"LIPID PEROXIDATION AND ANTIOXIDANT STATUS IN VEGETARIANS AND NON-VEGETARIANS- A CROSS SECTIONAL STUDY"

By

DR. MANJUNATH S SOMANNAVAR

DISSERTATION

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Under the guidance of
DR. M. V. KODLIWADMATH. M.D.

DEPARTMENT OF BIOCHEMISTRY

JAWAHARLAL NEHRU MEDICAL COLLEGE,

BELGAUM-590010.

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Date:
Place: **Belgaum.**

Signature of the Candidate
Dr. MANJUNATH.S. SOMANNAVAR.

KLE UNIVERSITY BELGAUM,
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Date:
Place: **Belgaum.**

Signature of the Guide
Dr. M. V. KODLIWADMATH.
MD
Professor,
Department of Biochemistry,
J. N. Medical College,
Nehru Nagar, Belgaum-590010.

KLE UNIVERSITY BELGAUM,
KARNATAKA.

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Seal & Signature of the
HOD
Dr. P. B. Desai M.D.
Professor and Head,
Department of Biochemistry,
J. N. Medical College,Nehru Nagar,
Belgaum-590010.

Date:
Place: **Belgaum**.

Seal & Signature of the
Principal
Dr.V. D. Patil MD
Principal,
J. N. Medical College,
Nehru Nagar,
Belgaum-590010.

Date:
Place: **Belgaum**.

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Signature of the Candidate
Dr. MANJUNATH S SOMANNAVAR.

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Date:

Place: Belgaum. **Dr. MANJUNATH S SOMANNAVAR**

LIST OF ABBREVIATIONS USED

CCl_4	-	Carbontetrachloride
$^{\bullet}CCl_3$	-	Trichloromethyl radical
Cl^-	-	Chloride
D/w	-	Distilled water
DNA	-	Deoxy ribonucleic acid
FAD	-	Flavin adenine dinucleotide
Fe	-	Iron
Fe^{2+}	-	Ferrous ion
Fe^{3+}	-	Ferric ion
FR	-	Free radical
g or gm	-	Gram
GR	-	Glutathione reductase
GSH	-	Reduced glutathione
GSH Px	-	Glutathione peroxidase
GSSG	-	Oxidised glutathione
H^+	-	Hydrogen ion
HCl	-	Hydrochloric acid
H_2O_2	-	Hydrogen peroxide
Hb	-	Hemoglobin
H_2O	-	Water
HOCl	-	Hypochlorous acid
IU	-	International units
KD	-	Kilodalton

Kg	-	Kilogram
L	-	Litre
$LOO^{\bullet}$	-	Peroxyl radical
mmol	-	millimol
MDA	-	Malondialdehyde
µmol	-	micro mol
µg/dL	-	micro gram / deci litre
mg/dL	-	milli gram / deci litre
min	-	minute
ml	-	milli litre
Mn	-	Manganese
MPO	-	Myeloperoxidase
nmol	-	nano mol
NAD	-	Nicotinamide adinine dinucleotide
NADH	-	Reduced nicotinamide adinine dinucleotide
NADP	-	Nicotinamide adinine dinucleotide phosphate
NADPH	-	Reduced nicotinamide adinine dinucleotide phosphate
ng	-	nanogram
$NO^{\bullet}$	-	Nitric oxide
nm	-	nano metre
$O_2^{\bullet-}$	-	Superoxide anion radical
$^{\bullet}OH$	-	Hydroxyl radical
$^1O_2$	-	Singlet oxygen
OFR	-	Oxygen free radical
OIs	-	Oxygen intermediates

$ONOO^-$	-	Peroxynitrite anion
PABA	-	Para-amino benzoic acid
PUFA	-	Polyunsaturated fatty acids
$R^\bullet$	-	Lipid radical
RNA	-	Ribonucleic acid
RBC	-	Red blood cell
ROM	-	Reactive oxygen metabolites
$ROO^\bullet$	-	Lipid peroxyl radical
ROOH	-	Lipid hydroperoxide
ROS	-	Reactive oxygen species
$RS^\bullet$	-	Thiyl radical
Se	-	Selenium
SOD	-	Superoxide dismutase
TBARS	-	Thiobarbituric acid reactive substances
TBA	-	Thiobarbituric acid
UV	-	Ultraviolet
Vit A	-	Vitamin A
Vit C	-	Vitamin C
Vit E	-	Vitamin E
Zn	-	Zinc

Abstract

Background and Objectives:

The diet is a key environmental factor implicated in health and disease. Oxidative stress, antioxidant status and their relation to diet is a subject of interest in recent years. The objective of the study was to compare lipid peroxidation and antioxidant status in healthy vegetarians and non-vegetarians.

Methods:

The present study comprises100 healthy individuals (50 vegetarians and 50 non-vegetarians) residing in Belgaum urban area. All the participants were in the age group of 40-60 years of both sexes. This cross-sectional study was done in one year period from April 2007 to March 2008. Malondialdehyde (lipid peroxidation product) was estimated by Thiobarbituric acid method, Glutathione Peroxidase by Beutler's method, Vitamin A and Vitamin E by Bessay et al and Quife et al methods respectively.

Results:

The blood MDA (in nmol/ml) in lacto-vegetarians was 3.76±1.57 while in lacto-ovo-vegetarians 3.97±1.28 where as 7.29 ± 0.86 in non-vegetarians. The erythrocyte Glutathione peroxidase (IU/g of Hb) in lacto-vegetarians was 19.70 ± 2.60 while in lacto-ovo-vegetarians 19.79 ± 2.62 and 13.21± 3.11 in non-vegetarians. The mean plasma vitamin A (µg/dl) in lacto-vegetarians was 35.61 ± 7.91 while in lacto-ovo-vegetarians 34.36 ± 5.79 and 30.83 ± 5.65 in non-vegetarians. The mean plasma Vit E (mg/dl) in lacto-vegetarians was 0.80 ± 0.13 while in lacto-ovo-vegetarians 0.80 ± 0.12 where as 0.73 ± 0.08 in non-vegetarians.

Interpretation & Conclusion:

Our study revealed that the Blood MDA level was significantly increased (P value <0.001) in non-vegetarians compared to lacto-vegetarians and lacto-ovo-vegetarians. There was significant decrease in the level of enzymatic antioxidant Glutathione Peroxidase and non-enzymatic antioxidants Vitamin A and Vitamin E in non-vegetarians compared to lacto-vegetarians and lacto-ovo-vegetarians (P value < 0.001).

Results of the present study indicate that there was increased Lipid Peroxidation and low antioxidant status in non-vegetarians compared to vegetarians. The study concludes that vegetarian nutrition provides adequate antioxidants which effectively prevent free radical generation.

Key words: Vegetarian nutrition, antioxidants, lipid peroxidation

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INTRODUCTION

Oxygen and diet are playing a vital role in human life. Oxygen is vital to provide energy through numerous metabolic reactions. A small fraction of the oxygen is diverted to form reactive oxygen species [ROS] either accidentally or deliberately.1

ROS are products of partial reduction of oxygen and are highly reactive. These are hydroxyl radical, superoxide anion, hydrogen peroxide, hydroperoxyl radical, lipid peroxide radical, singlet oxygen, nitric oxide and peroxy nitrite.[2]

SOURCES OF ROS IN VIVO

a. Oxidation of food stuffs due to leak in electron transport chain in mitochondria.
b. β-oxidation of fatty acids.
c. Microsomal cytochrome P-450 metabolism of xenobiotic compounds.
d. Respiratory burst in the inflammatory cell during phagocytosis.
e. Arginine metabolism in macrophages.
f. Tissue specific enzymes eg, xanthine oxidase in purine degradation.
g. Peroxidation in leukocytes and platelets.
h. Ionizing radiation.[2]

Damaging Effects of ROS

1. Lipid -------lipid peroxidation results in membrane damage and loss of membrane function.
2. Protein ----loss of function and inactivation of enzymes.
3. DNA-------- mutation, cancer.

Oxidative stress has been defined as a disturbance in the equilibrium of pro-oxidant/antioxidant systems in intact cells. This implies that cells have intact prooxidant/antioxidant systems that continuously generate and detoxify oxidants formed during normal aerobic metabolism.[3]

Oxidative stress is imposed on cells due to

1. Increased oxidant generation
2. Decreased antioxidant protection
3. Failure to repair oxidative damage

However, cells have multiple protective mechanisms against oxidative stress by enzymatic antioxidants such as superoxide dismutase, catalase, glutathione peroxidase etc which scavenge the free radicals and protect the cells against oxidative damage. Natural or dietary antioxidants like vitamin E and A act as chain breaking antioxidants and vitamin C is an important aqueous phase antioxidant.

Oxidative stress in human disease is a subject of intense interest. In recent years, oxidative stress due to ROS is implicated in the pathogenesis of wide variety of diseases like Cancer, Cataract, Diabetes mellitus, Rheumatoid arthritis, Atherosclerosis, Viral autoimmune diseases and Aging.[4]

The association of vegetarian diets with lower risk for several chronic diseases has been well documented. The mortality rate is low in vegetarians who consume more fruits, vegetables, cereals, pulses and nuts than the non-vegetarians[5]

In the earlier studies it was found that vegetarians have a higher concentrations of antioxidant enzymes; Catalase and superoxide dismutase as compared to non-vegetarians[6,7]. Plasma lipid peroxidation product (MDA) levels were lower in vegetarians compared to non-vegetarians.[6] It is well documented that

vegetarians have higher blood levels of beta carotene, Vit C and Vit A than non-vegetarians.[3,5,6,7] Vegetarians weigh less and have low blood pressure than their non-vegetarian counterparts. It is proved that plant sources contribute adequate proteins, less total fat, saturated fat and cholesterol and more carbohydrates and fiber.[5] Concentration of trace elements like Folate and Magnesium are generally high among vegetarians with low levels of Iron and variable levels of Selenium and Zinc[5,8]

The reduced risk of diseases found among vegetarians suggests that biologic processes are influenced by diet.[5, 8] Therefore much attention is currently focused on the beneficial effect of vegetarian versus non-vegetarian diet.[3, 5, 6, 7]

The present study is planned to compare the lipid peroxidation and antioxidant status in healthy vegetarians and non-vegetarians. Oxidative stress is assessed by estimating the Malondialdehyde [lipid peroxidation product] in the blood. Antioxidant status is measured by estimating Glutathione peroxidase [enzymatic preventive antioxidant] and Vit A and Vit E [dietary antioxidants] in plasma.

OBJECTIVES

1. To estimate Malondialdehyde (MDA) a lipid peroxidation product and antioxidants Glutathione Peroxidase, Vitamin A and Vitamin E in vegetarians and non-vegetarians.

2. To compare the above parameters in vegetarians and non-vegetarians.

REVIEW OF LITERATURE

Adequate nutrition is a fundamental requirement for survival of every individual and species. The diet is a key environmental factor implicated in health and disease.

1. A vegetarian is one who does not eat animal flesh (meat, poultry and fish) but includes eggs and dairy products in his/her diet.

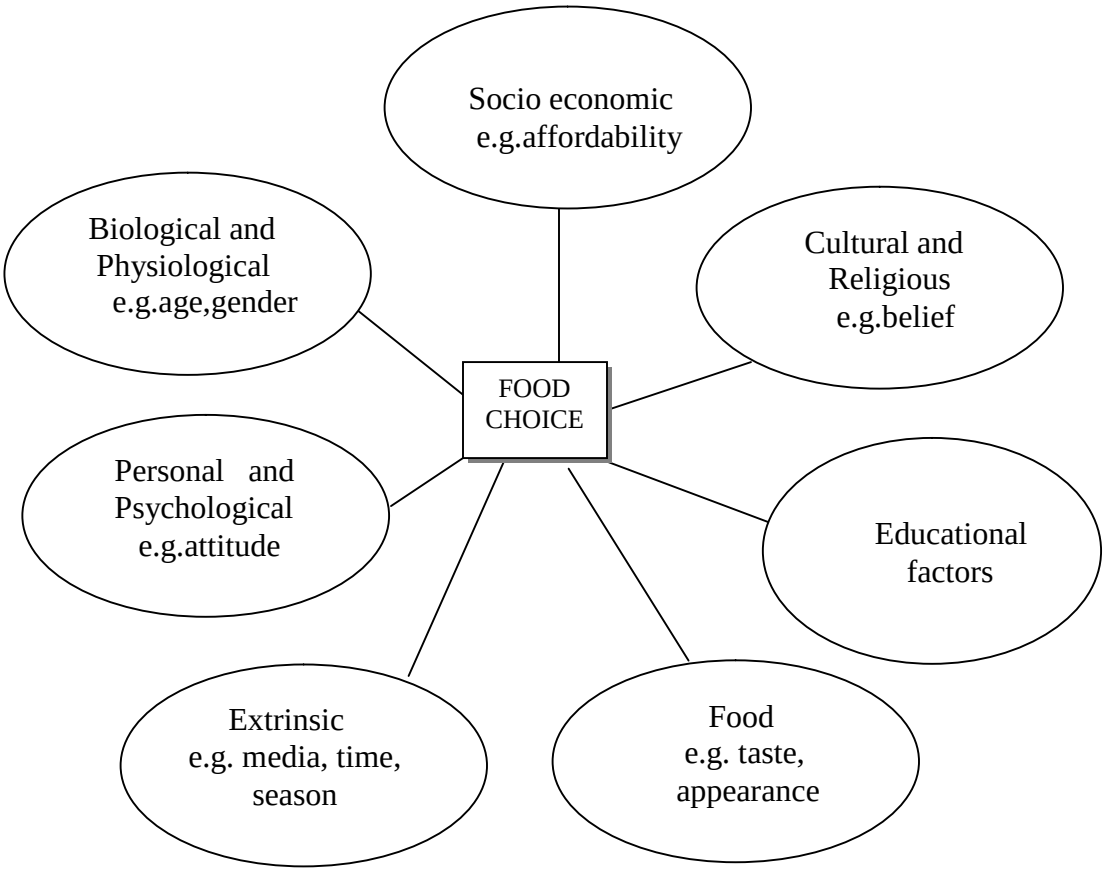
a. Lacto-vegetarians: include dairy products.

b. Lacto-ovo-vegetarians: include dairy products and eggs.

Cereals, grains, fruits, vegetables, legumes, nuts and seeds form the basis of vegetarian diets with or without eggs.

2. Non-vegetarian diet includes animal products such as meat poultry, fish and other sea foods.[5, 8]

Figure no.1 The factors affecting dietary variation[9]



Components of diet influencing various disorders [10]

DISORDER	Possible beneficial influences	Possible deleterious influences
Coronary heart disease	Complex carbohydrates, particular fatty acids (e.g. monounsaturated, polyunsaturated and w-3 fatty acids from fish) soluble fiber, antioxidants(vitamins E,C,β-carotene, selenium) folic acid, moderate alcohol, Soya protein, isoflavones	Saturated fat, cholesterol, excess calories, sodium, animal protein, abdominal distribution of body fat
Cancer	Fruits and vegetables ,fiber	Excess calories, fat, alcohol, red meat, salt and nitrite-preserved meats , abdominal distribution of body fat
Stroke	Potssium,calcium,w-3 fatty acids	Sodium ,alcohol
Diabetes mellitus	Fiber	Excess calories ,fat, alcohol, abdominal distribution of body fat
Chronic liver disease		Alcohol
Atherosclerosis	Particular fatty acids(for example monounsaturated and w-3 fatty acids) soluble fiber, antioxidant vitamins	Saturated fat, cholesterol
Obesity		Excess calories and fat
Hypertension	Potassium, calcium w-3fatty acids, fruits and vegetables	Sodium, alcohol, excess calories and saturated and total fat, abdominal distribution of fat
Osteoporosis	Calcium ,vitamin D, magnesium	Sodium, protein
Diverticular disease, constipation	Fiber	

Dietary constituents influencing the nutritional status of an individual:

Proteins; are the nitrogen containing macromolecules of paramount importance for biological systems. All the major structural and functional aspects of the body are carried out by proteins. They are the source of essential amino acids that are necessary for the synthesis of a wide variety of biologically important compounds like enzymes, hormones, cell membrane, neurotransmitters etc. 10-15% of total energy is derived from proteins. The recommended dietary allowance is 1g/kg body wt/day for a healthy adult.[8, 9]

Protein rich foods are obtained primarily from animal flesh/or products such as eggs and milk. Proteins of animal origin are generally of higher biological value than the vegetable ones, which are deficient in one or more of essential amino acids. Most plant foods are relatively poor in protein. Dairy products, whole grain, beans, nuts and seeds are important protein sources in lacto-vegetarians. Plant proteins in natural form are less digestible than animal protein. Protein intake is slightly low in vegetarians compared to non-vegetarians.[8, 11, 12, 13,]

Current evidence suggests that less intake of animal protein is beneficial and may lower urinary calcium excretion and slow the progression of renal disease.[14]

Carbohydrates; are the chief energy source of the body. In addition, they serve as structural components of cells and constituents of nucleic acids and nucleotides. Carbohydrates play vital role in lubrication, cellular intercommunication, immunity and also involved in detoxification. 60-80% of calorie requirement of a healthy person is provided by carbohydrates. The recommended intake in balanced diet is 300-400g/day.In addition to calories, they also provide fiber. Cereals, pulses and tubers are the major

sources of starch (complex carbohydrate) in vegetarian diets. Simple carbohydrate (sugar) intakes are similar in both groups. Intakes of dietary fiber (non-starch polysaccharide) e.g. cellulose, lignin, pectin, mucilage etc in vegetarians are 50%-100% higher than non-vegetarians. Vegetables and green leaves contain soluble fiber which is more beneficial. Dietary fiber requirement is about 30g/day.Dietary fiber improves bowel motility, prevents constipation, decreases reabsorption of bile salts and improves glucose tolerance. High fiber diet is associated with reduced incidence of coronary heart disease, colon cancer, diabetes mellitus, diverticulosis and haemorrhoids.[11, 15]

Dietary Carbohydrates.[11]

class	**components**	**examples**	**Source**
Free sugars	Monosaccharides Disaccharides	Glucose, Fructose Sucrose, lactose, maltose	Intrinsic: fruits , milk, vegetables Extrinsic: beet or cane sugar
Short-chain carbohydrates	oligosaccharides	Maltodextrins,fructo-oligosaccharides	
Polysaccharides	Slowly digestible	Starch Dextrin Glycogen	Cereals(wheat, rice),root vegetables(potato) legumes(lentils,beans,peas) Animal (Liver, muscle)
Nonstarch polysaccharides	Fibrous Viscous	Cellulose Hemi cellulose Pectin, gums	Plants

CARBOHYDRATE CONTENT OF FOODS (% OF WEIGHT) [13]

FOOD STUFF	**CARBOHYDRATE** (% of Weight)
Concentrated sweets: Sugar ;cane ,beet	99.5
Maple	90-96
Candies	70-95
Honey(extracted)	82
Syrup ;Table blends, molasses	55-75
Jams, jellies ,marmalades	70
Carbonated ,sweetened beverages	10-12
Fruits :Prunes ,apricots ,figs(cooked)	12-31
Bananas, grapes ,cherries ,apples, pears	15-23
Fresh pineapples ,oranges ,apricots , strawberry	8-14
Skimmed Milk	6
Whole Milk	5

CARBOHYDRATE CONTENT OF FOODS (% OF WEIGHT) [13]

FOOD STUFF	**CARBOHYDRATE** (% of Weight)
Grain products: Starches; corn, tapioca ,arrowroot	86-88
Cereals; wheat ,oat ,bran	68-85
Flour; corn , wheat(sifted)	70-80
Popcorn(popped)	77
Cookies; Plain, assorted	71
Saltines	72
Cakes ;Plain without icing	56
Bread; white, rye, whole wheat	48-52
Macaroni, spaghetti, noodles, rice(cooked)	23-30
Cereals(cooked);Oat, wheat, grits	10-16
Vegetables: Boiled ;corn, white and sweet potatoes ,lima and dried beans peas	15-26
Beets ,carrots, onions ,tomatoes	5-7
Leafy ;lettuce ,asparagus, cabbage ,greens, spinach	3-4

Lipids; are efficient storage forms of energy. They serve as structural components of biomembranes, provide insulation and protect internal organs, act as metabolic regulators, surfactants, detergents and emulsifying agents. They are the important source of fat soluble vitamins; Vit A and VitE which act as natural antioxidants protecting the human body from superoxides.[8, 15]

Dietary fats provide 25-30% of total calorie intake. The recommended intake of fat is 50-75g/day (20% of total energy). The requirement of essential fatty acids ranges from 3-5% of energy intake. Animal fat (meat, chicken, butter) is predominantly saturated. A significant fraction of fatty acids ingested is contributed by either long chain saturated fatty acids (palmitic and stearic acid) or monounsaturated fatty acids (oleic and palmitoleic acids).[15]

The polyunsaturated fatty acids(PUFA) [linoleic, linolenic and arachidonic acids] are present in vegetable oils and fish oils .Eicosapentaenoic and docosahexaenoic acids(ω-3 series of PUFA) occur in fish oils which serve as inhibitors of thrombosis. Fish oils lower plasma triglyceride levels and may prevent coronary heart disease.[13,]

The proportion of energy derived from fat is slightly lower in vegetarians than in non-vegetarians. The intake of PUFA is usually greater in vegetarians because of their preference for nuts, oilseeds and vegetable oils. Plant foods do not contain any cholesterol; instead they contain vegetable sterols (phytosterols) which inhibit cholesterol absorption. Saturated fats found in animal fat raise serum cholesterol while unsaturated fats lower it.[8, 14, 15]

Excess PUFA may lead to production of free radicals that may be injurious to the cell.[15]

Calcium: is a major mineral element of the body. Total calcium in human body is about 1to 1.5Kg, 99% of which is seen in bone and 1% in extra cellular fluid. There is a dynamic equilibrium between calcium in blood and that in the skeleton; this equilibrium is maintained by the interaction of vitamin D, parathyroid hormone and calcitonin.[15, 16]

Ionized calcium in the plasma has many vital functions including formation of bones and teeth, coagulation of blood, contraction of mucles, cardiac action, milk production, keeping cell membranes intact and in metabolism of enzymes and hormones. It also plays a crucial role in the transformation of light to electrical impulses in the retina. Recommended daily allowance for adults is 400-500mg /day. The physiological requirements are higher in children, expectant and nursing mothers.[16]

Sources of calcium are milk and milk products (cheese, curd, skimmed and butter milk), eggs and fish. Leafy vegetables cereals and millets also provide small amount of calcium. Bioavailability of calcium from cereals and leafy vegetables is poor because of presence of phytates and oxalates which form insoluble compounds with calcium.[16]

Lacto-ovo-vegetarians have calcium intakes that are comparable to or higher than those of non-vegetarians.[17]

Vitamin D: The nutritionally important forms of vitamin D are ergocalciferol (vitD2) and cholecalciferol(vitD3). Ergocalciferol may be derived by irradiation of plant sterol (ergosterol).Cholecalciferol is the naturally occurring (preformed) vit D which is found in animal fats and fish liver oils. It is also derived from exposure of human body to UV rays of the sunlight which convert the 7-dehydrocholesterol in the skin to vit D.

Functions of Vit D and its metabolites.

Intestine; promotes intestinal absorption of calcium and phosphorous.

Bone ; stimulates normal mineralization, enhances bone resorption, affects collagen maturation.

Kidney; increases tubular reabsorption of phosphate, variable effect on reabsorption of calcium.

Others ; acts to regulate cell proliferation in a variety of tissues and is involved in secretion of number of hormones.

Vit D is unique as it is derived both from sunlight and foods.Vit D is synthesized by the body by the action of UV rays of sunlight on 7-dehydrocholesterol in the skin.Liver,egg yolk,butter,cheese and some species of fish contain useful amounts of vitD. Fish liver oils are the richest source of Vit D. Dietary sources are relatively unimportant compared with endogenous synthesis in the skin; problems of deficiency arise when there is inadequate exposure to sunlight.[16, 17, 18]

Low vitamin D levels were reported in some elderly vegetarians.[5]

Iron: is an important mineral necessary for many functions in the body including formation of haemoglobin, brain development and function, regulation of body temperature, muscle activity and catecholamine metabolism. Iron is a component of haemoglobin, myoglobin, cytochromes, catalase, tryptophan pyrrolase etc. It is essential for binding oxygen to the blood cells; central function is oxygen transport and cell respiration.[16]

There are two forms of iron, haem-iron and nonhaem iron.Haem-iron is better absorbed than nonhaem iron .Foods rich in haem iron are liver, meat, poultry and fish.

Foods containing nonhaem iron are those of vegetable origin e.g. cereals, green leafy vegetables, legumes, nuts, oilseeds, jaggery and dried fruits. Bioavailability of nonhaem iron is poor owing to presence of phytates, oxalates, carbonates, phosphates and dietary fiber which interfere with iron absorption. Recommended daily allowance for iron for an adult Indian is 10-20mg.[5, 16]

Although biomarkers of iron status, serum ferritin and transferrin saturation are reduced in vegetarians, the prevalence of iron deficiency is not higher among vegetarians compared to non-vegetarians.[5, 12, 16]

Zinc: is primarily an intracellular ion present in small amounts in all tissues. It is involved in a multitude of diverse catalytic, structural and regulatory functions. Zn is found in numerous enzymes (SOD), is a component of biomembranes, is thought to be necessary for RNA, DNA and ribosomal stabilization, is involved in binding of a number of transcription factors, stabilizes some hormone-receptor complexes etc. Clinical manifestations of Zn deficiency in humans.[19]

Growth retardation	Delayed sexual maturation
Hypogonadism, hypospermia	Delayed wound healing
Alopecia	Skin lesions
Impaired appetite	Immune deficiencies
Behavioral disturbances	Eye lesions (photophobia and night blindness)
Impaired taste	

Shell fish, beef and other red meats are good sources of Zn. Nuts, legumes and cereals are the primary plant sources. Recommended daily allowance for adults is 10mg.The reported Zn contents of vegetarians and non-vegetarian diets are similar. However,

plasma and tissue Zn concentrations are significantly lower in vegetarians than in non-vegetarians because of high content of phytates in vegetable diet.[5, 12, 16]

Vitamin –B12: is a complex organo-metallic compound with a cobalt atom. Vitamin B12 cooperates with folate in the synthesis of DNA. Also essential in synthesis of fatty acids in myelin. Sources are liver, kidney, meat, fish and eggs. Curd is the only source in lacto-vegetarians.It is not found in foods of vegetable origin. Recommended intake is 1-2 microgram/day.[15, 16]

Although dairy products and eggs contain Vit B12, research suggests that lacto-ovo-vegetarians have low blood levels of Vit B12.[8, 12, 14]

Folic acid: it plays a role in the synthesis of nucleic acids. It is also needed for the normal development of blood cells in the marrow. Sources are yeast, green leafy vegetables, cereals, pulses, oil seeds and eggs. Intake and blood levels of folate are often higher in vegetarians than non-vegetarians because of their greater use of fruits and vegetables.[14, 16]

Vitamin A is involved in many biological activities in the body.

1.It is indispensable for normal vision. It contributes to the production of retinol pigments which are needed for vision in dim light.

2.Essential for maintaining the integrity and normal functioning of glandular and epithelial tissue which lines intestinal, respiratory and urinary tracts as well as the skin and eyes.

3. It supports growth especially skeletal growth.

4. It is anti-infective; there is increased susceptibility to infection and lowered immune response in Vit A deficiency.[16, 18]

Vitamin A is widely distributed in animal and plant foods; in animal foods as preformed vitamin A (retinol) and in plant foods as provitamins (carotenes).Liver and fish liver oils constitute the most concentrated sources of preformed vitamin A. Cheap vegetable sources are green leafy vegetables (spinach and amaranth).It also occurs in most green and yellow fruits and vegetables (e.g. papaya, mango, pumpkin and carrots). Recommended daily intake is 600-750 micrograms for adults.[15, 18]

In the earlier studies plasma vitamin A level was more in vegetarians compared to non-vegetarians.[7, 20]

Vitamin E is the generic name for a group of closely related and naturally occurring fat soluble compounds, the tocopherols. It functions in vivo as a chain breaking antioxidant that prevents propagation of free radical damage in biologic membranes. It is a potent peroxyl radical scavenger and especially protects PUFAs within phospholipids of biological membranes and in plasma lipoproteins. α-tocopherol inhibits platelet aggregation and vascular smooth muscle proliferation. It also modulates transcription of a number of genes including scavenger receptor for oxidized LDL in macrophages and smooth muscle.[16, 18]

Vitamin E is widely distributed in foods. Richest sources are vegetable oils, cotton – seed, sunflower seed, egg yolk and butter. Foods rich in PUFA are also rich in vitamin E. Recommended dietary intake is 15-20 mg/day. Requirements increase with higher intake of PUFA.[15, 16, 18]

In previous studies, it was shown that, vegetarian diet maintains higher Vit E level than non-vegetarian diet.[6, 7]

IMPLICATIONS OF VEGETARIAN/NON-VEGETARIAN DIET IN DISEASES:

Scientific data suggest positive relationships between a vegetarian diet and reduced risk for several chronic degenerative diseases and conditions including obesity, coronary artery disease, hypertension, diabetes mellitus and some types of cancer.[19]

Studies indicate that vegetarians often have lower morbidity and mortality rates from several chronic degenerative diseases than do non-vegetarians.[11, 15,1]

Death rate ratios with 95% confidence intervals for vegetarians versus non-vegetarians. Key et al (1998)

	Death rate	ratios
Ischaemic heart disease	0.76	0.62-0.94
Cerebrovascular diseases	0.93	0.74-1.17
Stomach cancer	1.02	0.64-1.62
Colorectal cancer	0.99	0.77-1.27
Lung cancer	0.84	0.59-1.18
Breast cancer	0.95	0.55-1.63
Prostate cancer	0.91	0.60-1.39
All causes	0.95	0.82-1.11

Positive health effects of vegetarian diets may be due to intake of fruits and vegetables which provide antioxidants that might act as beneficial supplements in humans.

Cancer: Nutrients, non-nutritive dietary constituents and nutritional status can influence the risk for cancer in a variety ways. Nutrients interact with each step of

carcinogenesis (carcinogen activation, tumor initiation, promotion and progression).Humans are exposed to countless potential carcinogens and to many anticarcinogens each day through dietary and other means. Excess calorie intake may favor the generation of free radicals and reduce the body's ability to detoxify carcinogens. Antioxidant nutrients scavenge the free radicals and other (pre)carcinogens and inhibit their activation and/or their ability to initiate mutations.[10]

Much evidence indicates that consumption of fruits and vegetables is inversely associated with lung cancer risk in smokers and nonsmokers .It is probable that many nutrients in fruits and vegetables are partly responsible for the protective effects.[10, 19]

Incidence of colorectal cancer is lower in vegetarians than non-vegetarians. Reduced risk is associated with increased consumption of fiber, vegetables and fruit .[19]

Cross-sectional data indicate that breast cancer rates are lower in populations that consume plant based diets.[19]

Coronary heart disease: lower risk of death from CHD among vegetarian population is well established. Vegetarian diets offer disease protection benefits because of their lower saturated fat, cholesterol and animal protein content and often higher concentration of folate (which reduces serum homocysteine levels), antioxidants such as vitamins C, E and carotenoids and phytochemicals.Vegetarian diets have also been successful in arresting coronary artery disease. Total cholesterol and LDL cholesterol levels are usually lower in vegetarians, but HDL cholesterol and triglyceride levels vary depending on type of vegetarian diet consumed.[10, 19]

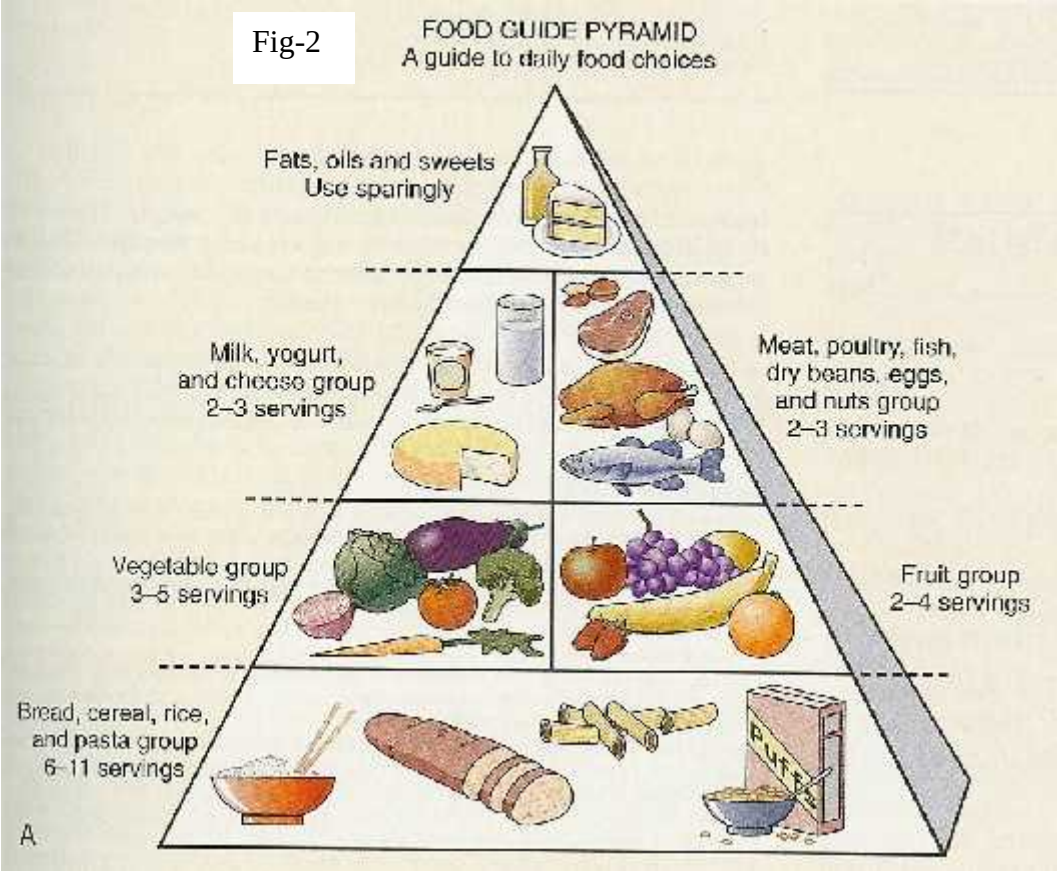
Hypertension: vegetarians tend to have a lower incidence of hypertension than in non-vegetarians. This effect appears to be independent of both body weight and sodium

intake. It is shown that a diet rich in fruits, vegetables and low dairy products and with reduced saturated and total fat content can decrease blood pressure levels.[10,15,19]The combined effects of several specific foods and or nutrients may be responsible for the lower blood pressure seen in vegetarians.

DIABETES MELLITUS: Vegetarians may be at less risk for developing diabetes than non-vegetarians. The various factors suggested as protective for diabetes found among vegetarians are lower serum cholesterol levels, high complex carbohydrate and fiber intake (improve glucose tolerance)and lower fat and animal protein intake.[15, 19]

It is also shown that type-2 diabetes mellitus is much less likely to be a cause of death in vegetarians than in non-vegetarians.[23]

Fig-2



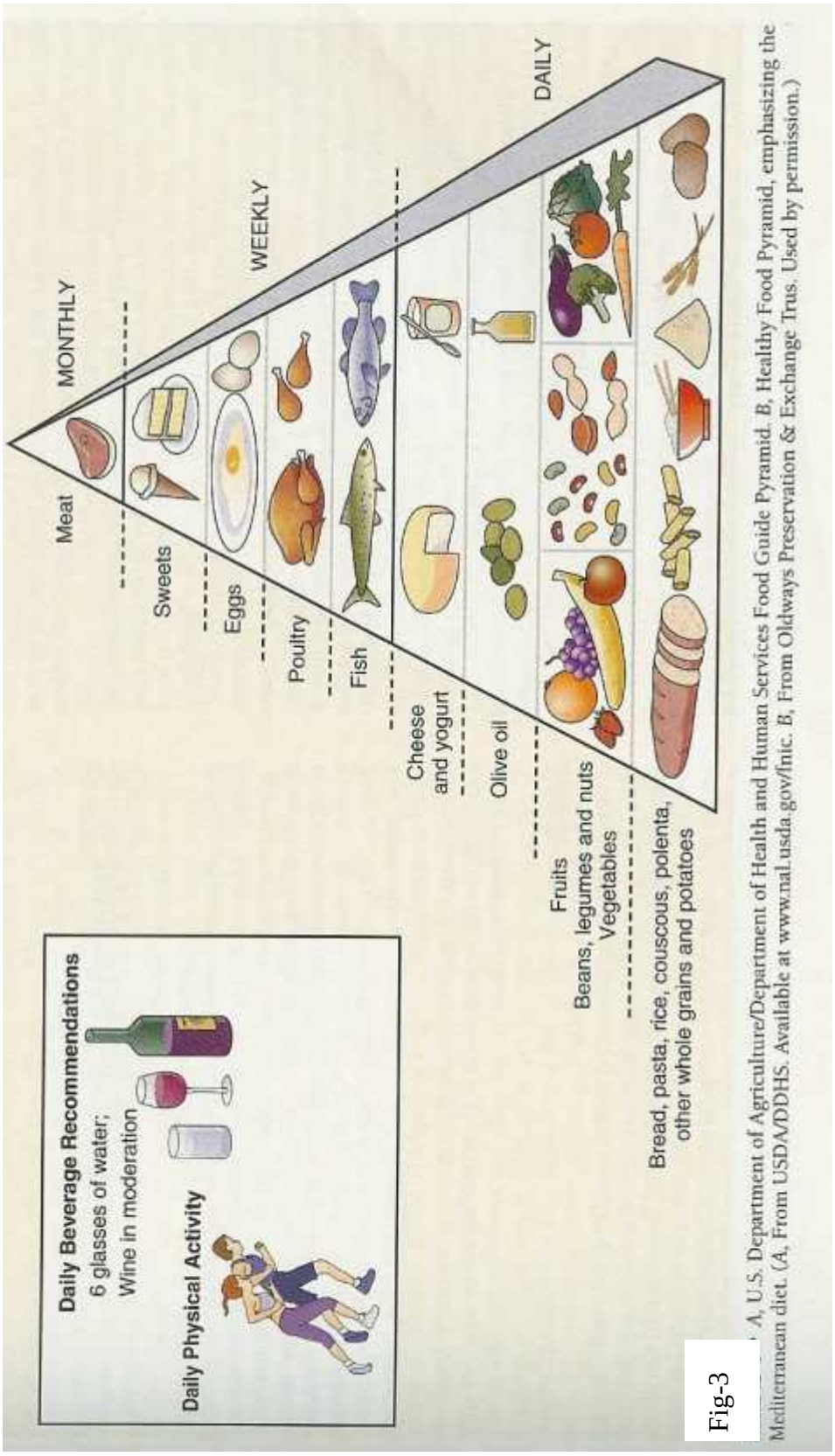


Fig-3

A, U.S. Department of Agriculture/Department of Health and Human Services Food Guide Pyramid. *B*, Healthy Food Pyramid, emphasizing the Mediterranean diet. (*A*, From USDA/DDHS. Available at www.nal.usda.gov/fnic. *B*, From Oldways Preservation & Exchange Trus. Used by permission.)

FREE RADICALS

A free radical is a molecule or molecular fragment that contains one or more unpaired electrons in its outer orbital.[21] A free radical is conventionally represented by a superscript dot ($R^{\bullet}$).

A compound becomes a free radical by gaining an additional electron, as in the case of reduction of molecular oxygen to superoxide anion radical ($O_2^{\bullet-}$). Other free radicals are hydroperoxyl radical ($HOO^{\bullet}$), hydroxyl radical ($^{\bullet}OH$), lipid peroxyl radical ($ROO^{\bullet}$). The sequential univalent reduction steps of oxygen may be represented as, [22]

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

Concept of Free Radical Generation:

a. Homolytic 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.

Characteristics:

Free radicals can be positively charged, negatively charged or electrically neutral

Extreme reactivity, Short life span

Generation of new ROS by chain reactions

Damage to various tissues.[23]

REACTIVE OXYGEN SPECIES (ROS)

The oxygen derived free radicals and related non-radical species are collectively known as reactive oxygen species (ROS).

Several reactive oxygen species (ROS) are known, among them the most prominent are:

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

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

Hydrogen Peroxide (H_2O_2):

Hydrogen peroxide is mainly produced by enzymatic reactions. These enzymes are located in microsomes, peroxisomes and mitochondria. Even in normal conditions, the hydrogen peroxide production is relatively important and leads to a constant cellular concentration between 10^{-9} and 10^{-7} M. In plant and animal cells, superoxide dismutase is able to produce H_2O_2 by dismutation of $O_2^{\bullet-}$, thus contributing to the lowering of

oxidative reactions. The natural combination of dismutase and catalase contributes to remove H_2O_2 and thus has a true cellular antioxidant activity. H_2O_2 is also able to diffuse easily through membranes.[26]

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

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

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

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

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

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

Singlet Oxygen ($^1O_2$):

This chemical form of oxygen is not a true radical but is reported to be an important ROS in reactions related to ultraviolet exposition (UVA 320-400 nm). Its toxicity is reinforced when appropriate photoexcitable compounds (sensitizers) are present with molecular oxygen.[29] Several natural sensitizers are known to catalyze

oxidative reactions such as tetrapyrroles (bilirubin), flavins, chlorophyll, hemoproteins and reduced pyridine nucleotides (NADH). Some of these sensitizers are also found in foods and cosmetics. Some others are used for therapeutic purposes (anti-cancer treatments) and are sensitive to visible light. The presence of metals contributes to increase the production of singlet oxygen, as well as anion superoxide, and thus accelerates the oxidation of unsaturated lipids generating hydroperoxides. It has been suggested that singlet oxygen may be formed during the degradation of lipid peroxides and thus may cause the production of other peroxide molecules. This singlet oxygen formation may account for the chemiluminescence observed during lipid peroxidation.[30]

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

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

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

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

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

Furthermore, they are able to oxidize NADH into NAD, ascorbic acid and to generate various free radicals ($^{\bullet}OH$ and $O_2^{\bullet-}$). These thiyl radicals may also be formed by homolytic fission of disulfide bonds in proteins.[32]

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 trichloromethyl radical ($^{\bullet}CCl_3$), which is able to react with oxygen to give several peroxyl radicals (i.e. $^{\bullet}O_2\ CCl_3$).[33]

Production of free radicals in cells

Free radicals are generally produced in cells by electron transfer reactions. These can be mediated by the action of enzymes or non-enzymatically, often through the redox chemistry of transition metal ions. Free radical production can either be accidental or deliberate.

ELECTRON TRANSPORT CHAIN; Free radicals are constantly produced during the normal oxidation of foodstuffs due to leaks in the electron transport chain in mitochondria. About 1-4% of oxygen taken up in the body is converted as free radicals.

ubiquinone ------ ubisemiquinone---- cytochrome c1 step

NADH dehydrogenase--- superoxides rapidly dismutate to form H_2O_2 . Some of the mitochondrial H_2O_2 may escape to cytosol.[34]

PURINE METABOLISM; Xanthine oxidase utilizes O_2 as the electron acceptor and produces superoxide while catalyzing the oxidation of hypoxanthine to uric acid.[35]

OXIDATIVE BURST IN PHAGOCYTES; NADPH oxidase located in the plasma membrane of neutrophils produces superoxides. Following spontaneous dismutation, superoxides are converted into H_2O_2 .Cytoplasmic azurophilic granules of neutrophils and to a lesser extent monocytes contain a hemoprotein peroxidase called myeloperoxidase, which are released during immune challenge or other stimuli; released myeloperoxidase complexes with H_2O_2 to form enzyme-substrate complex with an oxidizing potential. The complex oxidizes chloride (cl-) to produce hypochlorous acid (HOCL) that eliminates pathogenic infection. The oxidant force that kills pathogens is also cytotoxic to the host tissue and to the neutrophils. This accelerated generation of ROS by activated neutrophils is referred to as oxidative burst.[35]

DRUG METABOLISM; Microsomal and nuclear membrane electron transport systems, mainly involved in drug metabolism (via cytochrome P-450 and b5 systems) also host ROS production. These monooxygenases mediate the oxygenation of both endogenous products

(e.g. cholesterol, steroid hormones, fatty acids, prostaglandins) and foreign compounds including a large number of drugs, pesticides and environmental pollutants. Mechanism involves formation of oxy and subsequently peroxy intermediates. Breakdown of these intermediates yields ROS.[36]

NITRIC OXIDE SYNTHASE; $NO^{\bullet}$ is known to be implicated in a number of crucial physiological functions e.g. control of systemic blood pressure, respiration, digestion, penile erection ,platelet aggregation ,cerebral blood flow & neuronal synaptic plasticity. $NO^{\bullet}$ and its derivatives also contribute to microbicidal and tomoricidal activities of

macrophages and neutrophils. NO synthase in the endothelium and neurons is a calmodulin-activated enzyme that oxidizes arginine to citrulline in presence of biopterin, NADPH and oxygen. Cells like macrophages which are capable of producing both $NO^{\bullet}$ and superoxides are the likely host of a very powerful deleterious ROS, peroxy nitrite anion (.ONOO-). These are formed by reaction of $NO^{\bullet}$ with superoxide and they are relatively long lived ROS.[37]

TRANSITION METALS; Conditions such as plasma pH ≤6.0, haemolysis and ischemia perfusion etc lead to the release of transition metal ions (e.g. that of iron and copper) and remarkably amplify ROS toxicity. Iron and copper ions are capable of converting H_2O_2 to $^{\bullet}OH$. The hydroxyl radical is capable of initiating lipid peroxidation .In presence of free transition metal ions ascorbic acid functions as a pro-oxidant.[37]

CIGARETTE SMOKING AND ALCOHOLISM; enhance oxidative stress risk. Each puff of cigarette is estimated to contain 10^{14} free radicals in the tar phase and 10^{15} of them in gas phase. The vit E is consumed at a very high rate in the lungs of smokers and higher levels of lipid peroxidation have been observed in plasma of smokers.

The metabolism of ethanol produces acetaldehyde that is known to consume GSH. Ingestion of ethanol is associated with enhanced lipid peroxidation.[37]

OTHER SOURCES; other enzymes known to be responsible for generation of H_2O_2 or superoxide anion are[37]

Glycolate oxidase (peroxisome)

L-α-hydroxy acid oxidase (peroxisome)

L-gulonolactone oxidase(cytosol)

Aldehyde oxidase (cytosol)

D-amino acid oxidase (peroxisome)

Mono-amine oxidase (mitochondrial outer membrane)

Pyridoxamine oxidase (endoplasmic reticulum)

Diamine oxidase (ER)

Urate oxidase (peroxisome core)

Superoxide dismutase (cytosol and mitochondrial matrix)

PGH synthase dependent arachidonic acid metabolism generate superoxides in presence of NADH or NADPH.

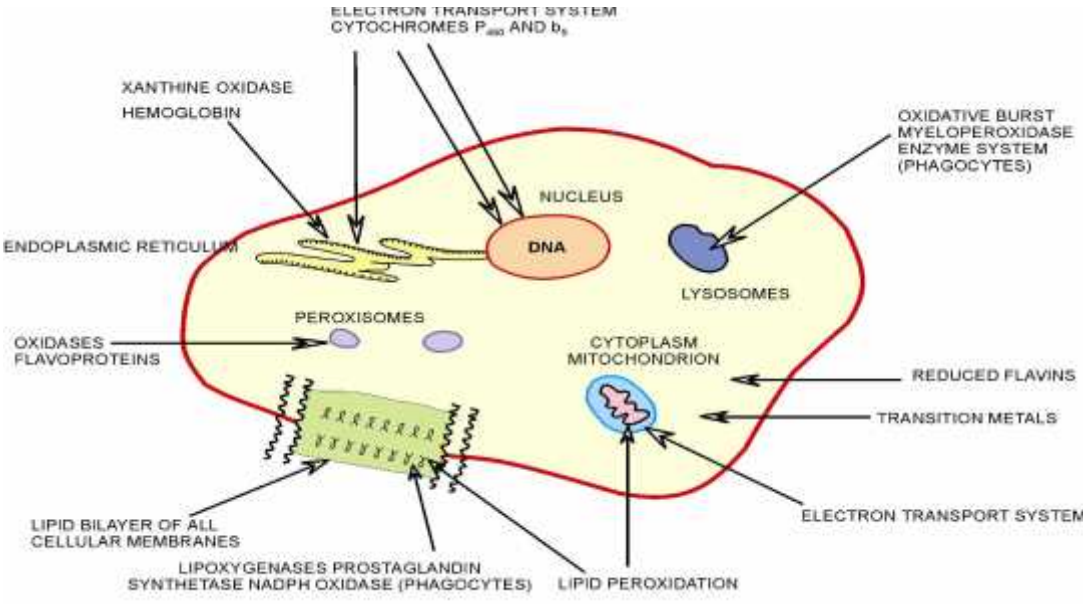


Figure no.4 The Cellular Sources of free Radicals

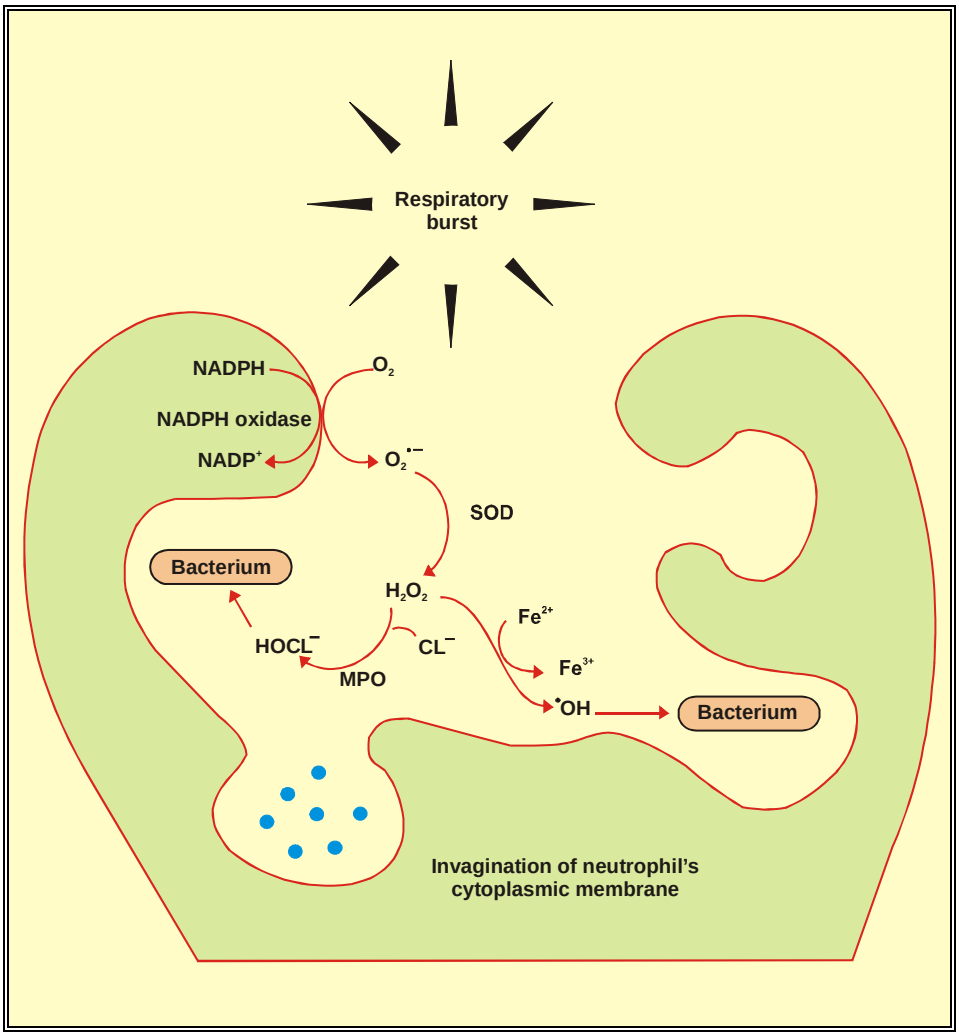


Figure no.5 Generation of Oxygen Free Radicals in Respiratory Burst

Sites of Free Radical Generation:

Main sites of free radical generation are mitochondria, lysosomes, peroxisomes, nuclei, endoplasmic reticulum, plasma membranes and the cytosol.[38]

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

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

ii) Plasma Membrane:

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

The interior of biological membranes is hydrophobic and $O_2^{\bullet -}$ produced in the environment could be extremely damaging. Much of the $O_2^{\bullet -}$ generated within cells comes from membrane bound systems and it is certainly possible that some of it is formed in the membrane interior.[39]

iii) Peroxisomes:

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

DAMAGE PRODUCED BY FREE RADICALS

Free radicals are extremely reactive. Their mean effective radius of action is only 30A^{o}. Their half-life is only a few milliseconds. When a free radical reacts with a normal compound, other free radicals are generated. This chain reaction leads to thousands of events. The peroxidation of polyunsaturated fatty acids (PUFA) severely damages the cell

membrane leading to loss of membrane functions like absorption, secretion etc. Almost all biological macromolecules are damaged by the free radicals, e.g.

a) peroxidation of PUFA in plasma membranes
b) Oxidative inactivation of sulfhydryl containing enzymes
c) Polysaccharide depolymerization and DNA breaks
d) DNA damage may directly cause inhibition of protein and enzyme synthesis; indirectly it also causes 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.[40]

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.[41] Enzymes undergo cross-linking with resulting increase in molecular weight, such enzymes cross-link with their neighbours in a random destructive reaction. The normal precision arrangement of protein and enzymes in subcellular membranes and organelles is badly disrupted and their biological properties are lost or impaired.[42]

Carbohydrates:

Advances in free radical chemistry indicate that no biological substance is impervious to free radical attack. Therefore, it is not surprising that glucose and other related monosaccharides undergo oxidation when conditions are appropriate. Hyaluronic acid undergoes polymer fragmentation following exposure to free radical systems, which leads to destabilization of connective tissue and loss of synovial fluid viscosity.[41]

Nucleic Acids:

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.[43]

Lipids:

All of the major classes of biomolecule may be attacked by free radicals but lipids are probably the most susceptible.[44] Cell membranes are rich sources of polyunsaturated fatty acids. Biomembrane and organelles are the major sites of lipid peroxidation damage. Major constituents of biological membranes are lipids and proteins. Lipid peroxidation can damage membrane proteins as well as lipids.[39] The membrane fluidity is due to the presence of polyunsaturated fatty acid side chain in many membrane lipids, which lower

the melting point of the interior membrane. Lipid peroxidation decreases membrane fluidity. Conditions, which favour lipid peroxidation are:

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

ii) A rich supply of oxygen and

iii) The presence of transitional metal catalysts.[45]

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.[46]

Figure no.6 Illustrates lipid peroxidation. The reaction is initiated by an existing free radical ($X^{\bullet}$), by light, or by metal ions. Malondialdehyde is only formed by fatty acids with three or more double bonds, and is used as a measure of lipid peroxidation together with ethane from the terminal two carbon of ω3 fatty acids and pentane from the terminal five carbon of ω6 fatty acids.[47]

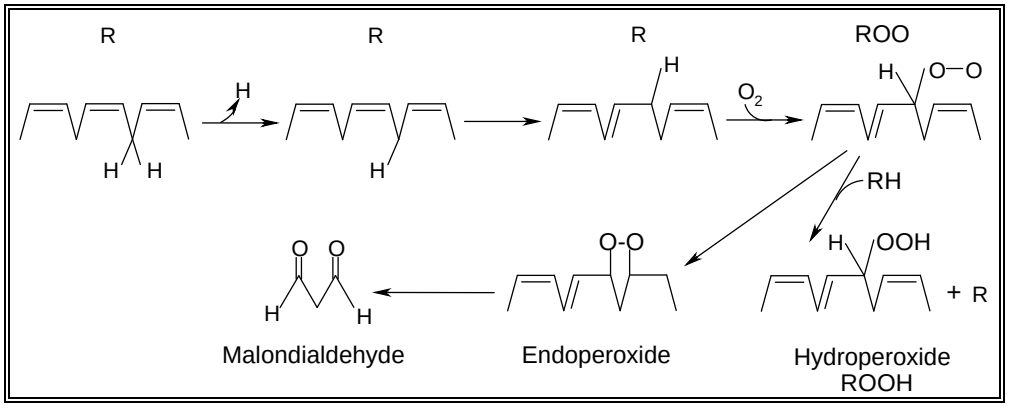


Figure no.6The lipid peroxidation process

Peroxidation of polyunsaturated fatty acids usually involves three operationally defined processes.[39]

Initiation Phase:

During this phase, the primary event is the abstraction of hydrogen atom from bis-allylic site of PUFA. Initiation of a peroxidation sequence in membrane or PUFA is due to the attack of any species that has sufficient reactivity to abstract a hydrogen atom from a methylene (CH_2) group.[41] This leaves behind an unpaired electron on the carbon, -CH-. The carbon radical tends to be stabilized by a molecular rearrangement to produce a conjugated diene, which then easily reacts with an oxygen molecule to give peroxy radical, R-OO$^{\bullet}$. The presence of the redox active metals such as iron or copper can facilitate the initiation process.[39]

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

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

Propagation Phase:

During this phase lipid peroxidation relies on the interaction of molecular oxygen with carbon-centered free radicals to form lipid hydroperoxides.[41] The peroxy radical abstract a hydrogen atom from another lipid molecule and once the process begins it tends to continue. The peroxy radical combines with the hydrogen atom that it abstracts to give a lipid hydroperoxides R-OOH. A probable alternative fate of peroxy radicals is to form cyclic peroxides. With the help of metal catalysts, the decomposition of

hydroperoxides results in the formation of alkoxyl or peroxyl radicals. These radicals are capable of further reactions and thus the propagation of lipid peroxidation continues.[39,41]

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

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

Termination Phase:

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

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

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

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

TOXIC EFFECTS OF LIPID PEROXIDATION

The uncontrolled peroxidation of bio-membranes can lead to profound effects on membrane structure and function and may be sufficient to cause cell death.[23] The toxic products generated during lipid peroxidation may be involved in damage to specific protein and transport systems critical to cell function.[48] Malondialdehyde produced by lipid peroxidation can cause cross linking and polymerization of membrane components. This can alter the intrinsic membrane properties such ion transport, enzyme activity.

Because malondialdehyde is diffusable, it will also react with nitrogenous bases of DNA[49] lipid hydroperoxides can directly inhibit enzymes.[48]

ANTIOXIDANT DEFENCE SYSTEMS

Detoxification of reactive oxygen species is one of the prerequisite of aerobic life. Many defense systems have evolved by providing an important antioxidant defense system of prevention, interception and repair consisting of non-enzymatic and enzymatic scavengers and quenchers.[50]

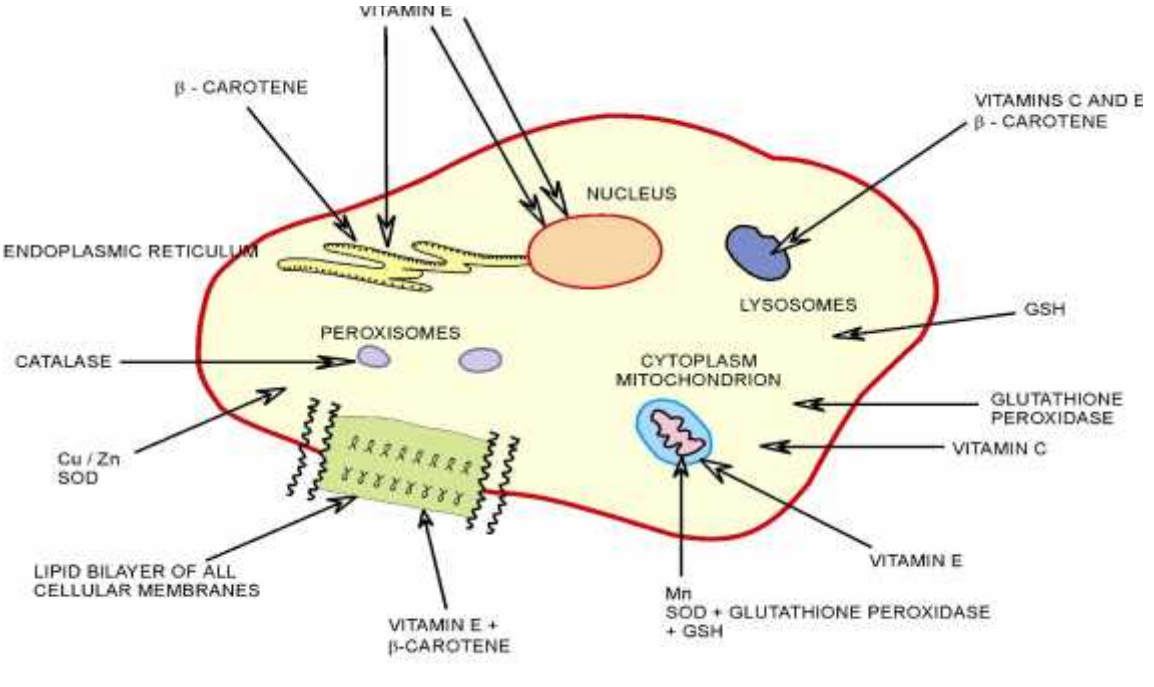


Figure no.7 Antioxidant protection within the cell

Prevention:

The important function of these antioxidant defenses is to prevent the generation of free radicals. Antioxidants defense system removes peroxides that are reacting with transition metal ions to produce reactive free radicals. These peroxides include both

hydrogen peroxide and also the lipid hydroperoxides, which are produced during lipid peroxidation. Catalase and glutathione peroxidases are the enzymes, their role is to safely decompose peroxides.[39] 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.[51,52] Ceruloplasmin an important extracellular antioxidant,[53] which oxidises Fe^{2+} to Fe^{3+} that swiftly binds to transferrin and any iron mobilized from serum ferritin.[54]

Interception:

The free radical scavenging enzyme is superoxide dismutase whose substrate is a free radical, other scavengers include lipid soluble vitamin E, vitamin A, ubiquinol and the aqueous phase compounds such as free radical scavengers like ascorbic acid glutathione, uric acid etc.[44]

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.[55] 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".[56] 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 as the preventive or the chain breaking. In the former category are metal chelators and enzymes like Superoxide dismutase, catalase and glutathione peroxidase. In the second category is the chain breaking agents such as alpha tocopherol, ubiquinone, beta-carotene, bilirubin and water-soluble substances such as ascorbate, GSH and urate. The defense of living eukaryotic cells against the damage caused by the activated oxygen is a complex process which involves a interrelated protective agencies for activated oxygen targets like DNA, proteins and polyunsaturated fatty acids. Peroxidation of polyunsaturated fatty acids will result in a disruption of membrane. So, the antioxidants lie functionally at the heart of this protective mechanism.

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

Tissue damage caused by singlet oxygen is counteracted by Vitamin- E and other carotenoids. This quenching ability depends upon the ability of these compounds to

absorb energy without chemical change so that the excited O_2 is returned to the O_2 ground state without detrimental to the biological system in which it occurs.

Other vitamins such as ascorbic acid, alpha-tocopherol and beta-carotene (precursor of vitamin A) function as the intracellular and the extra cellular antioxidants. Antioxidants by their quenching ability to absorb the free radical generated energy have been postulated to act as homeostatic buffer protecting the cell membranes against the free radical damage. Several antioxidant mechanisms serve to control lipid peroxidation.

ENZYMATIC ANTIOXIDANTS

SUPEROXIDE DISMUTASE

McCord and Fridorich described the metalloenzyme superoxide dismutase.[57] Superoxide dismutase is the major intracellular antioxidant enzyme, which is essential for the survival of aerobic cells. It catalytically scavenges the superoxide radical, which appears to be important agent for toxicity of oxygen and thus provides a defense against oxygen toxicity.

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

Superoxide dismutase catalyzes the dismutation of the superoxide anion free radical ($O_2^{\bullet -}$) to hydrogen peroxide and molecular oxygen at a rate 10^4 times faster than spontaneous dismutation at physiological pH resulting in no superoxide anion available to react with hydrogen peroxide to form hydroxyl radical through the iron catalyzed reactions.[58] Superoxide dismutase enzyme exists in several forms and is present in mitochondrial matrix, the cytoplasm and the extracellular fluid.

1) Cu and Zn containing SOD found in cytosol.

2) Mn containing SOD found in mitochondria.

1. Cu/Zn containing superoxide dismutase: 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/Zn SOD copper is catalytically active which oscillates cupric to the cuprous state, while Zn appears, primarily to play a structural role.[59]

2. Manganese containing superoxide dismutase: Manganese superoxide dismutase has been isolated from human liver mitochondria. It contains four subunits and has a molecular weight of 80,000.[59]

The hydrogen peroxide is damaging in living systems because it can give rise to the formation of $OH^{\bullet}$ radicals. It is therefore biologically advantageous for the cells to control the amount of H_2O_2 that is allowed to accumulate, there are two types of enzymes exist to remove H_2O_2 within cells. They are the catalases and the peroxidases.[39]

GLUTATHIONE PEROXIDASE

Mills established the presence of glutathione peroxidase in mammalian erythrocytes.[60] It has a molecular weight of about 85,000 and consisting of four apparently identical subunits and contains 4 atoms of selenium/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 glutathione peroxidase, which controls the concentration of hydrogen peroxide and

catalyzes the reduction of lipid peroxides with the formation of safely disposable hydroxy acids.

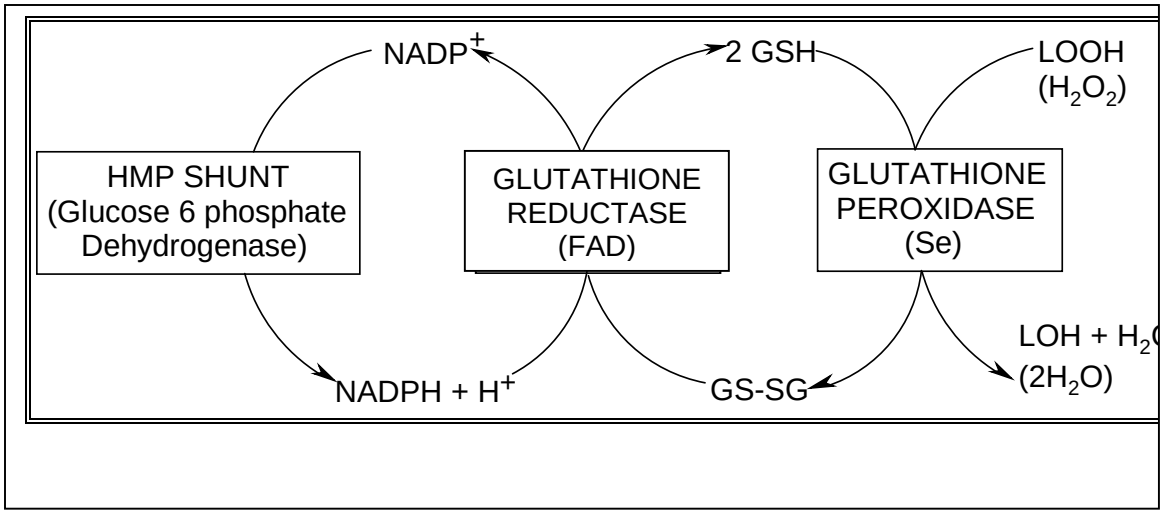
Glutathione peroxidase apparently reduces the selenium and reduced form of enzyme then reacts with hydrogen peroxide.[39] Both types of Glutathione peroxidase enzymes selenium dependent, and selenium independent have been shown to protect against radical damage by reducing peroxides. Glutathione peroxidase enzyme has been shown to catalyze with high specificity the invitro detoxification of H_2O_2 by the oxidation of reduced glutathione.[61]

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

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

Mills showed that glutathione peroxidase is one of the important components in the metabolic pathway of the glutathione system. [62]

In biological systems, high concentrations of H_2O_2 are disposed off by catalase and lower concentration by glutathione peroxidase (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 selenium as the metal prosthetic group.



The glutathione system and its components

Glutathione peroxidase is one of the primary antioxidants present in tissues that limit the amount of lipid peroxides.[63, 64]

Reduction of lipid peroxides to non-toxic hydroxyl fatty acids by glutathione peroxidase, 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 glutathione as its cofactor to convert lipid peroxides into relatively harmless hydroxylated fatty acids, water and glutathione disulfide. Therefore, if glutathione activity were deficient, lipid peroxides could increase in the tissue.

Mechanisms of action of GSH-PX:

a. Lack of specificity with respect to the hydroperoxide

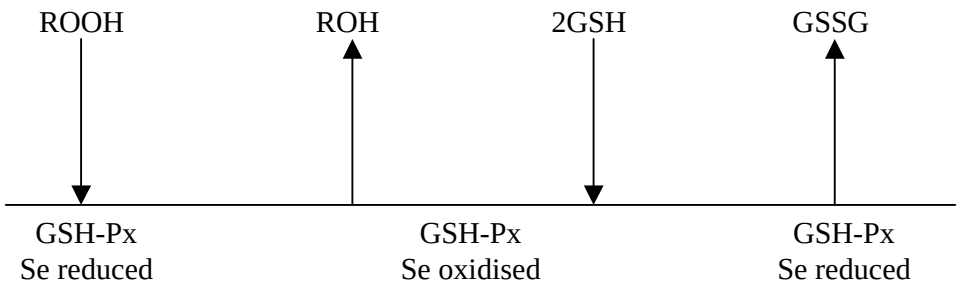
b. High specificity for GSH

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.

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



Glutathione Peroxidase Reactions

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

GLUTATHIONE REDUCTASE

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

Glutathione reductase is $NADPH+H^+$ dependent and in erythrocytes the main source of $NADPH+H^+$ is from Hexose monophosphate shunt activity.[65] The ratios of

GSH/GSSG in normal cells are kept high so there must be a mechanism for reducing GSSG back to GSH, which is achieved by glutathione reductase.

Glutathione reductase contains the FAD at its active site. NADPH reduces the FAD, which then passes its electrons on to a disulfide bridge (S-S) between two cysteine residues in the protein. The two-sulphydryl groups so formed then interact with GSSG and reduce it to 2GSH; reforming the protein disulfides.[39] Glutathione reductase is present in two forms.

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

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

NON-ENZYMATIC ANTIOXIDANTS

~-TOCOPHEROL (VITAMIN E)

Vitamin E or Alpha tocopherol is 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 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 phospholipid PUFA against peroxidative damage.

Vitamin-E is one of the most important chain breaking antioxidant, it protects the polyunsaturated fatty acids from peroxidative damage by donating hydrogen to the lipid peroxyl radical. Because of the lipophilic property of the tocopherol molecule, vitamin E is the major free radical chain terminator in the lipophilic environment. High levels of tocopherol are found in selected mammalian tissues e.g. adrenal glands, heart, testes, and liver and this preferential distribution may result from its high lipid solubility. Intracellularly, vitamin-E is associated with lipid rich membranes such as mitochondria and endoplasmic reticulum. Thus the antioxidant action of tocopherol is expected to be highly effective in protecting against membrane lipid peroxidation by reacting with lipid peroxyl and alkoxyl radicals.[41]

α-Tocopherol is known as a chain breaking antioxidant because it functions to intercept lipid peroxyl radicals ($LOO^{\bullet}$) and so terminates lipid peroxidation chain reactions. Peroxyl and alkoxyl radicals generated during lipid peroxidation preferentially combine with the antioxidant.[66]

$$LOO^{\bullet} + \alpha\text{-tocopherol -OH} \longrightarrow LOOH + \alpha\text{-tocopherol} - O^{\bullet}$$

The resultant tocopheryl radical is relatively stable and, in normal circumstances insufficiently reactive to initiate lipid peroxidation itself.[44] Evidence exists that this tocopherol radical can migrate to the membrane surface and reconvert to α-tocopherol by reaction with ascorbic acid.[67,68] Some thiol compounds, such as glutathione might also be involved in regenerating α-tocopherol from its radical[67,69] α-Tocopherol quenches and reacts with singlet oxygen and could therefore protect the membrane against this species. It also reacts with the superoxide radical.[70]

The chromanol head group of α-tocopherol is very close to the surface of the membrane while the flexible phytyl tail is believed to approximately align with the acyl chains in the interior of the membrane.[71] 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.[72] Although the two vitamins are completely sequestered and separated in their respective lipid and aqueous phases, a very significant extension of inhibition of peroxidation is obtained when both are present.[41]

RETINOL (VITAMIN-A)

Vitamin-A is a member of fat-soluble vitamin; it acts as free radical scavenger. Carotenoids have long been considered antioxidants because of their capacity to scavenge free radicals. β-Carotene a pigment found in all plants is the most efficient quencher of singlet oxygen known in nature and can also function as an antioxidant.[74] β-Carotene is the major carotenoid precursor of vitamin-A. Vitamin-A, however cannot quench singlet oxygen and has a very small capacity to scavenge free radicals. β Carotene has been found in cellular membranes, including those of lysosomes. Carotenoids protect lipids against peroxidation by quenching free radicals and other reactive oxygen species, notably singlet oxygen. The structural arrangement of β-carotene with their long chain of conjugated double bonds suggests that they are excellent scavengers for reactive free radicals.

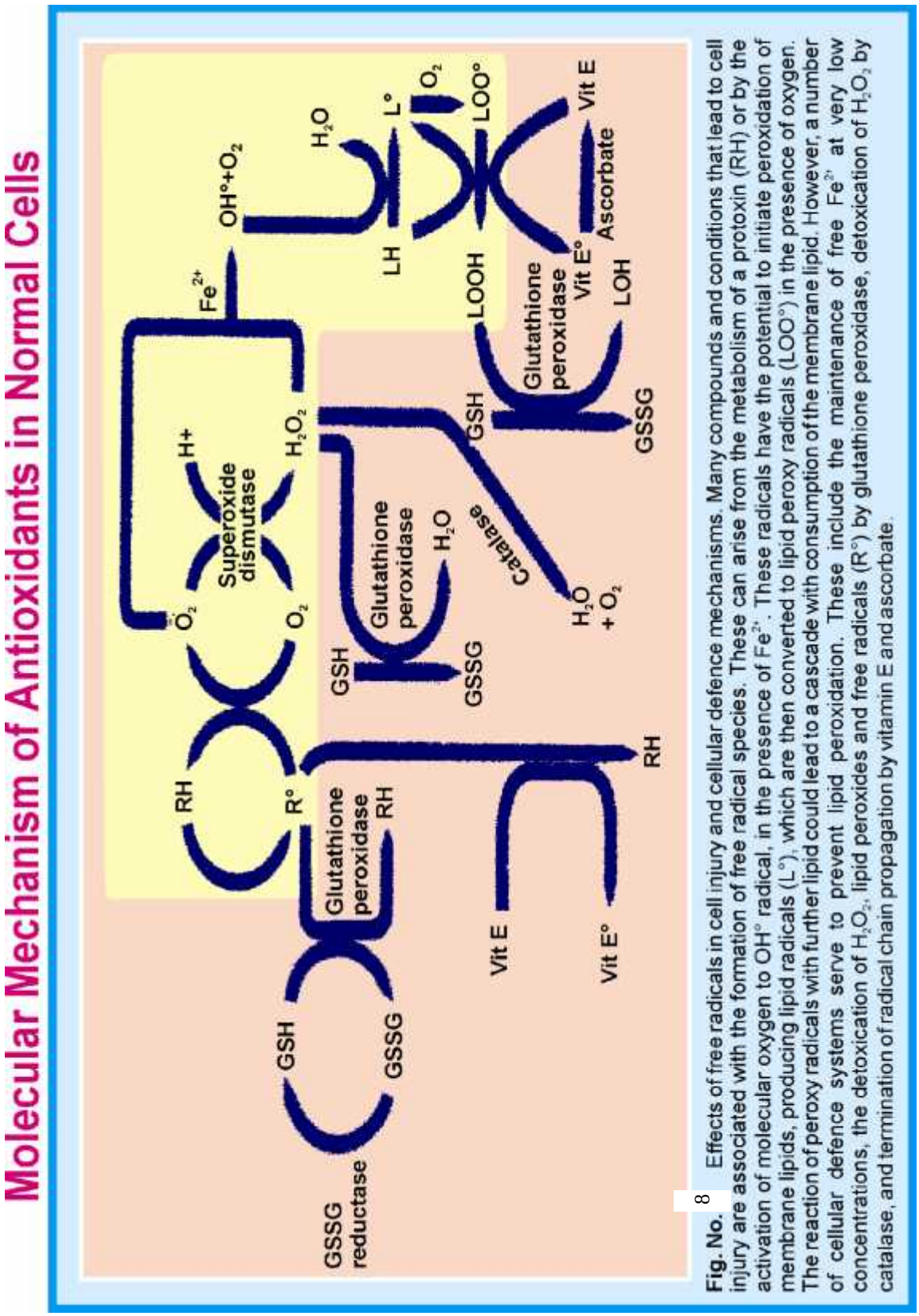


Fig. No. 8 Effects of free radicals in cell injury and cellular defence mechanisms. Many compounds and conditions that lead to cell injury are associated with the formation of free radical species. These can arise from the metabolism of a protoxin (RH) or by the activation of molecular oxygen to OH° radical, in the presence of Fe^{2+}. These radicals have the potential to initiate peroxidation of membrane lipids, producing lipid radicals (L°), which are then converted to lipid peroxy radicals (LOO°) in the presence of oxygen. The reaction of peroxy radicals with further lipid could lead to a cascade with consumption of the membrane lipid. However, a number of cellular defence systems serve to prevent lipid peroxidation. These include the maintenance of free Fe^{2+} at very low concentrations, the detoxication of H_2O_2, lipid peroxides and free radicals (R°) by glutathione peroxidase, detoxication of H_2O_2 by catalase, and termination of radical chain propagation by vitamin E and ascorbate.

ASCORBIC ACID (VITAMIN-C)

Vitamin-C or Ascorbic Acid is a water-soluble non-enzymatic antioxidant. Its role as an antioxidant is indicated by its known free radical scavenging action. As a reducing and antioxidant agent, it directly reacts with superoxide and hydroxyl radical and various lipid hydroperoxides. In addition it can restore the antioxidant properties of oxidized vitamin-E, suggesting that a major function of vitamin-C is to recycle the vitamin-E radicals. Ascorbic acid is widely distributed in mammalian tissues, but it is present in relatively high amounts in the adrenal and pituitary glands, lesser amounts are found in the liver, spleen, pancreas and brain. Ascorbic acid serves as both an antioxidant and a pro-oxidant. As an antioxidant, vitamin-C exerts a sparing effect on the antioxidant actions of vitamin-E and selenium. On the other hand, excess amount (≅1mM) may act as a pro-oxidant in the presence of transition metals Fe^{3+} or Cu^{2+}. Many studies have shown that ascorbate's, pro-oxidant action, which induces lipid peroxidation, resides in its ability to reduce Fe^{3+} to the Fe^{2+} state, Fe^{2+} is known to be a potent free radical inducer.[41]

Ascorbic acid appears to trap virtually all peroxyl radicals in the aqueous phase before they can diffuse into the plasma lipids. The ability of ascorbate to show antioxidant properties is related to its fast reaction with many reactive oxygen species (peroxyl radicals) and to the fact that the resulting semidehydroascorbate radical is a poorly reactive. Enzymatic systems exist invivo to reduce semidehydroascorbate back to ascorbate at the expense of NADH (NADH-semidehydroascorbate reductase enzyme).[73]

Materials and Methods

Source of data: Blood samples were collected from age and sex matched healthy Individuals (vegetarians and non-vegetarians) residing in Belgaum urban area. All the participants were in the age group ranging from 40-60years of both sexes.

Sample size: includes 100 subjects.

1) Lacto-Vegetarians - 25

2) Lacto-ovo-vegetarians – 25

3) Non-vegetarians – 50

Sampling Technique: Volunteers involved in the study group were selected randomly from Belgaum urban population.

Inclusion criteria:

Healthy persons aged between 40-60years

a) Lacto- vegetarians – vegetarians since birth but consuming milk and its products.

b) Lacto-ovo-vegetarians - vegetarians since birth but including dairy products and eggs.

c) Non-vegetarians- consuming animal products such as meat, poultry, fish& other sea foods at least twice a week.

Exclusion criteria:

Smokers and Alcoholics
Hypertension, Diabetes Mellitus
Coronary artery disease or hyperlipidemia
Suffering from any other systemic diseases (Liver and renal diseases.)

The study was approved by the ethical and research committee of J.N.Medical College, Belgaum.

Sample Collection: 8 ml of blood was drawn by venepuncture from vegetarians and nonvegetarians and was collected in heparinized tube (5 units/ml of blood) under

aseptic precautions. Parameters were analysed from whole blood, plasma and hemolysate.

1. 1ml of sample was processed immediately for hemolysate preparation which was used for estimation of enzymatic antioxidants.
2. 0.75ml of sample was used for estimation of MDA
3. Remaining sample was centrifuged at 3000 rpm for 20 min. The supernatant plasma was used for estimation of non-enzymatic antioxidants.

The study involved following analysis

1. Whole blood;

   Malondialdehyde—Thiobrbituric acid method.[75]

2. Hemolysate;

   Glutathione peroxidase—Beulter's E.[76]

3. Plasma;

   A. Vitamin A (Retinol)—Bessay et al method.[77]

   B .Vitamin E (a-tocopherol)—quife et al method.[78]

Statistical analysis

Mean and standard deviation (SD) for each of the outcome variable was computed. Comparison of gender distribution among groups was done by Chi-square test. Comparison of Mean MDA, Glutathione peroxidase, Vit A and Vit E levels among groups by Analysis of Variance (ANOVA) followed by Tukey HSD multiple comparison test.

ESTIMATION OF MALONDIALDEHYDE (MDA): [75]

Principle:

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

Thiobarbituric acid regent:

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

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

Procedure:

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

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

Calculation:

Malondialdehyde (nano moles /ml)

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

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

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

PREPARATION OF HEMOLYSATE

Isolation of Red Blood Cells: [79]

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

α - Cellulose and microcrystalline cellulose in 1:1 (W/W) was mixed with isotonic (9.0gm/L) sodium chloride solution. 5ml plastic disposable syringe without barrel was taken. It was placed in vertical position with the outlet pointing

downwards. A small piece of filter paper was placed at the bottom of the syringe. The well-mixed cellulose slurry was poured to the 2-ml mark. The bed was washed with 5-ml isotonic sodium chloride and 1 ml of whole blood was allowed to flow through the column. To ensure efficient removal of WBCs and platelets, the volume of cellulose mixture used was at least twice that of the blood sample.

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

LYSING OF THE RBCS: [80]

Reagents:

Stabilizing solution: 2.7 mM EDTA (pH 7.0) and 0.7mM β- mercaptoethanol: This solution was prepared by dissolving 100 mg of disodium salt of EDTA in D/W and 5 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, 1 volume of the RBC suspension was mixed with 9 volumes of the stabilizing solution. The hemolysate was frozen rapidly at – 20^0C to – 25^0C in a freezer. Then it was thawed in a water bath at 20^0C to 25^0C. This hemolysate was then ready for the assay.The hemoglobin estimation was performed on hemolysate using Drabkin's reagent, in order to express the enzyme

activities per gram of hemoglobin of the hemolysate. The red cell suspension and its hemolysate were prepared on the day of the assay.

The hemolysate was used to determine

Glutathione peroxidase (GSHPx)

Haemogobin

DETERMINATION OF HEMOGLOBIN[81]

Principle:

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

Reagents:

1) Drabkin's reagent: The prepared reagent was purchased (Span Diagnostics).
2) Cyanomethemoglobin 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
Cyanmethemoglobin standard	---	5.0	---
Haemolysate	---	---	0.02

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

Calculations:

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

GLUTATHIONE PEROXIDASE[76]

Principle:

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

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

Where ROOH is peroxide. t-Butylhydroperoxide 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 340nm.

To ascertain that the auxillary enzyme Glutathione reductase 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 distilled water and pH was adjusted

to 8.2 with concentrated hydrochloric acid and further till 8.0 with 1N HCl and final volume was made to 100 ml of distilled water.

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/2ml were diluted to 1ml with EDTA stabilizing solution.

3) Nicotinamide adenine dinucleotide phosphate reduced (NADPH) 2mM: 2.3mg tetrasodium salt of NADPH (SRL. Mwt 833.36) was dissolved in 1ml of D/W. To 0.85ml of D/W, 0.1ml 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 0.05ml of 2.3mg/ml

4) NADPH was added and the optical density was measured at 340nm 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 2mM concentration, 2ml of NADPH (2.3mg/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 (t-BHP): 7mM: 0.01ml of t-BHP (Sigma 7M) was dilutedto10ml.

Procedure:

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

	Blank$_1$ («l)	**Blank$_2$ («l)**	**Test («l)**
Tris HCl; 1M EDTA, 5mM, pH 8.0	100	100	100
Reduced Glutathione, 0.1 M	20	20	20
Glutathione reductase, 10 U/ml	100	100	100
NADPH, 2mM	100	100	100
1:20 hemolysate	10	---	10
EDTA – stabilizing solution	---	10	---
D/W	670	660	660
Pre-incubated at 37^0C for 10 minutes			
t-BHP, 7mM	--	10	10

The decrease in the optical density of the test at 340nm was measured against Blank at 0 minute and after 10 minutes.

Calculations:

The number of enzyme units per ml:

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

Where

Δ OD : Change in optical density per minute.

$$\Delta OD ; \frac{Test_{0\ min} - Test_{10\ min}}{10 \text{ minutes}}$$

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 units /g Hb

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

A: The number of enzyme units / ml

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

Estimations from Plasma

~-TOCOPHEROL[78]

Principle:

This method is based on the Emmerie Engel reaction. Xylene extract of plasma containing α-tocopherol when reacts with ferric chloride, reduces ferric ions to ferrous ions. The ferrous ions then react with α, α' – dipyridyl 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' Dipyridyl reagent: 120 mg %: 120 mg of dipyridyl were dissolved in small quantity of n – propanol and volume was made to 100 ml with n- propanol.
5) Ferric chloride reagent 120 mg %: 120 mg of ferric chloride hexahydrate ($FeCl_3$. $6H_2O$) were dissolved in small quantity of absolute ethanol and volume was made to 100 ml with absolute ethanol.
6) α- Tocopherol, stock standard 280 mg %: 280 mg of α - tocopherol were dissolved in small quantity of absolute ethanol and volume was made to 100 ml with absolute ethanol.
7) α- Tocopherol, working standard: 1.4 mg %: 0.1 ml of the stock standard of α - tocopherol was diluted to 20 ml with absolute ethanol.

Procedure:

A set of test tubes was prepared as follows:

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

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

Calculations:

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 %

(VITAMIN A) RETINOL[77]

Principle:

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

Reagents:

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

Procedure:

A set of test tubes was prepared as follows:

	Blank (ml)	Standard (ml)	Test (ml)
Plasma	-	-	2.0
Distilled Water	-	-	1.0
Ethanol	-	-	4.0
n-heptane	2.0	-	4.0
Std. Retinol	-	2.0	-

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

Calculations:

Concentration of retinol is expressed as μg % of retinol palmitate

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

RESULTS

The present study comprises 100 subjects, out of which 25 were lacto-vegetarians, 25 lacto-ovo-vegetarians and 50 non-vegetarians. All the values are expressed as Mean ±S.D. Comparison of the studied parameters among groups was done by analysis of variance (ANOVA) followed by Tukey HSD multiple comparison test. P value < 0.05 was considered significant. Results are given in table no.4,5 and 6.

- Mean age for lacto-vegetarians was 44.20 (± 6.95), for lacto-ovo-vegetarians 43.60 (± 7.53), for non-vegetarians 46.38 (± 6.34). There was no difference in the mean age of the three groups (P value 0.186).
- Out of 100 subjects 74 were of male sex (lacto-vegetarians -16, lacto-ovo-vegetarians -20 and non-vegetarians-38) while 26 were females (lacto-vegetarians -9, lacto-ovo-vegetarians -5 and non-vegetarians -12).There was no difference in the sex distribution of the three groups (P value 0.392).
- The blood MDA (in nmol/ml) in lacto-vegetarians was 3.76±1.57 while in lacto-ovo-vegetarians 3.97±1.28 where as 7.29 ± 0.86 in non-vegetarians .The mean MDA level was significantly increased in non-vegetarian group compared to other two groups (P value < 0.001). There was no significant difference in the mean MDA between lacto-vegetarians and lacto-ovo-vegetarians (P value 0.795).
- The erythrocyte Glutathione peroxidase (IU/g of Hb) in lacto-vegetarians was 19.70 ± 2.60 while in lacto-ovo-vegetarians 19.79 ± 2.62 and 13.21± 3.11 in non-vegetarians. Glutathione peroxidase level was significantly decreased in non-vegetarians compared to lacto-vegetarians and lacto-ovo-vegetarians (P value < 0.001). There was no significant difference of

Glutathione peroxidase between lacto-vegetarians and lacto-ovo-vegetarians (P value 0.994).

- The mean plasma vitamin A (µg/dl) in lacto-vegetarians was 35.61 ± 7.91 while in lacto-ovo-vegetarians 34.36 ± 5.79 and 30.83 ± 5.65 in non-vegetarians. The plasma Vit A level was significantly decreased in non-vegetarians compared to lacto-vegetarians (P value 0.007). There was no significant difference between lacto-vegetarians and lacto-ovo-vegetarians (P value 0.765). No significant difference was observed between lacto-ovo-vegetarians and non-vegetarians (0.063).
- The mean plasma Vit E (mg/dl) in lacto-vegetarians was 0.80 ± 0.13 while in lacto-ovo-vegetarians 0.80 ± 0.12 where as 0.73 ± 0.08 in non-vegetarians. The plasma Vit E level was significantly decreased in non-vegetarians compared to lacto-vegetarians and lacto-ovo-vegetarians (P value 0.025). There was no significant difference between lacto-vegetarian and lacto-ovo-vegetarian groups (P value 0.997).

Table no.4 Age distribution

Subject category	Mean ± SD (in years)
Lacto-vegetarians (n=25)	44.20 ± 6.95
Lacto-ovo-vegetarians (n=25)	43.60 ± 7.53
Non-vegetarians (n=50)	46.38 ± 6.34

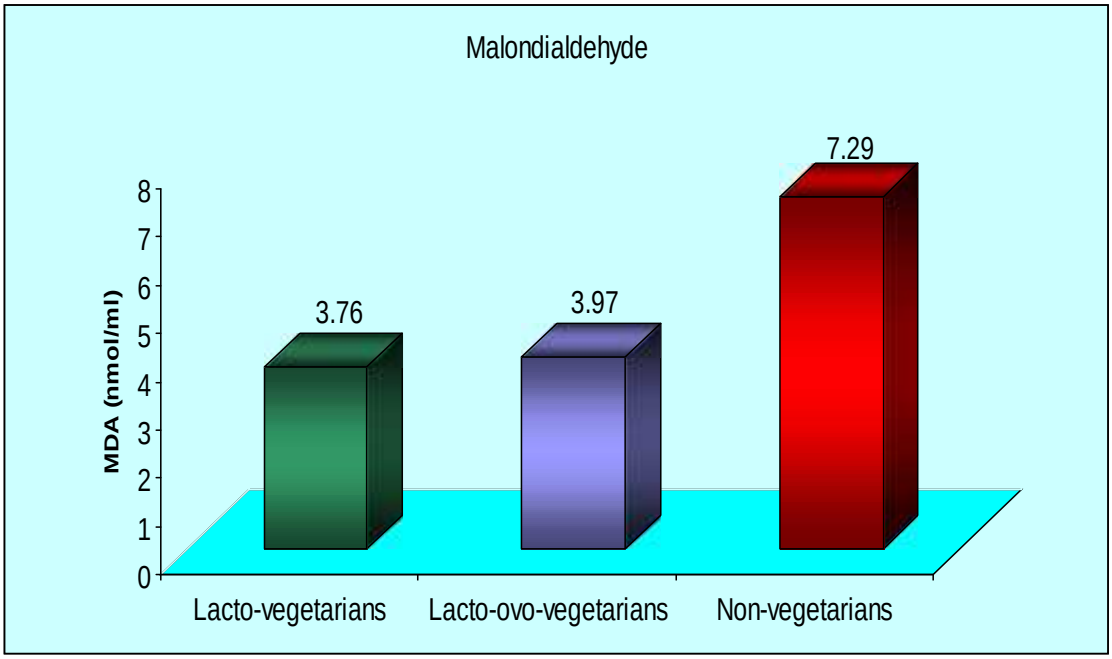
Table no. 5 Sex distribution

Subject category	Male (%)	Female (%)	Total
Lacto-vegetarians	16 (64)	09 (36)	25
Lacto-ovo-vegetarians	20 (80)	05 (20)	25
Non-vegetarians	38 (76)	12 (24)	50
Total	74 (74)	26 (26)	100

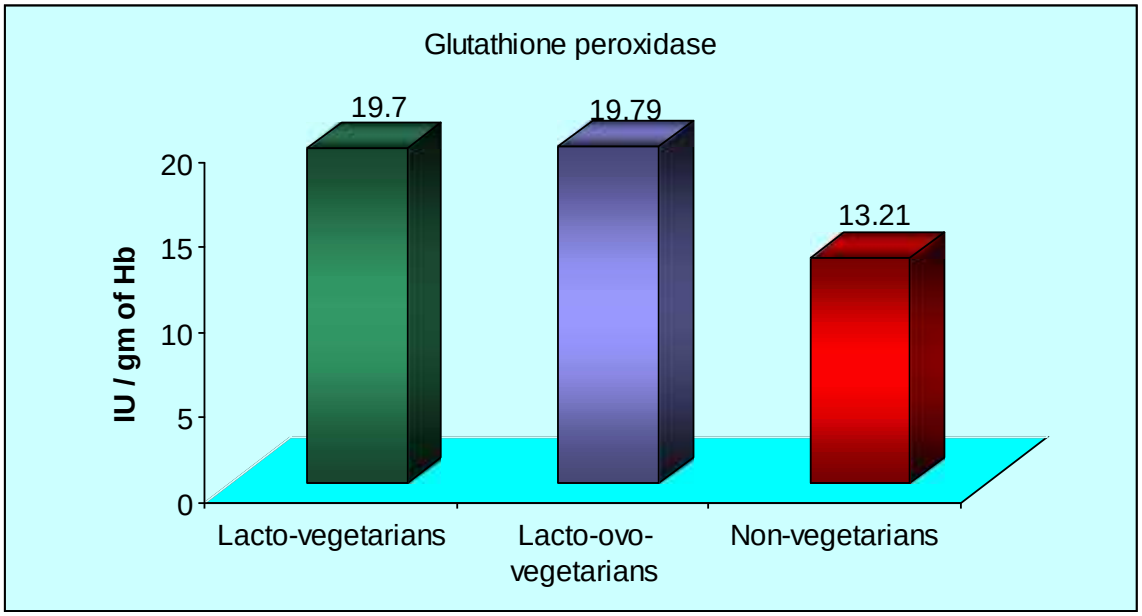
Table no. 6. Malondialdehyde and antioxidant levels in Vegetarians and Non-vegetarians.

Subject category	Malondialdehyde (MDA) nmol / ml Mean ± SD	Glutathione peroxidase IU/ g of Hb Mean ± SD	Vitamin A (µ g/dl) Mean ± SD	Vitamin E (mg/dl) Mean ± SD
Lacto-vegetarians n-25	3.76 ± 1.57	19.70 ± 2.60	35.61 ± 7.91	0.80 ± 0.13
Lacto-ovovegetarians n-25	3.97 ± 1.28	19.79 ± 2.62	34.36 ± 5.79	0.80 ± 0.12
Non-vegetarians n-50	7.29 ± 0.86	13.21 ± 3.11	30.83 ± 5.65	0.73 ±0.08
ANOVA $F_{2,97}$ P value	105.301 < 0.001	64.285 < 0.001	5.645 = 0.005	5.440 = 0.006

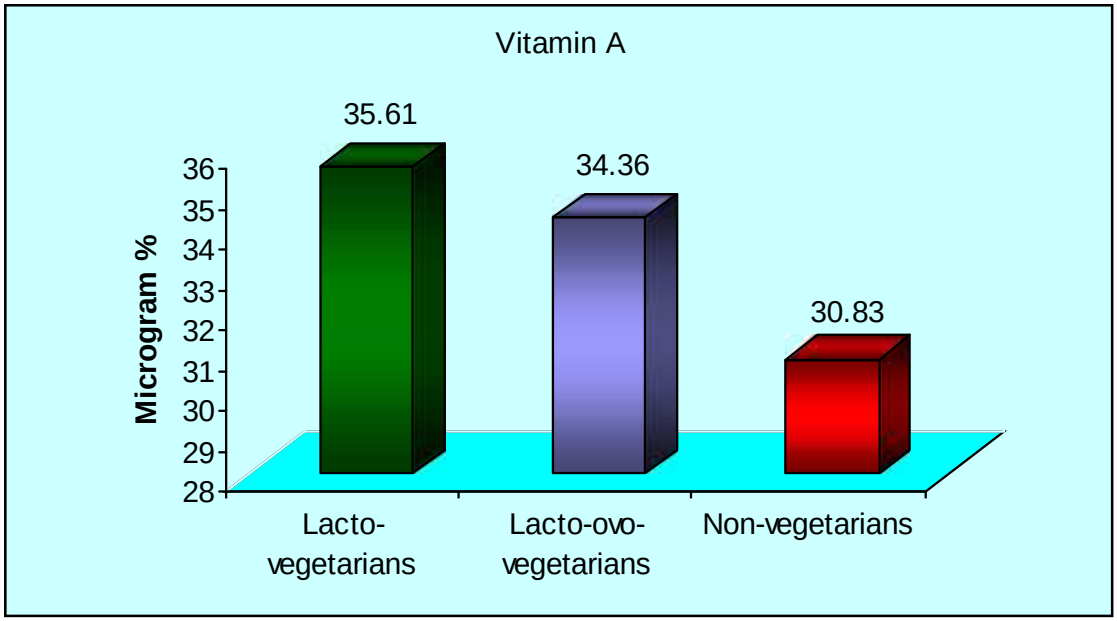
Graph No.1: Malondialdehyde levels in vegetarians and non-vegetarians.



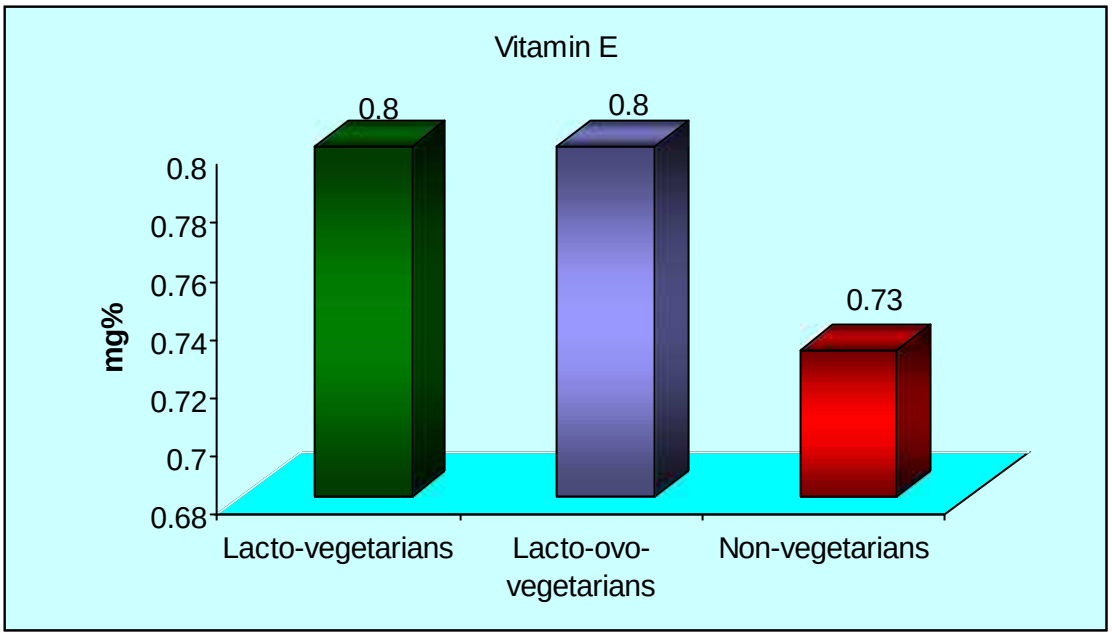
Graph No.2: Glutathione Peroxidase levels in vegetarians and non-vegetarians.



Graph No.3: Vitamin A levels in vegetarians and non-vegetarians.



Graph No.4: Vitamin E levels in vegetarians and non-vegetarians



DISCUSSION

Lipid peroxidation: In the present study there was a significant increase of MDA in non-vegetarians compared to lacto-vegetarians and lacto-ovo-vegetarians.

Our findings are in accordance with the findings of Krajcovicova et al[82],V Manjari et al[83], Anna Nagyova [84]et al who observed significantly increased levels of MDA in non-vegetarians compared to vegetarians. They concluded that lipid peroxidation was increased in non-vegetarians due to higher intake of triacylglycerol resulting in generation of free radicals. Iron utilization from non-vegetarian food is approximately 5 times more as compared to plant food. Iron released from the protein bond might serve as a stimulator of lipid peroxidation.

N.Dieckx[85] et al showed that intakes of Iron and Copper are related to MDA levels. They suggested that transition metals in the diet have important role in the initiation and progression of lipid peroxidation. They concluded that lipid peroxidation was significantly increased in non-vegetarians as their diet is good source of Iron and Copper. They also found that intakes of cholesterol and saturated fat correlated positively with increased lipid peroxidation in non-vegetarians. M.Krajcovicova-Kudlackova et al have shown significant age dependence (increase in elderly individuals) of lipid peroxidation in non-vegetarians.

The findings in the present study are contradictory with study of Y.T. Szeto[86] et al who shown that there is no change of MDA levels between vegetarians and non-vegetarians.

Glutathione Peroxidase: A selono-protein enzymatic antioxidant that removes H_2O_2 and organic hydroperoxides. In our study there was significant increase in Glutathione peroxidase levels in vegetarians compared to non-vegetarians.

Findings of our study are contradictory to Kovacikova Z et al[87], Bonnie Bruce et al[88] who showed that activity of selenium dependent Glutathione peroxidase was lower both in plasma and erythrocytes of vegetarians. They suggested it may be due to low dietary selenium intake/bioavailability in vegetarians which leads to low Glutathione Peroxidase activity. This may be due to higher content of phytic acid in vegetarian diet which is a well known inhibitor of mineral absorption.

V.Manjari et al[83], Haldar et al[89], Rauma et al[90] have found that there was no significant difference in the activity of Glutathione Peroxidase between vegetarians and nonvegetarians.

Non-enzymatic antioxidants

Vitamin E: (α-tocopherol): It is a fat soluble vitamin which converts, $O_2^{\bullet -}$, $^{\bullet}OH$ and lipid peroxyl radicals to less reactive forms and acts as a chain breaking antioxidant. In the present study plasma concentration of vitamin E was significantly increased in lacto-vegetarians and lacto-ovo-vegetarians compared to non-vegetarians due to higher intake of vegetable oils e.g. cotton seed oil and sunflower oils.

Our findings are in accordance with M.krajcovicoa et al[82], Rauma et al[90], Anna Nagyova et al[84] and Millet et al[91] who found significantly increased Vitamin E levels in vegetarians compared to nonvegetarians. They also found that serum vitamin E concentration was strongly associated with blood lipids. They suggested that reduced level of α-tocopherol in nonvegetarians may be due to enhanced lipid peroxidation and increased utilization of vit E. Rauma et al[90] and Bonnie Bruce et al[88] found that vegetarian diet provided more than twice the amount of vitamin E than the non-vegetarian diet. This may be due to higher consumption of nuts and seed oils by vegetarians. They also suggested that flavonoids which occur

naturally in fruits, vegetables and in some beverages such as tea might have reduced oxidative stress in vegetarians.

Haldar et al[89] found that there was no significant difference in the levels of α-tocopherol between vegetarians and non-vegetarians. Results of our study are contradictory to Y.T.Szeto[86] and Proniczok et al[92] who found that plasma α-tocopherol concentrations were lower in vegetarians compared to non-vegetarians.

Vitamin-A: is a fat soluble vitamin that scavenges $O_2^{\bullet -}$ and reacts directly with peroxy radical. In our study the plasma vitamin-A levels were significantly increased in lactovegetarians and lacto-ovo-vegetarians compared to non-vegetarians may be due to higher intake of carrot, fruits and vegetables.

Our results are in accordance with M. Krajcovicova et al[82], Rauma et al[90], Ingrid Kiefer et a[l93] and Millet et al[91] who found higher serum β-carotene concentration in vegetarians compared to non-vegetarians. They suggested that it may be due to higher dietary β-carotene intake in vegetarian diet and action of β-carotene in presence of other food factors such as non-nutritive phytochemicals could have played a role. They proposed that additive and synergistic effects of phytochemicals in fruit and vegetables are responsible for their antioxidant activity.

Haldar et al[89] have found that overall antioxidant status was similar between vegetarians and non-vegetarians. They also found that total intake of fruits and vegetable was positively associated with increased plasma levels of carotenoids.

CONCLUSION

We conclude from our study that the antioxidants present in vegetarian food are responsible for preventing the generation / damaging effect of free radicals as compared to non-vegetarian diet. So vegetarian diet is superior to non-vegetarian diet in protecting the individual from deleterious effect of ROS.

SUMMARY

The results of the present study indicate that there is an increased oxidative stress in non-vegetarians compared to vegetarians, as evidenced by increased lipid peroxidation product i e Malondialdehyde (MDA) and low antioxidant status as evidenced by decreased levels of enzymatic and non-enzymatic antioxidants i e Glutathione Peroxidase, Vitamin A and Vitamin E levels . This is due to higher and regular consumption of fruit and vegetables , dark and whole grain products , grain sprouts , plant oils and oil seeds rich in trace elements like Zinc, Copper and Selenium, mono and polyunsaturated fatty acids, antioxidant vitamins, fibers, complex carbohydrates and flavonoids by vegetarians. Our results clearly indicate that vegetarian nutrition provides adequate antioxidants which effectively prevent free radical generation and thus responsible for better antioxidant status.

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ANNEXURE – I

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 lipid peroxidation and antioxidant status in vegetarians and non-vegetarians- a cross-sectional study.

Participation in this study is completely voluntary. About 50 healthy vegetarians and 50 non-nonvegetarians as volunteers will be enrolled in this study at J. N. Medical College, Belgaum under the supervision of Dr. M.V.Kodliwadmath Professor, Department of Biochemistry, J. N. Medical College, K.L.E. University, Belgaum and will be carried out by Dr. M.S.Somannavar., P. G. Department of Biochemistry, K.L.E University , Belgaum for his M.D. Thesis to be submitted to KLE University, Belgaum.

PURPOSE OF THE STUDY

The diet is a key environmental factor implicated in health and disease. Oxidative stress, antioxidant status and their relation to diet is a subject of interest in recent years. The objective of the study was to compare lipid peroxidation and antioxidant status in healthy vegetarians and non-vegetarians.

PROCEDURE

For both Vegetarians and non-vegetarians 8 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 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.

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. 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 J. N. Medical College will provide, within the limitations of the laws of the Karnataka state, 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. M.V.Kodliwadmath, 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 renumeration for participating in this study.

VOLUNTARY PARTICIPATION/WITHDRAWAL

If you decide not to participate in this study, 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 Dr. V. D. Patil, Principal, J. N. M. C., K.L.E. University, Belgaum and Chairman of J. N. M. C. Institutional Ethics Committee of Human Subjects Research, Telephone No. 0831-2471701.

EMERGENCY PROVISION

If you have questions as a participant in our study, you can contact the study investigator Dr. M.S.Somannavar, Mobile No. 9480344394 or the research guide Dr. M.V.Kodliwadmath, Phone No. 0831-2473777 (Extension) 1522.

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
PROFORMA

LIPID PEROXIDATION AND ANTIOXIDANT STATUS IN VEGETARIANS AND NON-VEGETARIANS- A CROSS-SECTIONAL STUDY

Sl. No. :

Name. :

Age. :

Sex. :

Occupation. :

Address. :

Dietetic history :

Past history **:**

Family history **:**

General Physical Examination:

Pulse rate	B.P.	Height
Resp.Rate	Temperature	Weight

Systemic Examination:

- Respiratory system
- Cardiovascular system
- P/A
- Central Nervous system

Investigations

- Routine
- Special

LACTO-VEGETARIANS

Sl.no.	Name	Age	Sex	MDA	Glu.Px	Vit A	Vit E
1	V S S	36	M	10.6	14.3	57	0.87
2	V G	38	F	5.51	21.4	41.6	0.85
3	S S S	50	F	2.88	18.86	32.16	0.97
4	S B I	58	M	4.74	18.87	28.57	0.91
5	A K	38	M	2.85	21.17	30.7	0.77
6	P Y	42	M	3.62	23.51	35.7	0.66
7	NY	32	M	2.56	21.15	27.85	0.61
8	S K	39	M	2.82	20.35	31.42	0.61
9	V B J	30	F	3.91	22.96	28.57	0.61
10	S P	37	M	3.39	20.69	28.57	0.59
11	D M	40	M	3.27	20.74	27.14	0.78
12	K H	45	M	3.24	21.77	30	0.75
13	A B P	42	F	3.33	22.49	32.14	0.77
14	S V S	57	M	3.07	17.97	31.42	0.82
15	S K	48	F	3.11	20.41	37.85	0.83
16	K H	51	F	2.69	14.5	38.57	0.81
17	K G	47	F	4.71	22.56	37.14	0.81
18	K F T	45	F	3.3	19.05	39.28	0.82
19	R M	45	M	3.46	22.12	34.28	0.82
20	K K	51	M	3.23	21.08	36.42	0.79
21	K H	47	F	3.46	15.21	40	0.85
22	C S	48	M	3.4	18.47	38.57	0.77
23	V D	47	M	3.49	19.19	39.29	0.85
24	M K	49	M	3.41	16.7	37.82	0.83
25	V P	43	M	3.96	17.1	38.2	0.78

LACTO-OVO-VEGETARIANS

sl no	name	age	sex	MDA	Glu.Px	Vit A	Vit E
1	G B	36	M	9.6	19.3	54.16	0.94
2	B I D	45	M	4.61	17.2	33.33	0.86
3	S W	40	M	3.04	18.12	26.47	0.78
4	R A	38	M	2.72	23.95	28.26	0.82
5	S S	35	M	3.24	13.39	29.71	0.7
6	S B	45	M	4.67	20.78	31.42	0.76
7	M S	27	M	4.13	20.69	27.14	0.8
8	K P	28	M	3.26	18.57	31.42	0.6
9	I K	48	M	3.75	21.87	38.57	0.64
10	S U	40	M	4.35	23.89	30.02	0.68
11	M S S	37	M	2.91	25.45	36.42	0.82
12	B V	43	M	3.74	22.12	30	0.71
13	P J	53	M	3.33	18.27	34.28	0.79
14	S D	48	M	3.68	20.74	37.14	0.84
15	D K	43	F	3.42	19.48	30.71	0.71
16	K G	46	F	3.01	18.75	34.28	0.76
17	A T	47	M	3.75	18.91	38.57	0.83
18	K H	52	M	4.13	16.15	40	0.79
19	A G	44	F	4.01	17.03	27.14	0.79
20	S K	43	F	3.94	20.41	37.14	0.86
21	D B	43	F	4.07	20.74	35	0.83
22	M K	45	M	3.78	17.41	35.71	0.78
23	A A	58	M	3.92	21.1	36.18	0.84
24	B D	52	M	4.32	19.8	38.1	0.85
25	S I	54	M	4.02	20.6	37.9	0.82

NON-VEGETARIANS

sl.no.	name	age	sex	MDA	Glut.Px	Vit A	Vit E
1	P B D	42	M	6.57	29.7	55.83	1.06
2	A P	40	M	6.1	12.42	35	0.84
3	S M	37	M	5.28	15.13	29.28	0.81
4	V M	41	M	5.89	5.36	25.67	0.74
5	R J	38	M	5.44	16.34	29.28	0.79
6	S S	38	M	6.37	14.69	49.28	0.84
7	S A	40	M	6.66	15.13	44.28	0.83
8	S N	42	F	6.82	9.23	25.71	0.66
9	G C	47	M	6.53	12.24	27.85	0.6
10	RG	56	M	7.27	15.41	25.71	0.59
11	B K	43	M	6.21	15.82	29.28	0.78
12	P E	48	F	5.86	16.07	30.71	0.8
13	B R B	45	M	7.11	13.86	29.28	0.76
14	R K	43	F	6.37	12.78	31.42	0.69
15	A B	48	F	6.63	13.66	32.85	0.73
16	R K	48	F	6.63	13.44	26.42	0.73
17	K K	45	F	6.82	14.33	27.14	0.68
18	M M	52	F	6.69	12.61	28.57	0.67
19	M K	47	M	7.59	16.15	32.85	0.73
20	I S	50	M	6.95	13.8	33.57	0.7
21	A K	52	M	6.41	14.29	27.85	0.67
22	B C	52	M	7.37	14.05	32.14	0.67
23	M K	40	M	6.85	12.25	30	0.71
24	V K	45	M	7.15	12.79	25	0.8
25	P R	51	F	7.92	15.13	29.28	0.78
26	U M	49	M	7.43	14.48	34.28	0.74
27	M A K	49	F	8.04	14.29	32.85	0.77

sl.no.	name	age	sex	MDA	Glut.Px	Vit A	Vit E
28	C H	40	M	6.76	12.25	35	0.62
29	N M	47	M	7.63	12.07	30	0.79
30	S G	53	M	7.78	10.71	26.42	0.76
31	R B	46	M	7.88	11.74	27.85	0.64
32	D M	48	M	7.63	14.6	29.28	0.7
33	M K	46	M	7.53	14.49	31.42	0.82
34	I S	53	M	7.98	13.36	27.14	0.84
35	S R	54	M	7.31	12.92	25.71	0.77
36	I H M	52	M	8.01	13.09	30.71	0.74
37	A M	54	M	7.85	11.58	32.14	0.68
38	J B M	46	F	7.88	12.42	30.71	0.74
39	S P	44	M	8.33	10.57	27.14	0.65
40	G B	46	M	8.21	12.57	28.57	0.68
41	K P	41	F	8.17	11.18	25.71	0.68
42	A F	49	M	8.05	11.58	32.86	0.74
43	M S	46	M	8.3	12.41	33.57	0.73
44	G M	45	M	8.42	11.52	29.62	0.69
45	K S	51	M	8.51	10.21	30.04	0.71
46	M M	49	M	7.98	10.44	31.04	0.72
47	M K	55	M	8.41	11.12	32.24	0.66
48	M K	53	M	7.92	12.32	26.72	0.67
49	N H	57	M	8.64	10.88	27.34	0.68
50	N K	47	M	8.32	11.45	28.45	0.69