increase in the MDA levels in the group I cases compared to the controls. There was significant decrease in the SOD, GSH Px, catalase, vitamin C and E levels in the group I cases compared to the controls.

Table 9. Concentration of malondialdehyde, superoxide dismutase, glutathione peroxidase, catalase, vitamin E and C levels in controls and cases with pulmonary tuberculosis with HIV (n=25)

Parameters	Control		Cases (With HIV)		'p' value
	Mean	SD	Mean	SD	
MDA (nmol/ml)	5.2	1.42	15.1	1.75	<0.001
SOD (IU/gmHb)	882.1	116.81	429.1	60.41	<0.001
GSH Px (IU/gmHb)	19.2	1.67	7.4	1.74	<0.001
Catalase (IU/gmHb)	5.9	1.4	3.7	1.21	<0.001
Vitamin E (mg/dL)	0.92	0.16	0.22	0.07	<0.001
Vitamin C (mg/dL)	1.01	0.18	0.41	0.18	<0.001

The mean MDA, SOD, GSH Px, catalase, vitamin C and E levels in the controls were 5.2 ± 1.42 nmol/ml, 882.1 ± 116.81 IU/gmHb, 19.2 ± 1.67 IU/gmHb, 5.9 ± 1.4 IU/gmHb and 1.01 ± 0.18 mg/dL and 0.92 ± 0.16 mg/dL and in group II it was 15.1 ± 1.75 nmol/ml, 429.1 ± 60.41 IU/gmHb, 7.4 ± 1.74 IU/gmHb, 3.7 ± 1.21 IU/gmHb, 0.41 ± 0.18 mg/dL and 0.22 ± 0.07 mg/dL respectively. There was significant increase in MDA levels in the group II cases as compared to controls. There was significant decrease in SOD, GSH Px, catalase, vitamin C and E levels in the group II (pulmonary tuberculosis with HIV) as compared to controls.

Table 10. Concentration of malondialdehyde, superoxide dismutase, glutathione peroxidase, catalase, vitamin E and C levels in cases with pulmonary tuberculosis without and with HIV (n=25)

Parameters	Pulmonary tuberculosis without HIV		Pulmonary tuberculosis with HIV		'p' value
	Mean	SD	Mean	SD	
MDA (nmol/ml)	13.2	2.23	15.1	1.75	<0.001
SOD (IU/gmHb)	578.9	110.63	429.1	60.41	<0.001
GSH Px (IU/gmHb)	10.2	1.68	7.4	1.74	<0.001
Catalase (IU/gmHb)	3.7	0.83	3.7	1.21	0.999
Vitamin E (mg/dL)	0.39	1.8	0.22	0.07	<0.001
Vitamin C (mg/dL)	0.44	0.14	0.41	1.8	0.810

The mean MDA levels in group I was 13.2 ± 2.23 nmol/ml and in group II it was 15.1 ± 1.75 nmol/ml. There was significant increase in MDA levels in Group II patients with pulmonary tuberculosis coinfected with HIV than group I without HIV infection. The mean SOD, GSH Px levels, catalase levels in group I were 578.9 ± 110.63 IU/gmHb, 10.2 ± 1.68 IU/gmHb and 3.7 ± 0.83 IU/gmHb respectively and in group II it was 429.10 ± 60.41 IU/gmHb, 7.4 ± 1.74 IU/gmHb and 3.7 ± 0.83 IU/gmHb respectively.

There was significant decrease in SOD and GSH Px levels in group II compared to group I. However there was no significant difference in catalase levels was observed in both the groups.

The mean vitamin C and E levels in group I were 0.44 ± 0.14 mg/dL and 0.39 ± 0.18 mg/dL respectively and in group II it was 0.41 ± 0.18 mg/dL and 0.22 ± 0.07 mg/dL respectively.

There was no significant decrease in the vitamin C levels in both the groups whereas vitamin E levels were significantly decreased in group II patients as compared to group I.

DISCUSSION

Tuberculosis was announced by WHO as a global health emergency in the year 1993. It is still a worldwide health problem and one of the leading cause of death in young adults. Tuberculosis is a chromic granulomatous disease caused by Koch's bacillus called M. tuberculosis.[5]

Mycobacteria are intra cellular pathogens and replicate in the host macrophages. In an attempt to kill mycobacteria, host cells generate huge amounts of ROS which also contribute to inflammatory injury to host cells.[3]

These ROS cause membrane lipid peroxidation leading to oxidative stress which can be assessed by measuring MDA levels.[8]

Malondialdehyde

The rise in MDA levels as a marker of oxidative stress is due to free radicals generated which cause injury to the cell membrane leading to lipid peroxidation.[8]

In the present study the Mean MDA levels in controls were 5.2 ± 1.42 nmol/ml and in the study subjects that is patients with pulmonary tuberculosis it was 14.2 ± 2.21 nmol/ml. There was a significant increase in the MDA levels in the study group compared to controls that is normal healthy individuals.

Results of the present study were in accordance with previous studies[1,3,8,9,10,79] which showed elevation in the MDA levels as a marker of oxidative stress in untreated pulmonary tuberculosis patients compared to healthy

controls. One of the study[79] suggested particularly high concentration of MDA in patients who had pulmonary tuberculosis co infected with HIV infection compared to patients who had pulmonary tuberculosis without HIV infection. Another follow-up study[10] showed elevated levels of MDA as a marker of oxidative stress in pulmonary tuberculosis patients before treatment and the levels decreased after treatment with anti tuberculosis therapy (ATT) for two months.

Tuberculosis has been reported to enhance HIV replication and the progression to AIDS in dually infected patients, possibly involving enhancement of inflammatory cytokines such as tumor necrosis factor α.[80,81] The present study showed the patients with tuberculosis co-infected with HIV had higher concentrations of MDA than with HIV seronegative tuberculosis possibly reflecting increased oxidative stress in the co-infected patients that the seronegative patients. Oxidative stress has shown to enhance HIV replication,[82] to induce the production of several inflammatory cytokines[82,83] and to promote lymphocyte apoptosis[84] and T cell dysfunction.[85] Hence these could contribute to increased viral replication and progression of immunodeficiency in patients with dually infected with HIV and tuberculsis.

To defend the oxidative stress, cells have endogenous enzymatic antioxidants like SOD,[10] catalase,[10] GSH Px,[11] and exogenous non enzymatic antioxidants like vitamin C, vitamin E.[8,12]

Superoxide dismutase

Superoxide dismutase, one of the antioxidant enzyme which protect lung against oxidants by converting $O_2^{\bullet -}$ to H_2O_2.

In this study the mean SOD levels in control were 882.11 ± 116.81 IU/gmHb and in patients with primary pulmonary tuberculosis it was 504.10 ± 116.24 IU/gmHb. There was a significant decrease in the SOD levels in primary pulmonary tuberculosis. These results were in accordance to the various studies reported in the literature.[3,10,86,87] A study[10] showed decreased levels of SOD levels in all tuberculosis patients but increased gradually with clinical improvement following ATT for two months.

In the present study, it was observed that free radicals activity was increased leading to oxidative stress. Superoxide dismutase was near completely utilized to scavange the $O_2^{\bullet -}$. These findings are contradictory to a study[88] which showed compensatory increase in SOD levels in response to increase in lipid peroxidation.

Another study[89] assessed Cu and Zn containing SOD and mitochondrial Mn SOD activities in serum and pleural fluid of patients with carcinoma lung, tuberculosis and chromic heart failure. Superoxide dismutase activities were found to be higher in all the patients compared to the control. The study[89] concluded that, this enzyme can be used as a non specific prognostic marker in assessing cellular and mitochondrial tissue destruction.

Glutathione peroxidase

Glutathione peroxidase is one of the primary antioxidant present in tissue that limit the amount of lipid peroxides.[86]

In the present study the mean levels of GSH Px in the control group were 19.2 ± 1.67 IU/gmHb and in the case of pulmonary tuberculosis it was 8.8 ± 2.22 IU/gmHb. There was a significant decrease in the GSH Px levels in cases with pulmonary tuberculosis compared to control group. Similar findings were reported in the literature.[11,86]

Glutathione peroxidase essentially utilizes GSH as a reductant in disposing the free radicals. The enzyme is metallozyme and remains associated with selenium as the metal prosthetic group. Vitamin E mediated protection of lipid membranes may spare the requirements for GSH Px by reducing the free radicals at the membrane and thereby preventing leakage of the free radicals into the cytosol and maintaining intracellular killing capacity of the cell, conversely GSH Px activity in the cystosol may spare the requirement for Vitamin E in the membrane.[90,91,92]

These findings were contradictory to a study[88] which demonstrated that with focal tuberculosis, lipid peroxidation rises and compensatory rise in GSH Px takes place.

Catalase

Catalase is an enzyme present in animal tissue, characterized by its power to decompose H_2O_2 with formation of water and the evolution of O_2. Catalase, is

constitutively expressed especially in type II pneumocytes along with Mn SOD in the alveolar regions suggesting their protective role against damage caused by free radicals.[93,94]

In this study the mean levels of catalase in controls were 59.9 ± 1.4 IU/gmHb and in cases it was 3.7 ± 1.02 IU/gmHb. There was a significant decrease in catalase levels in cases of pulmonary tuberculosis compared to normal healthy individuals. These findings were in accordance to the previous studies.[10,86] Whereas, a study[95] suggests that mycobacteria are resistant to H_2O_2 and organic peroxide because mycobacteria also release catalase which neutralizes the hydrogen peroxide and hence increase the infectivity of mycobacteria which is contradictory to the present study.

Another animal study[96] conducted on mice suggested that, if they were infected with mycobacterium tuberculosis there were elevated levels of catalase which significantly decrease on administration of green tea.

Vitamin E

Vitamin E a micronutrient mediated protection of lipid membranes may decrease the requirements for GSH Px by reducing free radicals at the membrane and thereby preventing leakage of the free radical, into the cytosol and maintaining intracellular killing capacity of the cell.[92] It has been demonstrated that vitamin E acts as a mobilizable antioxidant, being released from the tissue stores and diverted to the lung of pulmonary tuberculosis patients during oxidative stress radical mediated pulmonary fibrosis.[97]

In this study the mean vitamin E levels in the control group were 0.92 ± 0.16 mg/dL and in the study group it was 0.31 ± 0.16 mg/dL. There was a significant decrease in the vitamin E levels in case of pulmonary tuberculosis compared to controls. These findings were in accordance with previous studies[1,12,79,86,90,92,98] reported in the literature.

A study[79] reported decreased levels of vitamin E in patients with pulmonary tuberculosis with and without HIV infection. Supplementation of vitamin E and selenium with antitubucular therapy improved the microbiological and radiological outcomes in patients with pulmonary tuberculosis after treatment.[93] Another study[99] showed improvement in vitamin E levels in patients with pulmonary tuberculosis after they were treated with ATT for two months. Whereas a study[12] reported contradictory results with these findings and reported no significant changes in Vitamin E levels before and after two months of treatment with ATT.

Vitamin C

Vitamin C is the first antioxidant to be depleted upon exposure to both environmental and inflammatory oxidants suggesting that it is the ultimate antioxidant directly scavenging these oxidants (free radicals) or trapping their intermediates. Hence supplementation may be beneficial in pulmonary tuberculosis patients for fast recovery from the disease.[8]

In the present study the mean Vitamin C levels in controls were 1.01 ± 0.18 mg/dL and in cases of pulmonary tuberculosis it was 0.43 ± 0.16 mg/dL. There was significant decrease in the vitamin C levels in pulmonary tuberculosis

cases compared to the healthy controls. These findings were in accordance with previous reports.[3,8,12,91,99,100]

Hypovitaminosis is a rule among patients with pulmonary tuberculosis. The degree of hypovitaminosis parallels the extent and actively of tuberculosis involvement. Two factors are responsible for the hypovitaminosis; first abnormal chemistry of the intestinal tract and second, increased requirement by the tissues of vitamin C.[100]

A study[101] reported that antitubercular drugs induce reactive oxygen species formation, thus increasing risk of drug toxicity. Another study[102] showed that antimicrobial chemotherapy supplemented with antioxidants like Vitamin C, reduce the toxicity of due to drugs, improve the cure rates, reduce the period of treatment and shortens the period of cavity closure.

Vitamin E scavenges lipid peroxy free radicals and interrupts the chain reaction of lipid peroxidation, becoming oxidized itself in the process. Vitamin C present in aqueous compartments functions as a water soluble chain breaking antioxidant, converts the tocopheroxy radical back to active tocopheral, thereby replenishing the antioxidant activity of vitamin E.[103]

In an Indian study,[104] 250 mg of natural vitamin C was given daily to patients with pulmonary tuberculosis. The study showed improvement of blood picture among these patients.

The findings of present study were contradictory to a study[105] which suggested that hypovitaminoses is encountered in many infection. It is assumed

to be the result of toxemia and therefore non specific, hence it is of no value in treatment of pulmonary tuberculosis patients and study also concluded that saturation with vitamin C neither contributes to recovery nor retards retrogression.

In this study low levels of vitamin E and C were observed in pulmonary tuberculosis. This might be due to malnutrition and exhaustion in attempt to neutralize heavy load of free radicals in these patients. Therefore supplementation with antioxidant vitamins like vitamin E and C may be necessary in the management of pulmonary tuberculosis.

Overall findings of the study suggest that there was significantly increased oxidative stress in patients with pulmonary tuberculosis assessed by raised MDA and significant decrease in the antioxidant enzymes like erythrocyte SOD, GSH Px and catalase. Also non enzymatic antioxidants like vitamin E and C in the cases of pulmonary tuberculosis were significantly decreased when compared to healthy controls. Low levels of enzymatic and non-enzymatic anti-oxidants and high levels of lipid peroxidation product like MDA may be due to heavy load of free radicals which cause the damage leading to oxidative stress. Decreased levels of Vitamin E and C may be due to malnutrition and exhaustion in an attempt to neutralize the heavy load of free radicals in these patients.

Nutritional supplementation may represent a novel approach for fast recovery among these patients with tuberculosis and hence the therapeutic benefit of exogenously administered antioxidant like vitamin C, tocopehrols further need to be assessed under carefully controlled clinical setup on a large sample.

CONCLUSION

This study was conducted in the Department of Biochemistry, KLES Dr. Prabhakar Kore Hospital and Medical Research Centre, Belgaum on 50 healthy individual and 50 cases of pulmonary tuberculosis.

The findings of the study suggest that there was significantly increased oxidative stress in patients with pulmonary tuberculosis assessed by raised MDA and significant decrease in the antioxidant enzymes like erythrocyte SOD, GSH Px and catalase. Also non enzymatic antioxidants like vitamin E and C in the cases of pulmonary tuberculosis were significantly decreased when compared to healthy controls. These findings suggest a significant association between oxidative stress and antioxidant defense mechanism.

SUMMARY

The objectives of the present study were to find out the oxidative stress in genesis of pulmonary tuberculosis, assess the role of antioxidant defense in the pathophysiology of the disease process and to correlate the oxidative stress and antioxidant defense mechanism in pulmonary tuberculosis.

The present case control study was conducted at Department of Biochemistry, KLES Dr. Prabhakar Kore Hospital and Medical Research Centre, Belgaum between February 2009 to January 2010 on 50 cases of primary pulmonary tuberculosis and 50 healthy controls in the age of group of 25 to 50 years of both sexes. Ten ml of blood was collected from the patients immediately after admission. Samples were collected under aseptic precautionary measures by using disposable syringe and transferred to heparin containing vials. Out of this one ml of whole blood was used for estimation of MDA and one ml was used for the preparation of hemolysate. Enzymes like SOD, GSH Px and catalase were analyzed from the hemolysate within one hour and the remaining sample of blood was centrifuged immediately and plasma was obtained for estimating vitamin E and vitamin C.

The mean age of the controls was 33.7 ± 5.61 years and in cases it was 34.8 ± 6.39 years. The mean BMI in the control group was 24.3 ± 3.06 kg/m^2 and 21.1 ± 2.53 Kg/m^2 in patients with pulmonary tuberculosis. The mean MDA levels in the control were 5.2 ± 1.42 nmol/ml and in the cases it was 14.2 ± 2.21 nmol/ml. The enzymatic antioxidants SOD, GSH Px and catalase levels in controls was 882.1 ± 116.81 IU/gmHb, 19.2 ± 1.67 IU/gmHb and 5.9 ± 1.40

IU/gmHb and in cases it was 504.1 ± 116.24 IU/gmHb, 8.8 ± 2.22 IU/gmHb and 3.7 ± 1.02 IU/gmHb respectively. The non enzymatic antioxidant vitamin E and C levels in the control group were 0.92 ± 0.16 mg/dL and 1.01 ± 0.18 mg/dL, and in cases it was 0.31 ± 0.16 mg/dL and 0.43 ± 0.16 mg/dL respectively. There was a significant increase ($p<0.001$) in the MDA levels in the cases compared to the controls. There was a significant decrease in enzymatic antioxidants SOD, GSH Px and catalase levels in the cases compared to controls ($p<0.001$). Also non enzymatic antioxidants like vitamin E and C in the cases of pulmonary tuberculosis were significantly decreased when compared to healthy controls ($p<0.001$).

The findings of this study suggest a significant association between oxidative stress and antioxidant defense mechanism among patients tuberculosis.

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ANNEXURE I - CONSENT FORM

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

Mr./Mrs/Ms. …… you are invited to participate in our research study that is a study to know the oxidative stress and anti oxidant status in primary pulmonary tuberculosis.

Participation in this study is completely voluntary. About 50 patients and equal number of healthy volunteers will be enrolled in this study at J. N. Medical College, Belgaum under the supervision of Dr. **** ***** Professor, Department of Biochemistry, Jawaharlal Nehru Medical College, Belgaum. The study will be carried out by Dr. ***** ******* Post Graduate Student, Department of Biochemistry, KLE University, Belgaum for her M.D. dissertation is to be submitted to KLE University, Belgaum.

PURPOSE OF THE STUDY

The incidence and mortality cases due to pulmonary tuberculosis are increasing in India. Hence this work is undertaken to know the oxidative stress and anti oxidant status in primary pulmonary tuberculosis.

PROCEDURE

For both the groups that is newly diagnosed pulmonary tuberculosis patients (Cases) and healthy subjects (Controls) 10 ml of venous blood will be collected under aseptic precautionary measures using sterile disposable syringe.

RISKS

Since the blood is drawn under aseptic precautionary measures by trained persons there is no scope for any risks. Further only small volume of blood is

collected which will be spontaneously replenished in the body. However there may be minor risks associated with having blood drawn that may include bruising, redness, discomfort or bleeding at the puncture site.

BENEFITS

No direct benefit is guaranteed to you from participating in our study. You can make use of blood levels of studied parameters if desired.

OPTIONS

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

NEW INFORMATION

Does not apply to this research.

PRIVACY AND CONFIDENTIALITY

All information collected about you during the course of the study will be kept confidential to the extent permitted by law. You will be identified in this research record by the code numbers. Information which identifies you personally will not be revealed without your written permission. However your records may be revealed to the sponsor of the study. Information from this study may be published but your identity will be confidential in any publication.

INSTITUTIONAL POLICY

In the event that you are physically injured as a result of participating in this research emergency care will be available. There is no commitment to provide any compensation for research related injury. The Jawaharlal Nehru Medical College will provide, within the limitations of the laws of the state of Karnataka, facilities and medical attention to subjects who suffered any harm as the result of your participation in this study. In the event you believe that you

have suffered any how as a result of your participation in this study you may contact research guide Dr. **** ****** Professor, Department of Biochemistry.

COST FOR PARTICIPATION

You will not be charged for the test to be carried out on your blood sample.

FINANCIAL INCENTIVE FOR PARTICIPATION

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

VOLUNTARY PARTICIPATION/WITHDRAWAL

If you decide not to participate in this study, it will not affect the quality of the medical care you receive at this institution.

You may withdraw from the study anytime. The researchers might use the information learned from the study in scientific journal articles or in presentations.

In case you have any questions regarding your rights as a study participant, you may please contact Principal, Jawaharlal Nehru Medical College, KLE University, Belgaum and Chairman of Jawaharlal Nehru Medical College, Institutional Ethics Committee of Human Subjects Research, Telephone No. *********.

EMERGENCY PROVISION

If you have questions as a participant in our study, you can contact the study investigator Dr. *** *****, or the research guide Dr. **** ***** Professor, Department of Biochemistry, Phone No. ****** ******.

CONSENT TO PARTICIPATE IN A RESEARCH TRIAL

I voluntarily agree to take part in this study. If I choose to take part in the study, I may withdraw at anytime. I am not giving any of my legal right by signing this form. My signature below indicates that I have read, or had read to me, this entire consent form including the risks and benefits. I may ask questions at any time.

_____ _____

Signature of participant Date

_____

Participants Name (Printed):

_____ _____

Name and Signature of witness-1 Date

_____ _____

Name and Signature of witness-2 Date

_____ _____

Signature of researchers or Person obtaining consent Date

ANNEXURE II

QUESTIONNAIRE (PROFORMA) USED FOR COLLECTING THE DATA

"Oxidative stress and antioxidant status in primary pulmonary tuberculosis" – A case control study.

Name : Sex :

Age : Occupation :

IP No : DOA :

Address:

Presenting Complaints:

C/o fever, evening rise of temperature

C/o of Cough with expectoration

Onset : Duration :

Aggravating and relieving factors

C/o weight loss

C/o dyspnoea

Palpitations

Giddiness / Syncope

C/o haemoptysis

Past history

Similar complaints

Family history

Similar complaints in any of family members.

Personal history

Smoking and alcohol consumption

Drug intake

Sleep

Appetite

Bowel and bladder habits

General Physical Examination

Pallor :

Pedal oedema :

Nail clubbing :

Cyanosis :

Icterus :

Lymphadenopathy:

Vitals

Pulse rate :

Blood pressure:

Respiratory rate:

Temperature :

SYSTEMIC EXAMINATION

Respirator System

Inspection :

Palpation :

Percussion :

Auscultation :

CVS - Peripheral

Pulse :

Blood pressure:

Other signs of CCF:

CVS – Central

Inspection :

Palpation :

Percussion :

Auscultation :

Central nervous system

Inspection :

Palpation :

Per abdomen

Inspection :

Palpation :

Percussion :

Auscultation :

Investigations

Blood

Hb% : RBS :

TC : FBS :

DC : PPBS :

ESR :

Urine

Sugar :

Microscopy

Chest X-ray PA view

HIV ELISA

Sputum examination

Final diagnosis

CONTROL GROUP

Sl. No.	Name	Sex	Age (Years)	BMI (Kg/m2)	Hb (gm%)	Malon-dialdehyde	Super oxide dismutase	Glutathione Peroxidase	Catalase	Vitamin C	Vitamin E
1	Preeti	F	26	20.18	12.37	5.89	609.04	18.19	4.63	1.18	1.03
2	Shikha	F	28	28.32	11.25	6.39	931.36	19.07	5.62	0.92	0.61
3	Tejaswini	F	27	27.36	12.31	3.85	846.12	19.07	6.33	0.96	0.67
4	Bharati	F	32	23.43	12.50	5.51	1011.28	18.15	5.43	0.86	0.62
5	Kasturi	F	36	25.70	11.22	6.22	862.13	23.89	3.47	1.13	0.93
6	Ranjeeta	F	30	2.64	12.34	5.37	821.31	21.17	4.92	1.07	0.88
7	Poornimka	F	28	22.20	12.11	6.93	801.23	20.19	6.11	1.06	1.07
8	Rukmini	F	40	25.70	13.06	4.49	921.12	21.87	5.13	0.83	0.98
9	Girija	F	42	19.80	12.24	6.91	1001.36	17.64	5.21	0.83	0.73
10	Arati	F	44	22.10	13.12	3.95	1063.35	20.19	4.67	0.80	0.61
11	Shilpa	F	29	19.35	15.16	4.43	996.12	19.76	5.66	1.13	0.89
12	Rohini	F	30	24.80	12.23	4.27	932.18	16.87	6.33	1.23	0.99
13	Bhavana	F	32	26.25	12.36	6.32	766.23	17.28	7.67	1.11	1.10
14	Jayashree	F	36	28.12	12.28	5.51	852.12	20.96	8.07	1.13	1.01
15	Neetymangala	F	47	19.96	13.04	6.12	896.13	18.82	5.83	0.96	0.93
16	Deepa	F	34	22.12	12.36	6.25	852.10	18.36	5.41	0.93	1.10
17	Seema	F	36	23.43	11.14	6.11	698.20	20.10	7.62	0.96	0.89
18	Survarna	F	30	27.36	12.40	3.39	821.23	20.21	6.63	0.99	0.92
19	Harshita	F	29	29.81	12.10	4.93	691.21	19.86	8.12	1.21	0.96
20	Kalpana	F	34	26.22	10.12	3.86	593.36	17.96	8.17	1.31	1.21
21	Parvathi	F	46	25.70	12.06	2.56	932.40	18.63	5.89	0.67	0.97
22	Urmila	F	28	20.11	10.38	6.11	890.12	17.36	6.78	0.51	0.93
23	Neeta	F	27	23.60	10.36	5.12	932.80	17.96	5.31	0.81	0.86
24	Sulochana	F	32	27.13	11.62	4.32	839.10	16.28	5.32	0.93	1.24
25	Geeta	F	36	20.18	12.13	2.68	826.06	15.15	4.36	1.21	1.20
26	Manjunath	M	28	22.89	12.75	5.28	980.00	18.12	5.51	1.18	1.00
27	suresh W	M	43	26.23	13.34	5.89	925.00	17.39	7.67	0.71	0.61
28	B Desai	M	42	29.32	14.37	7.37	1011.20	19.29	3.07	0.83	0.66
29	Chanappa	M	39	28.16	11.25	6.39	927.00	21.36	8.07	1.00	0.62
30	Musah	M	28	25.70	12.37	7.28	998.00	20.12	8.06	1.07	0.93
31	Basangouda	M	36	24.13	13.31	3.85	1111.00	18.19	5.83	1.10	0.88
32	Ramnath	M	34	26.12	14.12	5.51	890.00	19.26	5.41	0.96	1.02
33	Ramesh	M	29	20.23	12.49	6.22	937.00	20.12	7.62	0.83	0.97
34	Gautam	M	27	23.48	13.11	6.93	1015.00	20.14	6.63	0.83	1.07
35	Aditya	M	30	26.12	11.22	4.43	932.40	17.67	8.12	0.89	0.98
36	Shivanand	M	28	19.68	15.00	4.57	835.40	19.81	8.17	0.96	0.73
37	Sidingesh	M	32	28.64	14.31	6.32	830.10	19.64	5.89	1.02	0.61
38	Arjun	M	34	27.32	12.11	5.51	845.10	20.21	6.78	1.12	0.89
39	Hanumanth	M	36	28.26	13.16	6.12	609.04	20.96	5.31	1.26	0.99
40	Anand	M	32	26.41	14.20	3.81	786.61	18.89	5.32	0.96	1.03
41	Nagraj	M	27	22.35	13.06	4.63	933.41	18.36	4.31	0.80	1.01
42	Monesh	M	31	21.64	13.61	5.12	859.10	20.11	5.73	1.13	0.93
43	Dhanraj	M	41	22.44	14.12	3.39	931.13	19.89	4.63	1.23	1.10
44	Sanjay	M	42	20.18	12.81	6.42	996.67	17.96	6.33	1.31	1.00
45	Prakash	M	39	26.22	12.33	3.82	1012.10	18.63	6.79	0.93	0.93
46	Imran	M	36	24.28	14.12	2.68	939.32	17.36	7.12	1.34	0.93
47	Vikram	M	29	20.16	13.61	3.49	693.78	20.01	5.43	1.29	0.76
48	Ravishankar	M	30	22.22	14.62	6.83	763.99	19.07	6.76	0.96	0.82
49	Bhushan	M	38	27.37	12.93	8.92	941.11	18.15	3.39	1.13	0.99
50	Shekhar	M	34	20.25	13.67	2.62	912.12	23.87	2.83	1.21	1.12

CASES GROUP

Sl. No.	Name	Sex	Age (Years)	BMI (Kg/m2)	Hb (gm%)	Malon-dialdehyde	Super oxide dismutase	Glutathione Peroxidase	Catalase	Vitamin C	Vitamin E
1	Hemant	M	36	23.48	11.30	14.12	676.17	11.49	3.96	0.42	0.41
2	Basantray	M	28	26.12	10.23	13.98	496.21	9.96	3.94	0.21	0.23
3	Malappa	M	29	19.67	9.32	12.81	438.12	11.13	4.12	0.31	0.49
4	Devendra	M	31	26.38	11.47	10.96	432.11	9.62	3.83	0.21	0.29
5	Shivrudrappa	M	48	27.32	10.16	14.62	511.13	9.64	3.64	0.36	0.21
6	ganesh	M	38	28.66	10.38	12.16	492.11	9.43	4.83	0.41	0.26
7	Devappa	M	36	22.35	10.08	10.86	496.20	9.69	4.21	0.42	0.36
8	Sharanbasappa	M	43	21.26	10.69	11.43	556.50	8.96	3.12	0.41	0.28
9	Hanumantappa	M	40	20.18	12.31	12.13	532.20	8.81	4.31	0.61	0.31
10	Shivkumar	M	42	19.89	11.93	16.54	683.10	9.12	3.43	0.51	0.41
11	Phakirapa	M	47	18.86	12.12	12.13	491.20	12.16	2.89	0.63	0.29
12	Hiremath	M	29	20.13	10.61	14.61	381.21	8.93	3.96	0.53	0.19
13	Basavaraj	M	30	22.16	11.11	15.67	812.10	10.93	4.12	0.49	0.39
14	Dananjaya	M	40	21.38	9.68	13.62	569.28	12.32	2.96	0.31	0.29
15	Narayan	M	28	20.86	9.70	16.15	697.11	10.11	2.98	0.56	0.39
16	Gouramma	F	28	22.36	9.80	16.12	624.34	9.61	3.12	0.42	0.41
17	Savitri	F	32	19.68	9.60	14.72	697.31	9.12	2.91	0.56	0.21
18	Basawwa	F	42	19.77	8.60	15.42	624.11	8.67	3.14	0.39	0.24
19	Devakibai	F	47	18.68	9.70	13.96	697.17	9.96	2.98	0.56	0.62
20	Geeta D	F	29	20.12	12.10	15.89	717.14	12.19	6.32	0.46	0.47
21	Gayatri	F	34	22.38	9.60	8.67	620.15	16.32	3.38	0.32	0.41
22	Shreedevi	F	38	26.38	12.30	10.93	438.12	10.13	4.97	0.21	0.51
23	Sharanawwa	F	29	20.18	9.68	12.68	683.20	9.61	2.98	0.67	0.92
24	Rekawwa	F	26	19.18	7.30	9.93	512.34	8.31	3.12	0.71	0.46
25	Mahadevi	F	34	18.36	12.30	9.96	596.12	10.12	4.66	0.39	0.82
26	Tukaram	M	41	20.16	9.80	17.61	369.38	9.23	4.12	0.31	0.21
27	Harinath	M	36	18.36	9.90	18.21	256.32	10.26	3.23	0.34	0.19
28	Rajendra	M	32	19.38	11.43	16.22	521.21	6.21	2.21	0.29	0.21
29	Somanath	M	29	20.28	10.12	17.93	401.22	10.23	2.21	0.21	0.18
30	Lakshmikanth	M	43	19.96	10.93	15.28	425.28	7.23	3.19	0.41	0.15
31	Klrishnakumar	M	46	23.48	10.68	14.67	493.39	6.96	3.23	0.61	0.32
32	Ravi Pujar	M	34	22.16	11.42	13.39	521.12	7.12	4.16	0.52	0.12
33	Hanumanthappa	M	35	19.28	9.63	16.26	502.10	5.47	4.91	0.53	0.13
34	Phirsab	M	39	26.24	8.96	13.96	565.23	5.12	5.26	0.71	0.21
35	Malangouda	M	41	22.36	8.12	14.33	335.21	8.93	3.12	0.33	0.19
36	Basavraj	M	28	19.36	9.17	18.21	432.18	12.15	2.37	0.39	0.31
37	Umesh	M	29	18.36	10.26	16.23	521.23	8.10	3.06	0.33	0.12
38	Chandrashekhar	M	26	20.12	10.43	14.67	368.13	6.28	4.46	0.62	0.31
39	Irappa	M	30	19.56	11.04	16.53	415.21	6.66	2.31	0.61	0.31
40	Andaneppa	M	31	18.38	12.31	13.21	450.33	6.54	5.32	0.69	0.21
41	Veeresh	M	34	22.16	9.10	10.96	430.21	6.89	4.21	0.21	0.12
42	shankar	M	37	19.42	10.24	14.31	492.11	6.39	2.09	0.12	0.23
43	Malapa	M	44	20.38	10.10	15.21	379.36	7.92	6.97	0.71	0.21
44	Kashawwa	F	32	20.26	9.12	13.96	384.23	7.01	4.93	0.61	0.33
45	Razia	F	39	20.69	8.96	16.12	412.15	9.21	3.23	0.21	0.23
46	Savita	F	29	20.72	7.98	14.38	405.12	5.09	2.91	0.43	0.30
47	Gulabi	F	27	21.38	8.31	13.39	368.23	5.63	3.63	0.31	0.31
48	Bharati	F	29	19.16	7.63	14.45	393.12	6.62	3.06	0.12	0.11
49	Roopmati	F	40	19.68	9.24	15.16	399.00	6.69	4.91	0.43	0.21
50	Mallawwa	F	26	18.12	9.36	13.93	496.12	7.84	4.21	0.21	0.22

ANNEXURE III

KEY TO MASTER CHART

BMI	-	Body Mass Index
F	-	Female
Hb	-	Haemoglobin
Kg	-	Kilogram
M	-	Male
m	-	Meter
Sl. No.	-	Serial Number