APPENDICES

APPENDIX 1

Free radicals and reactive oxygen species: oxidative damage and pathogenesis—biochemistry in brief[213]

In the sequential univalent process by which O_2 undergoes reduction, several reactive intermediates are formed such as superoxide ($^{\cdot}O_2^-$), hydrogen peroxide (H_2O_2) and the extremely reactive hydroxyl radical ($^{\cdot}OH$): collectively termed as the reactive oxygen species (ROS). The process can be represented as:

$$O_2 \xrightarrow{\bar{e}} {}^{\cdot}O_2^- \xrightarrow{\bar{e}} H_2O_2 \xrightarrow{\bar{e}} {}^{\cdot}OH \xrightarrow{\bar{e}} H_2O$$

In normal respiring cells, the cytochrome oxidase of mitochondrial electron transport reduces O_2 by four electrons to H_2O without releasing either $^{\cdot}O_2^-$ or H_2O_2. But due to the probable 'leak' of single electron at the specific site of the mitochondrial electron transport chain resulting in inappropriate single electron reduction of oxygen to $^{\cdot}O_2^-$,[214,215] When the electron transport chain is highly reduced and the respiratory rate is dependent on ADP availability, leakage of electrons at the ubisemiquinone and ubiquinone sites increases so as to result in production of $^{\cdot}O_2^-$ and H_2O_2.[216,217] For the production of $^{\cdot}OH$, requires the presence of trace amount of transition metals like iron and copper. A simple mixture of H_2O_2 and Fe^{2+} salt forms $^{\cdot}OH$.

$$O_2 + H_2O_2 \xrightarrow{Fe^{2+}} OH + {}^{\cdot}OH + O_2$$

$^{\cdot}O_2^-$ is toxic to a cell growing under aerobic conditions, and superoxide dismutase (SOD) which scavenges $^{\cdot}O_2^-$, offers cellular defence against this toxicity.[218] An indirect deleterious action of $^{\cdot}O_2^-$ is mediated by its dismutation to H_2O_2, which is sensitive to catalase. Since H_2O_2 is long lived and membrane permeable, it may diffuse considerable distance away from its site of generation and also it shows limited toxicity. But $^{\cdot}OH$ is extremely reaction having a very short half life, and with a very limited diffusion capacity. It can attack and damage almost every molecule in its vicinity at a diffusion controlled rate.[217]Thus the extent of damage to cells by $^{\cdot}O_2^-$ and H_2O_2 increases in presence of the transition metal ions due to the generation of more powerful $^{\cdot}OH$.

Primary defence against ROS: catalytic removal of ROS by antioxidant enzymes and free radical scavengers (antioxidants)

Superoxide dismutase (SOD), catalase and peroxidases constitute mutually supportive team of defence against ROS. While SOD lowers the steady state level of $^{\cdot}O_2^-$, catalase and peroxidases do the same for H_2O_2.

Free radical scavengers(antioxidants) mainly include reduced glutathione (GSH) and ascorbic acid

Superoxide dismutase (SOD)

This enzyme catalyses the reaction

$$2O_2 + 2H \xrightarrow{SOD} H_2O_2 + O_2$$

SOD is a metalloprotein found in both prokaryote and eukaryotic cells. The iron containing (Fe-SOD) and the manganese containing (Mn-SOD) enzyme are characteristic of prokaryotes.[218] In eukaryotes cells the predominant forms are the copper containing enzyme and the zinc containing enzyme

located in the cytosol. The second type is the manganese containing SOD found in mitochondrial matrix. The biosynthesis of SOD is controlled by its substrate- the $^{\cdot}O_2^-$.

Glutathione peroxidase

Glutathione peroxidase[219] catalyses the reaction of hydroperoxidase with reduced glutathione (GSH) to form glutathione disulphide (GSSG) and the reduction product of the hydroperoxide.

$$ROOH/H_2O_2 + GSH \xrightarrow{GSH\text{-}peroxidase} ROH/H_2O + GSSG$$

Catalase

Catalase present in almost all mammalian cells is localised in the peroxisomes.[215] It is a haemoprotein and catalyses the decomposition of H_2O_2 to water and oxygen and thus protects the cell from oxidative damage by H_2O_2 and $^{\cdot}OH$.

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

Antioxidants

Antioxidants mainly include reduced glutathione (GSH) and ascorbic acid. Reduced glutathione readily interact with free radicals notably hydroxyl radicals by donating a hydrogen atom to give glutathione oxidised (GSSG).[220] It is the major cellular antioxidant in the different line of defence against oxidation damage.

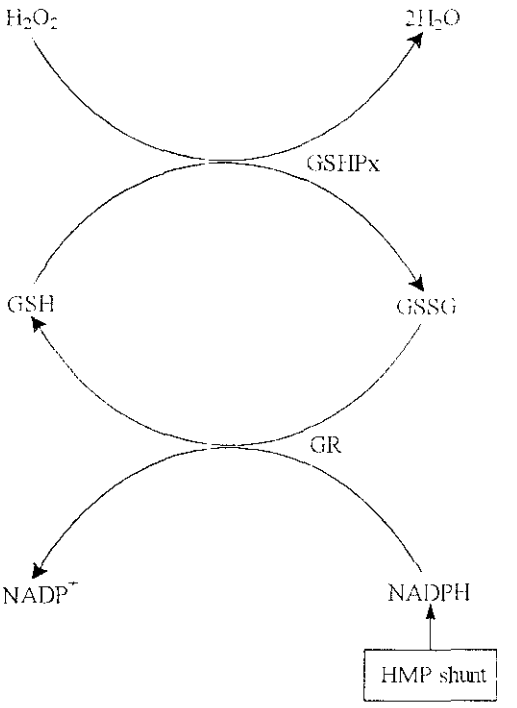

Figure 1. Free radical scavenging pathway of Glutathione (GSH)

H_2O_2	-	Hydrogen peroxide
H_2O	-	Water
GSSG	-	Oxidised glutathione
GSH	-	Glutathione
GHSPx	-	Glutathione peroxicase
GR	-	Glutathione reductase
NADPH	-	Reduced Nicotinamide Adenine dinucleotide phosphate
$NADP^+$	-	Odixised nicotinamde Adenine dinucleotide phosphate
HMP shunt	-	Hexose monophosphate pathway

Ascorbic acid directly reacts with $^{\cdot}O_2^-$ and $^{\cdot}OH$. When compared to other water soluble antioxidants, vitamin C offers the most effective protection against free radical damage.[133]

Secondary defenses against ROS

This includes some auxiliary enzymes like glutathione reductase (GR), glutathione-s-transferase (GST) and Gamma glutamyl transpeptidase (GGT).

Glutathione reductase converts the oxidised GSSG to GSH with the help of NADPH. While glutathione-S-transferase helps in the conjugation reactions of GSH.[220] Gamma glutamyl transpeptidase (GGT) hydrolyses the γ-glutamyl bond of glutathione and transfers the γ-glutamyl amino acid into the cell where it is resynthesised into GSH.[221]

The whole reaction of reaction O_2 metabolism in combination with glutathione metabolism is represented below.

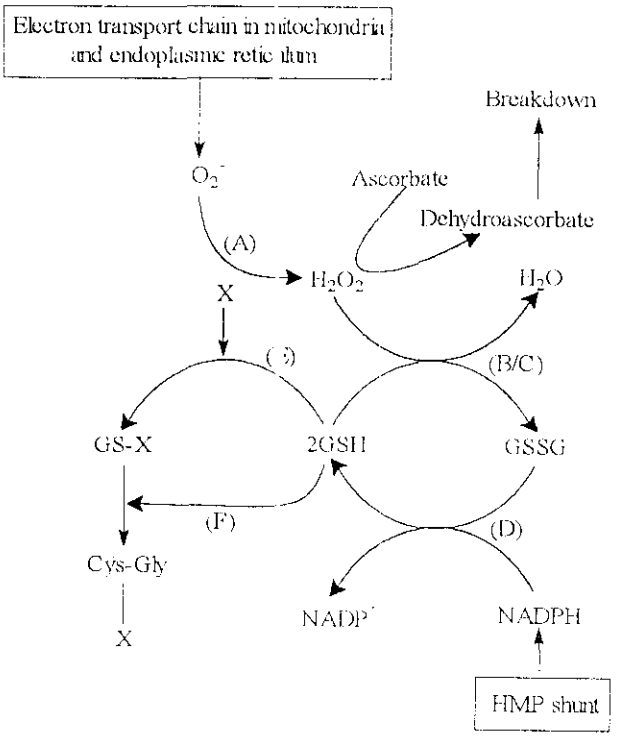

Figure 2. Free radical pathway in combination with glutathione metabolism is given below

(A) -Superoxide dismutase, (B) – Catalase, (C) - Glutathione peroxidase, (D) - Glutathione reductase, (E) - Glutathione-S-transferase, (F) - γ-Glutamyl transpeptidase

Biological effects of free radicals[222]

The free radical produced nterfere with the cellular function thus causing cellular damage. Free rac ical usually interact with protein, nucleic acid and lipids.

Interaction with protein

Free radicals have high affinity to molecules with nucleophilic characters and so it easily interacts with protein to form covalent linkage, thus damaging the quaternary structure of protein. Free radical attack on the quarternary structure of proteins results in the oxidation of amino acids in the proteins which may be easily degraded by the proteolytic enzymes[133,153]. But this damage is repaired in normal cells.

Interaction with nucleic acids

Certain xenobiotics were found to interact with nucleic acids, resulting in their damage. $\cdot O_2^-$ radicals can damage nucleic acids in two ways. On one hand the hydroxyl radicals can react with the sugar phosphate backbones of nucleic acids resulting in strand scissions and possibly chromosome breakage. On the other hand, reactive $\cdot O_2^-$ species may oxidatively modify the sugar moiety or the different bases. Oxidative damage may result in cell death (mainly through apoptosis).

Interaction with lipids—lipid peroxidation[133]

Lipid peroxidation arising from the reaction of free radicals with lipids is considered as prevalent, important feature of cellular injury brought about by free radical attack. This progresses by three operationally defined processes; initiation, propagation and termination. The initiation phase of peroxidation

usually proceeds with the formation of conjugated diene bonds generated by abstraction of hydrogen atoms.

Propagation of lipid peroxication relies on the interaction of molecular oxygen with carbon centred free radicals to form lipid hydroperoxides. These lipid hydroperoxides can form free radicals to propagate the lipid peroxidation. Lipid peroxidation usually affects membrane lipids. In addition to the self destructive nature of membrane lipid peroxidation arising from free radical generation by other membranes, lipid peroxidation is a major source of other cytotoxic products such as aldehydes produced from the decomposition of lipid hydroperoxides. Also LDL chloesterol component may be affected by the peroxidation where LDL gets oxidised to form oxLDL which is toxic to the body. These oxLDL some times disturbs the cholesterol absorption to give a pathological condition called atherosclerosis characterised by high levels of LDL in the blood.

APPENDIX 2

Cholesterol and lipid metabolism[223]

Cholesterol and triacylglycerols are transported to target cells by lipoproteins. There are mainly four types of lipoproteins which are classified according to their density. They are

Chylomicrons: Transport cholesterol and other lipids in the diet (exogenous) away from the intestine to the tissues.

Very low density lipoproteins (VLDL): VLDL or Pre β lipoprotein, derived mainly from liver for the export of triacyl glycerol.

Low density lipoprotein (LDL): LDL or β lipoprotein representing the final stage in the catabolism of VLDL and chylomicrons.

Intermediate density lipoprotein (IDL): Also help in the metabolism of VLDL and chilomicrons.

All the three VLDL, IDL and LDL transport endogenous triacyl glycerol and cholesterol from the liver to the tissues.

High density lipoprotein (HDL): HDL transports endogenous cholesterol from the tissues to the liver.

Chylomicrons loaded with cholesterol and the triacylglycerols reach various tissues to deliver these components to the tissues. Triacylglycerols are delivered to the muscles and adipose tissue. While the cholesterol is delivered to liver for breakdown.

VLDL, which are synthesised in the liver as lipid transport vehicles, are also degraded to IDL and then to LDL, almost all their protein are removed and much of their cholesterol is esterified.

Michael Brown and Joseph Gold Stein have demonstrated that cells obtain cholesterol mainly throughthe endocytosis of LDL particles. Cholestryl esters are hydrolysed to yield cholesterol which is subsequently incorporated in the cell membranes. Any excess intracellular cholesterol is reesterified to cholestryl ester for storage within the cells. Some cholesterol is used for the syntheiss of bile acids (cholic acid, etc.), vitamin D, Steroid hormones (Androgens, estrogens, etc.), prostaglandins (PGE, PGA, etc.), and faecal sterols (cholesterol, coprostanol, etc.).

Triacylglycerols whichare absorbed into the body are broken down into fatty acids and glycerol, where glycerol enters the glycolytic pathway or for esterification of fatty acids in phospholipid biosynthesis. Fatty acids are oxidised into acetyl CoA in the mitochondria. This acetyl CoA either enters the tricarboxylic acid cycle (TCA cycle) or cholesterol biosynthetic pathway. In the TCA cycle it generates electrons and then ATP molecules with the help of mitochondrial electron transport chain. In cholesterol biosynthesis acetyl CoA condenses with two other molecules of acetyl CoA and then undergoes a series of reactions to produce cholesterol. This usually happens in the liver.

If there is excess of cholesterol storage in the liver, then the acetyl CoA enters another pathway for the production of ketone bodies (acetone, β-hydroxy butyrate and acetoacetate) which results in a diseased condition called Ketosis.