REVIEW OF LITERATURE
3.1 Methotrexate

3.1.1 Introduction

Methotrexate (amethopterin) is an antimetabolite of folic acid. Being a structural analogue of folic acid, it comes under the antifolate class of drugs. Food and Drug Administration (FDA) had approved its clinical use in 1953. Methotrexate is now widely used for the treatment of leukemia (33), lymphoma (4), sarcoma (5), carcinoma (3), ectopic pregnancy (8), psoriasis (7) and eczema (34). It is also useful in the treatment of psoriasis, rheumatoid arthritis, dermatomyositis, Wegener's granulomatosis and sarcoidosis (35).

3.1.2 Chemistry

Methotrexate is 4-amino-4-deoxy-N-10-methylpteroylglutamic acid with a molecular weight of 454 daltons. Its molecular structure differs from folic acid only in that folic acid has a hydroxyl group in place of the 4-amino group on the pteridine ring and there is no methyl group at the N¹⁰ position. Methotrexate is a bicarboxylic weak acid with pKₐs in the range of 4.8 to 5.5 (36). Methotrexate is ionized and lipid insoluble at physiological pH. Its solubility in human urine is directly proportional to pH.
Figure: 3.1. Structure of methotrexate (MTX)

Figure: 3.2. Structure of folic acid
3.1.3 Mechanism of action

Methotrexate blocks the de novo nucleotide synthesis primarily by depleting cells of reduced tetrahydrofolate cofactors through competitive inhibition of dihydrofolate reductase (DHFR), thus decreasing the availability of folate required for DNA synthesis and cell proliferation (6). MTX binds with high affinity and inactivates the dihydrofolate reductase (DHFR), resulting in the depletion of metabolically active intracellular folates with subsequent inhibition of the synthesis of thymidylic acid and inosinic acid which are of special importance in cellular proliferation (37). Being a high affinity inhibitor of dihydrofolate reductase, methotrexate is a pro-oxidant compound that causes depletion of the dihydrofolate pool and directly affects the synthesis of thymidylate, suppressing DNA synthesis (2). Moreover, the cytosolic NAD(P) – dependent dehydrogenases (38) and NADP – malic enzyme are inhibited by methotrexate, suggesting that the drug could decrease the availability of NADPH in cells (39).

The drug is highly cell cycle dependent, acting primarily during DNA synthesis (S-phase). As a result, the tissues that are undergoing rapid cellular turnover with a high fraction of the cells in cycle are the most susceptible to the drug’s cytocidal effects (40). Reduced forms of folates are important for the metabolic transfer of 1- carbon units in a variety of biochemical reactions. They are also required as co-factors in the conversion of glycine to serine and of homocysteine to methionine. By preventing this amino acid inter conversions; methotrexate may also interfere with protein synthesis. Methotrexate also inhibits 5–aminoimidazole–4–carboxamide ribonucleotide (AICAR) formoyltransferase that leads to increased adenosine levels. Adenosine binds with A2α receptors and increases cAMP levels that produce a range of anti-inflammatory effects (6, 41).
3.1.4 Pharmacokinetics of methotrexate

i) Bioavailability of MTX
MTX is mainly absorbed in the proximal jejunum (42). The absorption of MTX from intramuscular injection is rapid and complete. One third of the absorbed MTX is subjected to metabolism by intestinal bacteria prior to absorption (43). The absorption of orally administered MTX is dose dependent and is 30% less at higher doses compared to lower doses (44). The absorption of MTX from the gastrointestinal (GI) tract is reduced in the case of intestinal pathology, such as inflammatory bowel disease, shortened bowel, antibiotic treatment or other malabsorption syndrome and is not affected by concomitant food intake. The bioavailability of intramuscular (im) and subcutaneous (sc) MTX is similar to that of intravenous (iv) MTX.

ii) Metabolism of MTX
MTX enters the cell via the proton coupled folate transporter/ heme carrier protein-1 (PCFT/HCP-1) (45) and undergoes polyglutamation catalyzed by folylpolyglutamate synthetase. Once polyglutamated, MTX is retained in cells for prolonged periods of time (35). Once inside the cell, it enters a deeper compartment from which it will be released slowly. After intravenous administration of MTX, its decay is triphasic – the distribution phase, initial elimination phase and a prolonged elimination phase. The slow release of polyglutamated MTX from cells may contribute to the prolongation of the third phase of elimination (46). MTX polyglutamates and dihydrofolates (that accumulate as a result of DHFR inhibition) also inhibit thymidylate synthase and other enzymes involved in the purine biosynthetic pathway (47-49). Less than 10% of the dose of MTX is oxidized to 7 – hydroxymethotrexate (7-OH-MTX) by aldehyde oxidase (50-52). MTX is metabolized to 7-OH-MTX, as well as polyglutamates of MTX and 7-OH-MTX. These polyglutamates are further hydrolyzed by γ – glutamyl hydrolase and may serve as a reservoir of methotrexate (53). MTX excreted in the bile is converted to 2, 4-diamino–N¹⁰–methylpterio
acid (DAMPA) by carboxy peptidase in gut flora (54). The polyglutamate metabolites are stored in the liver and in erythrocytes for long periods.

**iii) MTX clearance**

65–80% of the drug is excreted by renal clearance and 20–35% is secreted with the bile and metabolized or transferred to other compartments. In renal elimination, the glomerular filtration plays a major role, while the tubular secretion and re-absorption play a minor role. Biliary secretion of MTX is a minor pathway, but may assume importance if the renal clearance or metabolism were saturated. In case of impaired renal function, MTX clearance from plasma will be decreased and thus might cause a greater risk of toxicity. The terminal serum half-life of MTX is approximately 7–10 h (55). MTX is highly ionized at physiological pH and extremely low levels were reported in saliva and breast milk (56).

**iv) MTX Distribution**

MTX is 35 to 50% albumin-bound, while 7–hydroxymethotrexate (7–OH–MTX), the principal metabolite, is 91–95% albumin-bound. Transport of MTX and 7-hydroxymethotrexate into cells occurs both passively and actively (by folate receptors PCFT/HCP-1), and by facilitated diffusion (57, 58).

**3.1.5 Therapeutic uses**

In the 56 years since Farber et al first described clinical remissions in children with acute leukemia after treatment with the folate antagonist aminopterin (59), antifolate drugs dominated by MTX, have been used to treat millions of patients with malignant and autoimmune diseases (60). MTX is classically used as antifolate or antimetabolite in the treatment of malignancies
like leukemia (33), lymphoma (4), sarcoma (5), carcinoma (3) in large doses. Later it is also used in the treatment of rheumatoid arthritis and other rheumatic disorders (6), refractory inflammatory bowel disease (61), ectopic pregnancy (8), psoriasis (7), eczema (34), urticaria (62), ankylosing spondylitis (63), idiopathic hypertrophic cranial pachy meningitis (64), chronic cholestatic disorder (65), wegener’s granulomatosis (66), primary biliary cirrhosis (67), systemic lupus erythematosus (68), inflammatory eye disease (69), and haemophagocytic lymphohistiocytosis (70). It is estimated that MTX is now prescribed to at least 5,00,000 patients with rheumatoid arthritis (RA) worldwide, making it by far the most commonly used disease-modifying antirheumatic drug (DMARD) (60). Recently MTX is suggested to have role in the treatment of malaria and clinical trials are on the way (71).

3.1.6 Drug dosage
High dose methotrexate (HDMTX) is used for its cytotoxic – antiproliferative action in the treatment of cancer has varied from 5000mg/m$^2$ to 12,000mg/m$^2$ per week (130 – 300 mg kg$^{-1}$) for several weeks (72). Low dose MTX (LDMTX) has been used in the range of 0.1 – 0.4 mg kg$^{-1}$ (7.5 – 30 mg/ adult) once weekly in the treatment of rheumatic diseases and psoriasis (73). MTX produces side effects such as mucositis and enterocolitis. The adverse effects were commonly seen after several weeks of LDMTX and somewhat with higher doses of MTX (more than 15 mg/m$^2$/week in adults) (73).
Figure: 3.3

Dose – response effect of drugs in humans, as per Paracelsus’ law using methotrexate (MTX) (72).
3.1.7 Drug Toxicity

The primary toxic effects of methotrexate are exerted against rapidly dividing cells of the bone marrow and gastrointestinal epithelium (74). Elevation of hepatic enzymes is a consistent finding with HDMTX, but usually is reversible. On the other hand, LDMTX may lead to cirrhosis after long-term continuous treatment.

LDMTX caused adverse events including gastrointestinal symptoms, hepatic enzyme abnormalities, pneumonitis and severe leucopenia (75). The enhanced toxicity of methotrexate also includes alopecia (76), dermatitis (77), interstitial pneumonitis (78), neurotoxicity (79), nephrotoxicity (80), defective oogenesis or spermatogenesis (81), abortion (82), and teratogenesis (83). Folic acid supplementation had decreased the incidence of elevation of hepatic enzymes during MTX therapy, but seems to have no effect on the incidence, severity, and duration of other adverse events, including gastrointestinal and mucosal side effects (11, 84).

HDMTX toxicity includes hepatotoxicity, myelotoxicity and death due to infections and hemorrhage, nephrotoxicity, gastrointestinal toxicity including mucositis, pulmonary toxicity, myocardial toxicity, and skin problems including alopecia. The toxicity of LDMTX was observed when it is used at doses >7.5 mg per week and several weeks after the first drug administration (72). 72.9% of patients with rheumatoid arthritis who received on average a low dose of MTX (8.8 mg/week) for a mean duration of 36.5 months, had at least one adverse event and the most common being the gastrointestinal and elevation of liver enzymes (85). Worldwide, it is estimated that 0.5 – 1 million adults and 50,000 – 100,000 children receive LDMTX weekly for the treatment of RA and juvenile idiopathic arthritis, respectively (86). In the case of multiple sclerosis, MTX at a dose of 7.5 mg per week for up to 2 years was not
associated with toxicity (87). The use of 7–30 mg per week LDMTX in the treatment of rheumatoid and juvenile arthritis and psoriasis was associated with an acceptable toxicity profile (73). Higher doses (<100 mg) were associated with toxicity, in the treatment of cancer.

3.2 SMALL INTESTINE

The small intestine has wide range of functions that include digestion and absorption of micro – and macro – nutrients, production of regulatory hormones and acting as a barrier against noxious environmental agents. Apart from these, the intestine takes part in other basic homeostatic processes such as maintenance of normal blood potassium concentration, acid – base balance and normal plasma volume. Thus, proper function of small intestine is essential for normal health and well being. The development of small intestine both structurally and organizationally is highly ordered and strictly regulated (88, 89).

3.2.1 Morphological and structural organization of the small intestine

The small intestine is made up of four layers. The mucosa, submucosa, muscularis externa and serosa.

The mucosa consists of three basic layers – the epithelium, lamina propria and muscularis mucosa (89). The epithelium is folded into finger-like projections (villi), and the lamina propria forms the fibro vascular core of the villi and the muscularis mucosa separates the mucosa from the submucosa. In the proximal small intestine, the villi appear as leaf-like protrusions, whereas, in the distal end, they appear as finger-like and shorter, thus reflecting differences in the absorptive function and capacity of these regions of the intestine. At the base of the villi are the crypts of Lieberkühn. The crypts are the proliferative regions of the small intestine containing the stem cells in which all cells of the epithelium are produced. These proliferative cells move
up from the crypts to the villi and cover the whole of its surface (90). Covering the luminal surface of the small intestine is the mucus layer that acts as a protective layer for the epithelial layer and is secreted continuously by specialized cells in the epithelium (91). The rate of cell turnover in the intestine is rapid (92).

The crypts are the proliferative zones of the small intestine. There may be four to six stem cells per crypt, located in cell positions 4–6 up from the base of the crypt (93). On an average, the stem cells divide once a day to produce rapidly dividing clonogenic stem cells. These clonogenic cells form a population of transit cells that might consist of about six successive cell generations. Cells from the fourth generation onwards stop dividing and differentiate into functional enterocytes (94, 95). The enterocytes, goblet cells and entero – endocrine cells differentiate and move up from the crypt to the villus. In contrast, the Paneth cells differentiate and move down to the base of the crypt. If any DNA damage is found in stem cells, they go into apoptosis immediately instead of attempting to repair it whereas; the clonogenic cells have a high capacity to repair their DNA (90, 93). Fully differentiated and functional enterocytes move out of the crypt and spread over the villus surface. Once they reach the apex of the villus, they are sloughed off into the lumen. This process is continuous and, as a result, the whole epithelial surface of the intestine is replaced every 3–7 days (96).

The lamina propria forms the connective tissue core of the villi and surrounds the epithelium of the crypts of Lieberkuhn. Within the lamina propria there is an extensive network of blood and lymphatic vessels and nerve fibers. Lamina propria contains numerous nodules of lymphatic tissue. These nodular aggregations which are large and numerous particularly in the ileum are called Payer’s patches.
Muscularis mucosa is the narrow smooth muscle layer that forms the base of the mucosa. Between villi the epithelial sheet dips down into the lamina propria to form the intestinal crypts or glands, which extend to the muscularis mucosa (97).

The epithelium of the small intestine consists of five cell types. They are the enterocytes, goblet cells, paneth cells, entero–endocrine cells and M (microfold) cells. The primary function of the enterocytes is the terminal digestion and absorption of nutrients. The goblet cells synthesize and secrete mucins that make up the mucous layer covering the entire epithelial surface. The goblet cells also secrete bioactive peptides that facilitate the movement of cells during restitution and repair of the small intestine. The Paneth cells are located in the base of the crypts. The Paneth cells secrete different antimicrobial peptides, enzymes and proteins into the lumen of the crypts to keep this metabolically active region of the gut sterile. Thus the Paneth cells play an important role in regulating the normal bacterial flora of small intestine. The entero–endocrine cells produce a wide array of hormones, including gastrin, secretin, cholecystokinin, enteroglucagon and peptide YY, which modulates the metabolism in the small intestine and associated tissues. The M cells overlie on the peyer’s patches and other large lymphatic nodules. M cells convey microorganisms and macro molecules from the intestinal lumen to peyer’s patches thus suggesting their role in immune function (98). During migration and differentiation of the intestinal epithelium, disaccharidase activity increases and alkaline phosphatase isoenzymes are modified (99). Enzymes, such as disaccharidases and peptidases as well as receptors and transport proteins, are distributed along the microvillus membrane (100). In contrast to mature absorptive cells at the villus tip, disaccharidases, alkaline phosphatase and dipeptidase activities are not found in crypt epithelium (101).
Submucosa is made up of dense connective tissue and localized sites that contain aggregates of adipose cells. The presence of submucosal glands or Brunner’s glands is a characteristic feature of duodenum. The highly alkaline secretions of submucosal glands serve to protect the proximal part of the small intestine by neutralizing the acidic chyme.

Muscularis externa consists of an inner circular layer and outer longitudinal layer of smooth muscle cells. These help in the muscular contractions occurring in the small intestine – the segmentation and peristalsis.

The serosa or the outermost layer is an extension of the peritoneum and consist of a single layer of flattened mesothelial cells overlying some loose connective tissue.
Schematic diagram of rat small intestine enterocytes depicting different zones, types, layers as well as direction of cell growth (102).
3.3 Methotrexate and small intestinal damage

The use of chemotherapy agents had demonstrated therapeutic benefits in a wide variety of cancers including gastrointestinal (GI) cancers (103-105). But, chemotherapy is known to be associated with various side effects, with toxicity to the GI tract being a major clinical concern. GI toxicity is often a major cause of cancer treatment – related morbidity (106).

3.3.1 Mechanisms suggested to play role in MTX induced small intestinal damage

Few studies have implicated ROS induced oxidative stress in the pathogenesis of methotrexate induced gastrointestinal toxicity (21). Increased production of free radicals has been shown to occur in the small intestine in response to administration of methotrexate (107). Fluid retention in the small intestine due to administration of MTX was suggested to induce fluid accumulation (entero pooling effect of PGE$_2$) that may lead to diarrhea (25).

3.3.2 Studies reported thus far on MTX induced small intestinal damage

i) MTX treatment in children with acute lymphoblastic leukemia (ALL) induced malabsorption (108), malnutrition (109), enteropathy (110), biological alterations in the mucosa of human proximal intestine (22) and reduced crypt mitosis and patchy degenerative abnormalities (19).

ii) The MTX – induced intestinal mucositis in rats is characterized by crypt loss, villus fusion and atrophy (20), resembling the gut mucositis experienced as a common side – effect by patients undergoing chemotherapy or radiotherapy (111).
iii) MTX caused severe jejunal injury, diarrhea, weight loss and damage to villus architecture. There was increased lysozyme expression and decreased sucrose – isomaltase and sodium/glucose transport 1 expression (24).

iv) MTX treatment caused neutrophil infiltration, increased ROS production followed by increase in mucosal permeability (107).

v) MTX deteriorated the barrier function of mucosa against intravascular bacteria (21), and the absorptive function is altered (23).

vi) MTX reduced the amounts of both AMP and GMP and in turn inhibited the DNA synthesis in the crypt cells (112).

vii) MTX administration resulted in epithelial apoptosis and increased villus damage and ulceration (2, 22).

viii) Methotrexate has been reported to cause fluid accumulation in the small intestine with a subsequent increase in mitochondrial size (25), and also cause oxidative damage to the mitochondrial lipids and DNA (113).

In other studies, MTX was known to inhibit state III and state IV respiration (114), decreases the membrane potential ($\delta \psi$) (115) of mitochondria in liver and to inhibit transplasma – membrane electron transport and ferricyanide-induced proton extrusion by HeLa cells (115). MTX was also shown to induce apoptosis in HeLa cells (116), activated T cells (117), rat intestinal epithelial cells (RIE-1) (118), and mouse embryonic fibroblasts (MEFs) (119). MTX is known to block the malate – aspartate shuttle and thus decreasing the transport of reducing equivalents from
cytoplasmic NADH into the respiratory chain of mitochondria that is required for cellular energetic gain in HeLa cells (120).

Although there are some preliminary clinical studies and reports, there does not appear to be an extensive examination of gastrointestinal toxicity of methotrexate. Since the mechanism of these side effects has not been completely clarified, cancer chemotherapy has to be accompanied by symptomatic therapy such as antibiotics and anti-diarrheal drugs. Therefore, it is important to clarify the mechanism of the MTX-induced intestinal damage to perform the cancer chemotherapy effectively by preventing the side effects.

3.4 Mitochondria

Mitochondria are called the power house of the cell, where ATP is generated. The structure of mitochondria consists of two membranes, an outer membrane which is permeable to most metabolites and an inner membrane, which is selectively permeable and thrown into a number of folds or cristae with the matrix enclosed within it. The inner membrane regulates movement of mitochondrial enzymes, cations (including calcium) and substrates into cytosol, as well as acting as a specific transport site for a variety of mitochondrial substrates that must be taken up from the cytosol. It also contains specialized transport sites for small molecules, the electron transport chain that accepts electrons generated in the citric acid cycle and ATP synthase that carries out oxidative phosphorylation and ATP synthesis. The mitochondrial matrix contains the enzyme of the tricarboxylic acid cycle, fatty acid oxidation and urea synthesis, as well as substrates, nucleotide cofactors and inorganic ions. The matrix also contains the mitochondrial genetic machinery (DNA, RNA and ribosomes) that generates several mitochondrial proteins.
3.4.1 The electron transport chain (ETC) and oxidative phosphorylation

The transduction of energy by the transfer of electrons from substrates of the tricarboxylic acid cycle (TCA cycle) (via NADH) and from the fatty acid oxidation cycle (via NADH and FADH$_2$) to oxygen is facilitated by the respiratory chain or ETC. This occurs with the help of five large protein complexes embedded in the inner mitochondrial membrane and ubiquinone (coenzyme Q). Mitochondrial oxidative phosphorylation occurs only in the presence of adequate amounts of its substrates, namely ADP, Pi and O$_2$ and an oxidizable metabolite that can generate NADH and FADH$_2$. The dependence of the process on ADP shows that respiration is tightly coupled to ATP synthesis. ATP synthesis is absolutely dependent on electron flow along the respiratory chain and this occurs in normal mitochondria only when ATP is being synthesized as well. This regulatory phenomenon is known as respiratory control and ensures that there is no wasteful oxidation of substrates. The electron transport chain (the oxidation of NADH and FADH$_2$ by O$_2$) and oxidative phosphorylation (synthesis of ATP) are normally tightly coupled. In the resting state, when oxidative phosphorylation is minimal, the electrochemical gradient across the inner membrane builds up to the extent that it prevents further proton pumping and therefore inhibits electron transport. The free energy generated from the step-wise redox reactions is converted into a transmembrane proton gradient by the extrusion of protons through the inner membrane at complexes I, III and IV. Because of the proton gradient, the protons are allowed to flow back into the mitochondrial matrix through complex V, and the released energy is used by ATP synthase to synthesize ATP by oxidative phosphorylation. However, if the permeability of the inner mitochondrial membrane to protons is increased, this would uncouple oxidative phosphorylation from electron transport by providing a route for the dissipation of the proton electrochemical gradient that does not require ATP synthesis (121).
3.4.2 Mitochondrial calcium flux

The calcium concentration within mitochondria in resting cells is believed to be low (122). However, isolated mitochondria can accumulate large quantities of Ca$^{2+}$. Elevated levels of Ca$^{2+}$ in the mitochondria results in increase in the activity of key enzymes of mitochondria. The reversible uptake of Ca$^{2+}$ into mitochondria is postulated to coordinate energy production to cellular needs (123). Thus, mitochondria have a unique role in rapidly accumulating and then releasing large quantities of Ca$^{2+}$, thereby actively participating in cellular Ca$^{2+}$ signaling.

3.4.3 The mitochondrial permeability transition (MPT)

The mitochondrial permeability transition (MPT) pore is an assembly of preexisting proteins of the inner and outer mitochondrial membranes into a large conductance channel permeable to small solutes of > 1,500 daltons in and out of mitochondria, resulting in mitochondrial swelling. A characteristic feature of mitochondrial damage is the opening of the permeability transition pore. Calcium, inorganic phosphate, and many oxidant chemicals induce this increased permeability, whereas Mg$^{2+}$, adenosine diphosphate (ADP), and low pH oppose the onset of the MPT pore opening. The post effects of the MPT pore opening are membrane depolarisation, uncoupling of oxidative phosphorylation, release of intra-mitochondrial solutes such as glutathione and pyridine nucleotides, and large amplitude mitochondrial swelling (124). MPT is comprised of the adenine nucleotide translocater (ANT) protein, VDAC (voltage-dependent anion channel), Cyclophilin – D, PBR (peripheral benzodiazepine receptor) (125,126). Other proteins from the matrix, in the inter-membrane space and outer membrane are proposed to associate with the MPT pore complex are creatine kinase (outer compartment), porin (outer membrane) and hexokinase (outer surface of outer membrane).
The components of mitochondrial permeability transition (MPT) pore. cyclophilin D (Cyp-D); ANT – adenine nucleotide translocase; PBR – peripheral benzodiazepine receptor; VDAC – voltage dependent anion channel; RSH – reduced thiol; RSSR – thiol disulfide (125).
3.4.4 Mitochondrial susceptibility to oxidative and nitrosative damage

Morphological and functional changes in the mitochondria are prominent features of irreversible cell damage caused by oxidative stress (127). About 90% of inspired oxygen is consumed by the mitochondria and 1 – 2 % of oxygen reduced in the mitochondria is constitutively converted to superoxide (O$_2^-$). Superoxide in the mitochondria is produced exclusively by the respiratory chain by the reaction of oxygen with the iron – sulphur centers in complex I, partially reduced ubiquinone and cytochrome b in complex III (128). Hydrogen peroxide is generated in mitochondria by dismutation of superoxide by mitochondrial superoxide dismutase (SOD) and succinate is considered to be the most effective substrate for generation of hydrogen peroxide by mitochondria (129). Mitochondria are susceptible to damage by reactive oxygen species (ROS) (128). Mitochondrial matrix enzymes and membrane-bound enzymes are affected by oxidative stress due to peroxidation of phospholipids. Mitochondrial respiration is shown to be uncoupled by oxidants. The NADH oxidizing site of the respiratory chain has been suggested to be the site of generation of these oxidants (130). Hydroxyl radicals produced from iron and copper found in mitochondria can attack nucleic acids, proteins and lipids resulting in lipid peroxidation (131). Mitochondrial DNA and protein have also been shown to undergo oxidative damage (132). Increased calcium uptake and decreased ATP supply are seen as a result of pyridine nucleotides oxidation in the mitochondria (133). Since mitochondria are prone to damage by various oxidants, antioxidant systems are present in the mitochondria to provide protection against such damage. One such antioxidant is reduced glutathione (GSH). The amount of glutathione in mitochondria accounts for 10 – 15% of total intracellular glutathione. Enzymes necessary for GSH synthesis are absent in mitochondria and hence GSH is imported into mitochondria from the cytosol. Mitochondrial GSH, in the presence of GSH peroxidase, is the only defence for
mitochondria against toxic oxygen intermediates. Oxidative stress-induced hepatocyte damage has been shown to be reduced by the supplementation of mitochondrial GSH with glutathione monoethylester, even when cytosolic GSH is depleted (134). Superoxide dismutase (SOD) acts as an antioxidant by scavenging the superoxide radicals that dismutes spontaneously to \( \text{O}_2 \) and hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) quite rapidly. Thus SOD prevents the reaction of superoxide with sensitive and critical cellular targets.

Mitochondria contain a constitutive nitric oxide synthase (NOS) called mNOS that catalyzes the formation of nitric oxide in the mitochondria by the conversion of arginine to citrulline (135). Mitochondria act as intracellular sinks of NO because of thiol groups and glutathione that can bind reversibly to NO (136, 137). NO in mitochondria can be reduced to nitrosyl anion by taking up electron from ubiquinol or cytochrome-c or cytochrome oxidase (138). Alternatively it can be oxidized with superoxide to form more potent peroxynitrite (139). Peroxynitrite reacts with a wide range of biomolecules, such as proteins (140), nucleotides (141), lipids (142) and antioxidant molecules (143). Mitochondrial membrane ubiquinol scavenges free radicals derived from peroxynitrous acid decomposition, thereby protecting mitochondrial proteins against nitration. Removal of superoxide by matrix Mn – superoxide dismutase yields \( \text{H}_2\text{O}_2 \) that is in turn reduced to \( \text{H}_2\text{O} \) by mitochondrial glutathione peroxidase (144).
Redox transitions of ubiquinol and the scavenging and formation of nitrogen – and oxygen – centred reactive species (145).
3.5 Free radicals

Free radicals are atoms or group of atoms with an odd or unpaired number of electrons in the outer most shell (146, 147). Free radicals are highly reactive due to the presence of unpaired electron(s). Oxygen free radicals are commonly seen in biological systems. Any free radical involving oxygen can be referred to as reactive oxygen species (ROS). Much of the behavior of molecular oxygen and its partially reduced species derives from their reduction potentials and molecular orbital structures.

\[
\begin{array}{c|c|c|c|c}
\sigma_x^* & \sigma_x & \pi_y & \pi_x & \pi_y, \pi_x^* \\
\hline
\hline
O_2 & \downarrow & \downarrow & \downarrow & \downarrow \\
O_2^- & \downarrow & \downarrow & \downarrow & \downarrow \\
H_2O_2 & \downarrow & \downarrow & \downarrow & \downarrow \\
{^1}O_2 & \downarrow & \downarrow & \downarrow & \downarrow \\
\end{array}
\]

Molecular oxygen has one unpaired electron in each of its two \(\pi^*\) outer antibonding orbitals and due to the parallel spin of these electrons, the reactivity of molecular oxygen is very low. Turning the two parallel spinning electrons into antiparallel by means of an energy input will increase the reactivity of oxygen, to produce singlet oxygen, which is highly reactive due to removal of the spin restriction (148).
Table: 3.1

<table>
<thead>
<tr>
<th>Reactive oxygen species</th>
<th>Reactive Nitrogen species</th>
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<tbody>
<tr>
<td>Superoxide anion radical [$O_2^{-}$]</td>
<td>Nitrosyl cation [NO$^{+}$]</td>
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<tr>
<td>Hydrogen peroxide [H$_2$O$_2$]</td>
<td>Peroxynitrite [ONOO$^{-}$]</td>
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<tr>
<td>Hydroxyl radical [HO$^\cdot$]</td>
<td>Nitrosothiols [RSNO]</td>
</tr>
<tr>
<td>Lipid peroxide radical [ROO$^\cdot$]</td>
<td>Nitroxyl anion [NO$^{-}$]</td>
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<tr>
<td>Singlet oxygen radical [$^{1}$O$_2$]</td>
<td>Dinitrogen trioxide [N$_2$O$_3$]</td>
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<tr>
<td>Hydroperoxyl radical HO$_2^\cdot$</td>
<td>Dinitrogen tetroxide [N$_2$O$_4$]</td>
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<td></td>
<td>Nitric oxide (NO$^\cdot$)</td>
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<td></td>
<td>Nitrogen dioxide (NO$_2^\cdot$)</td>
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Table: 3.1

Different types of reactive oxygen species and reactive nitrogen species (148, 149).
Oxidative stress is defined as the shift in the balance between the prooxidant: antioxidant towards the prooxidant. The amount of oxidative stress that an organism experiences depends upon the rate at which $\text{O}_2^-$ and $\text{H}_2\text{O}_2$ are made.

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are produced as by-products of normal metabolic processes in all organisms. In physiological conditions, the antioxidant defense system protects the cells against these species. When the generation of ROS/RNS exceeds the ability of antioxidant defense systems to remove them, such an imbalance can cause oxidative/ nitrosative damage to cellular constituents (DNA, proteins, lipids and sugars), which is defined as oxidative/ nitrosative stress (150).

### 3.5.1 Reactive oxygen species

**i) Superoxide radical**

The free radical, superoxide anion ($\text{O}_2^-$) is formed by the addition of one electron to ground state molecular oxygen. This very reactive chemical species is unstable due to its spontaneous reaction with another superoxide molecule, producing hydrogen peroxide and molecular oxygen (dismutation reaction) (151).

$$\text{O}_2^- + \text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$$

The charged nature of $\text{O}_2^-$ renders it membrane – impermeable unless there is a transmembrane channel through which it can move, such as the anion channel in erythrocytes (152). The reactivity of individual oxygen – derived molecules differs greatly. As a relatively strong reductant, superoxide can function either as a reductant or an oxidant, depending on the oxidation – reduction potential of the reacting molecule. Although it is a precursor to more reactive species, superoxide reacts with a limited repertoire of chemical targets, such as the
iron – sulphur centers that function as electron carriers in respiratory chains of bacteria and mitochondria (153).

ii) Hydrogen peroxide

The reduction, by two electrons of molecular oxygen produces the peroxide ion \([O_2^{2-}]\), which on protonation gives hydrogen peroxide. Hydrogen peroxide is very harmful to cells since it may cross biological membranes and it can give rise to the highly reactive hydroxyl radical (146). Hydrogen peroxide \((H_2O_2)\) is a more potent oxidant and is more reactive, although its targets are still rather limited, and include methionine and certain highly reactive cysteine residues such as those found in the active sites of some enzymes. Oxidation of such cysteine residues inactivates enzymes such as protein tyrosine phosphatases (154). \(H_2O_2\) can form highly reactive hydroxyl radicals \((OH^-)\) in the presence of transition metal ions, such as iron (155).

iii) Hydroxyl radical

The tri-electron reduction product of molecular oxygen is the hydroxyl radical. This is an extremely reactive chemical entity with a short half life and a small active radius, which can react with any biological molecule. Hydroxyl radicals are highly reactive with various biomolecules, initiating free-radical chain reactions that produce marked oxidative damage. In contrast, \(O_2^-\) and \(H_2O_2\) are less reactive oxidants but have a longer half life, which enables them to react with molecules at locations distal from their site of production (151). The main source of hydroxyl radicals is the metal – catalyzed Haber – Weiss reaction, which is the net result of two reactions involving superoxide radical, iron and hydrogen peroxide.
\[
\begin{align*}
O_2^- + Fe^{3+} & \rightarrow O_2 + Fe^{2+} \\
Fe^{2+} + H_2O_2 & \rightarrow Fe^{3+} + OH^- + OH^- \text{ (Fenton-type reaction)}
\end{align*}
\]

\[
\begin{align*}
O_2^- + H_2O_2 & \rightarrow O_2 + OH^- + OH^- \text{ (Haber-Weiss reaction)}
\end{align*}
\]

The anionic charge of \(O_2^-\) inhibits its effectiveness as an oxidant of electron-rich molecules. The reactivity of \(H_2O_2\) is diminished by the stability of its oxygen-oxygen bond. Neither of these features applies to the hydroxyl radical, and \(OH^-\) reacts at virtually diffusion-limited rates with most biomolecules (148).

### 3.5.2 Reactive nitrogen species

Nitric oxide (NO) also known as endothelial-derived relaxing factor (EDRF) has regulatory, protective and deleterious effects. Nitric oxide is an uncharged and hydrophobic radical molecule that can easily diffuse among the cellular compartments and transmit information as well as damage different targets. It can react with various molecules, e.g., molecular oxygen, superoxide anion (\(O_2^-\)), or transition metals, yielding reactive nitrogen species (RNS) and metal–nitrosyl adducts. NO can react directly with the target molecules or act through the reactive nitrogen species. Formation of nitrosamines by nitrosation of amines most probably takes place only through the interaction of \(N_2O_3\) with amino compounds, providing the best indicator of nitrosative stress. Most of the systemic effects of NO are mediated by its conversion to S–nitrosothiol that acts as reservoir of NO. S-nitrosothiol is readily formed by auto–oxidation of NO via the interaction between \(N_2O_3\) with certain thiols (156). NO and its derived RNS such as peroxynitrite and nitrogen dioxide causes nitration of proteins and lipids. The oxidant
reactivity of peroxynitrite is highly pH – dependent and both peroxynitrite anion (ONOO\(^{-}\)) and peroxynitrous acid (ONOOH) can participate in direct one – and two – electron oxidation reactions with biomolecules (e.g. transition metal and thiol oxidation, respectively), where ONOOH undergoes homolytic fission to generate one-electron oxidants hydroxyl and nitrogen dioxide radicals (with a 30 % yield, responsible of protein tyrosine nitration). This proton-catalyzed decomposition to form hydroxyl and nitrogen dioxide radicals may become relevant in hydrophobic phases resulting in the initiation of lipid peroxidation processes. The reaction of peroxynitrite-derived radicals with lipids leads to peroxidation (malondialdehyde, conjugated diene and lipid hydroperoxide formation) (140, 157) and formation of nitrito –, nitro –, nitrosoperoxo – and/or nitrated lipid oxidation adducts (142, 158). Nitrated fatty acids represent novel signaling mediators leading to secondary alteration or changes in protein function mostly via thiol-based modifications (159, 160). RNS causes nitration of protein tyrosine residues to 3 – nitrotyrosine that can result in modification, loss or gain of protein function (161).

Under nitro-oxidative stress conditions, the pathway of lipid nitration is unregulated accompanied with lipid oxidation and protein nitration. Thus, the nitration chemistry switches from signaling to damage, since lipid oxidation products and nitrated proteins start to affect normal cellular functions. This also depends on the fact that NO can serve an effective antioxidant mechanism (142, 162, 163) and that the processes leading to enhanced \(\mathrm{O}_2^{-}\) formation and activation of heme peroxidases reactions by \(\mathrm{H}_2\mathrm{O}_2\) divert the normal physiological routes of NO to oxidative pathways. When the production rates of peroxynitrite are high, the antioxidant mechanisms will be compromised; protein tyrosine nitration (and oxidation) will increase and thus participate in events such as mitochondrial cytochrome c release and apoptosis (164, 165).
3.5.3 Sources of free radicals in biological systems

3.5.3.1 Reactive Oxygen Species (ROS)

Cellular sources of "reactive oxygen species" (ROS) production include plasma membrane NADPH oxidase, intracellular cytosolic xanthine oxidase, peroxisomal oxidases, endoplasmic reticular oxidases and mitochondrial electron transport components. \( \text{O}_2^{-} \) and \( \text{H}_2\text{O}_2 \) are formed by accident when molecular oxygen adventitiously oxidizes redox enzymes that are designed to transfer electrons to other substrates. In order for enzymes to transfer electrons to oxygen, they must be adept at univalent redox reactions. The respiratory chain includes enzymes that meet this criterion and thus mitochondria is the major source of free radicals. Though the electron transport chain is designed to prevent the release of partially reduced intermediates, superoxide radicals can be formed, especially by auto-oxidation of ubiquinone and NADH dehydrogenase (166). Mitochondria account for the bulk of endogenously formed ROS (167). An unavoidable respiratory electron leak results in the formation of superoxide anion radicals, \( \text{O}_2^{-} \), which are toxic to mitochondrial enzymes such as aconitase, and also can reduce \( \text{Fe}^{3+} \) into \( \text{Fe}^{2+} \), a catalyst of Fenton-type chemistry. Although superoxide poorly crosses biological membranes, its uncharged protonated form, \( \text{HO}_2^{+} \), diffuses easily and is highly reactive (168). Mitochondria consume about 90% of inspired oxygen, of which about 1 – 2% of the total oxygen reduced in mitochondria is constitutively converted to superoxide. Superoxide is produced in the respiratory chain by the reaction of oxygen with the iron sulphur centers in complex I, partially reduced ubiquinone and cytochrome b in complex III (169, 170). Dismutation of superoxide by Mn – superoxide dismutase is the main mitochondrial source of \( \text{H}_2\text{O}_2 \) and succinate is considered the most effective substrate for \( \text{H}_2\text{O}_2 \) generation by mitochondria (129). Thus, the two main sites of \( \text{O}_2^{-} \) and \( \text{H}_2\text{O}_2 \) generation in the inner mitochondrial membrane seem to be ubiquinone at complex III and NADH dehydrogenase at complex I.
Figure: 3.7

Schematic diagram of mitochondrial processes that are the main free radical generators.

Oxygen radicals attack lipids, carbohydrates, proteins, and DNA. The products of lipid peroxidation include highly reactive molecules that can cause lipoxidative damage to mitochondrial DNA and proteins (171).
Superoxide dismutase – 2 (SOD2), manganese superoxide dismutase, reduces most mitochondrially produced superoxide into hydrogen peroxide (H$_2$O$_2$) while the cytoplasmic superoxide is reduced by copper-zinc superoxide dismutase, SOD1. Hydrogen peroxide, which readily diffuses throughout the cell, is eliminated by glutathione peroxidase and catalase. During Fenton reactions, hydrogen peroxide is partially converted into the extremely reactive hydroxyl radicals, HO·, which immediately attack surrounding macromolecules, including DNA, proteins and lipids. Hydroxyl radicals exert their pathogenic activity also indirectly, by reacting with unsaturated fatty acids under the formation of organic peroxides, which decompose to aldehydes. The latter are responsible for a number of toxic effects, including the formation of protein – protein aldehyde bridges (172). Xanthine oxidase forms another major source of superoxide radical. It catalyzes the oxidation of hypoxanthine and xanthine to form O$_2$·− (173, 174). Other sources of free radicals in cells include the auto-oxidation of small soluble molecules in the cellular cytoplasm, such as catecholamines, flavins, tetrahydropterins, quinones and thiols (151).

Peroxisomes contain many of the cellular enzymes that generate hydrogen peroxide such as glycolate oxidase, acyl CoA oxidase from the beta oxidation system. Hydrogen peroxide produced from those enzymes can undergo Fenton’s reaction and to give highly reactive hydroxyl radicals. Under normal condition the hydrogen peroxide and superoxide are degraded by catalase and Cu – Zn superoxide dismutase present in the peroxisomes.

Endoplasmic reticulum contains cytochromes collectively called cytochromes P$_{450}$. Cytochrome P$_{450}$ is involved in the oxidation of a wide range of substrates at the expense of molecular oxygen. One atom of oxygen enters the substrate and other forms water, such a reaction is known as monooxygenase or mixed function oxidase reaction. ROS arise from the cytochrome
P_{450} by two ways. First, the intermediates in the catalytic cycle can be short circuited in such a way that O\textsubscript{2} is reduced to O\textsubscript{2}·⁻ or H\textsubscript{2}O\textsubscript{2} instead of being added to the substrate. Second, electrons may escape to O\textsubscript{2} from the flavins in the NADPH – P\textsubscript{450} reductase enzyme to form O\textsubscript{2}·⁻ radical.

Yet another source of free radicals in the system is from infiltration of neutrophils. Neutrophils play a pivotal role in inflammatory conditions (175). These activated phagocytic cells are drawn to the site of injury, secreting superoxide and other oxidants derived from this free radical. Two of the enzymes, which are involved in the formation of free radicals, are membrane associated NADPH oxidase and heme – containing myeloperoxidase. NADPH oxidase catalyzes the oxidation of NADPH to NADP, the electron being used to reduce oxygen to superoxide radical. This occurs during the respiratory burst of phagocytic cells. The enzymes NADPH oxidase (NOX) and dual oxidase (DUOX) generate ROS in a regulated manner, producing reactive oxygen in various cells and tissues in response to growth factors, cytokines and calcium signals. This implies important biological functions for ROS and is consistent with some earlier studies that indicate roles for ROS in growth regulation (153). Myeloperoxidase forms hypochlorous acid, in presence of hydrogen peroxide and chloride ions (176). Hypocholorus acid is highly reactive and oxidizes many biological molecules. Other oxygen-derived species such as hydroxyl ions and hydrogen peroxide are also formed by this system. Peroxidases use H\textsubscript{2}O\textsubscript{2} to produce highly reactive oxidants either at the active site or as discrete diffusible oxidants such as hypochlorous acid (HOCl). Myeloperoxidase-generated HOCl carries out a wide variety of oxidative reactions, including chlorination of tyrosine and the oxidative modification of enzymes.
3.5.3.2 Reactive Nitrogen Species (RNS)

The main source of nitric oxide (NO) is the enzyme nitric oxide synthase (NOS). There are four isoforms of NOS. They are the eNOS (endothelial NOS), iNOS (inducible NOS), nNOS (neuronal NOS) and mNOS (mitochondrial NOS) (177). NOS catalyze the oxidation of L-arginine to nitric oxide and L-citruline. The NOS isoforms are probably active as homodimers and catalyze the oxidation of the guanidino nitrogen of L-arginine to nitric oxide. NO plays a variety of roles such as maintenance of vascular tone, neurotransmission, and host defense by destroying microbes (178).

NOS are classified into two types – Constitutive and Inducible forms. nNOS, eNOS and mNOS are constitutive enzymes and normally produce small (nanomolar) amounts of NO, whereas iNOS is induced as a part of immune response in many cell types by endotoxins or combinations of pro-inflammatory cytokines (e.g., interleukin-1, tumor necrosis factor) (179). NO produced by nNOS in central or peripheral neurons, may function as a neurotransmitter, particularly in NANC (nonadrenergic and noncholinergic) neurons. NO production by eNOS in endothelial cells has an important role on the effects of endothelial – dependent vasodilators on vascular relaxation and decreased platelet adhesion and aggregation. Thus various forms of NOS have the ability to alter a range of cellular and tissue functions.
Figure: 3.8

Oxygen and nitrogen-based free radicals and associated reactants that are generated in cells by various processes (180).

Arg: L-arginine; BH4: 5, 6, 7, 8-tetrahydro-L-biopterin; Cit: L-citruline; ETC: electron transport chain; FAD: flavin adenine dinucleotide (oxidized); FADH₂: flavin adenine dinucleotide (reduced); Gly: glycine; MOP: myeloperoxidase; NAD⁺: nicotinamide adenine dinucleotide (oxidized); NADH: nicotinamide adenine dinucleotide (reduced); P – 450: cytochrome P – 450.
3.5.4 Cytotoxicity of reactive oxygen and nitrogen species

Biomolecules in a cell, namely carbohydrates, proteins, nucleic acids and lipids are damaged by reaction with free radicals, giving rise to metabolic and cellular disturbances.

i) Carbohydrates

Free radicals oxidize monosaccharides and cause depolymerisation of polysaccharides. It has been demonstrated that overproduction of superoxide by the accumulated neutrophils in rheumatoid arthritis causes depolymerisation of hyaluronic acid, a glycosaminoglycan necessary for maintaining synovial viscosity in joints (181).

ii) Proteins

Oxygen free radicals can oxidize proteins, increasing their hydrophobicity and sensitivity to proteolysis. Since carbohydrates are also sensitive to damage, glycoproteins are especially sensitive to free radical-induced damage. These molecules may react with amino acids containing unsaturated or sulfur groups, leading to cross-linking and aggregation of proteins (182). It has also been suggested that free radicals may induce peptide bond hydrolysis following oxidation of proline residues by hydroxyl radical and superoxide radical. Oxidative stress can also result in protein carbonyl formation. Several ROS can attack amino acid residues in proteins (particularly histidine, arginine, lysine and proline) to produce products with carbonyl groups. For example, hydroxyl radical converts histidine to 2 – oxohistidine and singlet oxygen converts tryptophan to N – formylkynurenine.

NO is a relatively stable and highly diffusible free radical, O$_2^-$ is much shorter lived and has restricted diffusion across biomembranes. Peroxynitrite is formed by a diffusion-limited reaction between O$_2^-$ and NO. The biological half life of NO is 1 – 10 seconds whereas that of
peroxynitrite is 5 – 20 milli seconds (183). NO and the derived reactive nitrogen species react with protein’s tyrosine residues and produce 3-nitrotyrosine, thus modifying the protein and altering the biological activity of the protein. Nitration of protein tyrosine residues is usually seen in the biomembranes at specific proteins such as in plasma membrane (e.g. erythrocyte membrane), mitochondria (e.g. complex I of the inner membrane), sarcoplasmic reticulum (e.g. Ca$^{2+}$-ATPase) and microsome (e.g. glutathione-S-transferase) (184). NO reacts with iron containing proteins to form nitrosyl complexes (185). Nitration of specific tyrosines can potentially result in modification, loss, or gain of function (161).

iii) Nucleic acids

Oxidative radical-induced damage occurs to a large extent in nucleic acids. Strand scission has been demonstrated in DNA preparations exposed to high concentrations of oxygen free radicals (186). The most sensitive components of the DNA to free radical attack are the thymine and cytosine bases, followed by adenine and guanine. In double-stranded DNA, the deoxyribose moiety is modified more frequently than the bases, due to its external location in the helix. NO and the derived reactive nitrogen species act as mutagenic agents causing nitration, nitrosation and deamination reactions on DNA bases (187, 188).

iv) Lipids

Lipids are highly susceptible to oxygen free radical attack. Hydroxyl radicals can attack unsaturated fatty acids of phospholipids and other membrane lipids initiating lipid peroxidation. This results in extensive damage to membrane structure, causing changes in membrane fluidity and function. Malondialdehyde, a by-product resulting from peroxidation of polyunsaturated fatty acids, is a major indicator of lipid peroxidation. It causes cross-linking and polymerization of membrane components and also reacts with nitrogen bases of DNA (189). The membranes
that surround cells and cell organelles such as mitochondria, peroxisomes and microsomes contain large amounts of polyunsaturated fatty acids (PUFA) side chains. The oxidation of PUFA due to free radical attack is accompanied by the formation of conjugated diene structures with a double–single-double bond arrangement. NO$_2^-$ reacts with unsaturated fatty acids and lipid radicals producing isomerized, oxidized and/or nitro-allylic, nitroalkene, dinitro or nitrohydroxy lipid derivatives. ONOO$^-$, ONOOH and/or their derived radicals readily diffuse through membranes to mediate fatty acid oxidation and nitration (142, 158).

3.5.5 Oxidative/ nitrosative damage to sub cellular components

Oxidative damage to mitochondria is well known (166). It has been demonstrated that both mitochondrial matrix enzymes and membrane–bound enzymes are affected by oxidative stress along with membrane damage due to peroxidation of phospholipids. Oxidants also uncouple mitochondrial respiration. Studies have also suggested that the site of inhibition is the NADH oxidizing site of the respiratory chain (130). Mitochondria contain iron and copper, which favor the formation of hydroxyl radicals, which can attack nucleic acids and proteins as well as lipids, resulting in lipid peroxidation (131). Mitochondrial DNA and protein have also been shown to undergo oxidative damage (190, 191). Many oxidants are capable of altering calcium cycling in mitochondria. NO is known to inhibit cytochrome oxidase and mitochondrial respiration (192). Peroxynitrite formed extra–mitochondrially can diffuse in to the mitochondria, and react with abundantly present metalloproteins and fast-reacting thiols thus affecting adenine nucleotide translocase (ANT), creatine kinase (CK), nicotinamide nucleotide transhydrogenase, aconitase, Mn – SOD, glutathione peroxidase and reduced glutathione (164). Excess of mitochondrial reactive nitrogen intermediates lead to the nitration and release of cytochrome c and apoptosis (193,194).
Figure: 3.7

The actions of NO and peroxynitrite, and their consequences on mitochondrial respiration (195).
3.5.6 Poly (ADP – ribose) Polymerase (PARP)

Apoptosis, the programmed execution of cell death, is vitally important for development and maintenance of tissue homeostasis and is a tightly regulated process (196). Once apoptosis is triggered, caspases are activated by the cleavage of proenzymes (zymogens) into distinct subunits that rearrange to form active cysteine proteases. Caspases transduce and augment the apoptotic signal by activation of other caspases. They are thought to execute cell death by proteolysis of important functional and structural intracellular proteins, such as poly (ADP-ribose) polymerase (PARP; EC 2.4.2.30) (197) and lamin A (198, 199). PARP, an enzyme involved in DNA repair and genome surveillance, is cleaved by members of the CPP32 family, whereas lamin A is a caspase 6 – specific substrate (200).

Poly (ADP-ribose) polymerase (PARP) commonly referred to as PARP – 1, is a protein modifying and nucleotide polymerizing enzyme that is abundantly present in the nucleus. PARP consists of the DNA – binding N – terminal domain, the central auto modification domain, and the C-terminal catalytic domain. The DNA-binding domain utilizes two zinc fingers, which recognize breaks in double stranded DNA. The central, highly conserved domain can be poly (ADP) – ribosylated by PARP, which can result in auto – inhibition of the enzyme. The C – terminal catalytic domain is involved in the synthesis of poly (ADP – ribose) polymer (201, 202). Although this enzyme is very abundantly expressed in proliferating cells, its catalytic activity depends on DNA single – or double – strand breaks. Exposure of living cells to ionizing radiation, alkylating agents or oxidants immediately triggers formation of protein-conjugated poly (ADP-ribose), which is followed by rapid degradation of the polymer catalyzed by poly (ADP-ribose) glycohydrolase.
PARP activation is primarily induced by DNA single strand breaks, which can be triggered by a variety of environmental stimuli and free radical/oxidants, most notably hydroxyl radical and peroxynitrite (203, 204). In response to DNA damage, PARP becomes activated and, using NAD$^+$ as a substrate catalyzes the building of homopolymers of adenosine diphosphate ribose units. NAD$^+$ serves as a cofactor for glycolysis and the tricarboxylic acid (TCA) cycle, thus providing ATP for most cellular processes. NAD$^+$ also serves as the precursor for NADP, which acts as a co-factor for the pentose shunt, for bioreductive synthetic pathways, and is involved in the maintenance of reduced glutathione pools. Thus, activation of PARP leads to decreased cellular NAD$^+$ levels and affects cellular energetics and function leading to cell death (205-207). PARP pathway is suggested to play an important role in oxidant-mediated cell injury (208, 209). PARP activation is a key mediator of inflammation and inhibition of PARP activation was shown to decrease the inflammation (210).

3.5.6.1 DNA damage detection and repair

The covalent modification of proteins by PARylation is an immediate and dramatic biochemical response to DNA damage induced by oxidation, alkylation, and ionizing radiation. The binding of PARP – 1 to damaged DNA, including single-strand breaks (SSBs) and double strand breaks (DSBs), through its double zinc finger DNA – binding domain potently activates PARP – 1 enzymatic activity (as much as 500 – fold) (211). As such, PARP – 1 can function as a DNA damage sensor. With low levels of DNA damage, PARP –1 acts as a survival factor involved in DNA damage detection and repair. In contrast, with high levels of DNA damage, PARP – 1 promotes cell death (212). PARP – 1 has been implicated in multiple DNA repair pathways, including the SSB, DSB, and base excision repair (BER) pathways (213, 214).
3.5.6.2 PARP and Cell death pathways

In contrast to its role as a survival factor, in the presence of low levels of DNA damage, PARP-1 acts to promote cell death in the presence of extensive DNA damage. As such, chemical inhibition or genetic deletion of PARP – 1 can protect animals from several DNA – damage – dependent pathophysiological conditions leading to aberrant cell death, including [1] ischemia – reperfusion injury, [2] glutamate excitotoxicity in the central nervous system, [3] 1– methyl– 4– Phenyl – 1,2,3,6 – tetrahydropyridine (MPTP) – induced Parkinsonism, [4] cardiac infarction, [5] inflammatory injury, and [6] streptozotocin (STZ) – induced diabetes (215, 216). Although a role for PARP – 1 in these conditions has been well established, the mechanisms by which PARP – 1 activation leads to cell death are still under active debate in the literature. Several mechanisms have been proposed, including energy failure – induced necrosis and apoptosis inducing factor (AIF) – dependent apoptosis.

Hyper synthesis of PARP by PARP-1 in response to extensive DNA damage can promote cell death through necrosis, which occurs as a result of the depletion of cellular NAD$^+$ and ATP, and subsequent cellular energy failure (217, 218). In contrast, apoptosis is an ordered cell death process in which the cell is systematically dismantled within membrane – enclosed vesicles that are engulfed by phagocytes, preventing the release of intracellular components into the surrounding tissue (219). Studies from Yu et al. (220) show that PARP – 1 can play a role in caspase – independent apoptotic cell death through AIF.

The mechanisms underlying the choice of PARP – 1 dependent cell death pathways (i.e., necrosis vs. apoptosis) in response to genotoxic stimuli have not been determined, but may be influenced by the type, strength, and duration of the stimuli, as well as the cell type (221). One
feature of apoptosis is its dependency on ATP for the ordered degradation of cellular structures and maintenance of membrane integrity (219). Thus, in cells whose ATP pools have been depleted due to PARP – 1 activation, cell death occurs by necrosis.

PARP – 1 protein is cleaved by caspase-3 during the course of apoptosis, and detection of the cleaved form of PARP – 1 serves as an early marker of apoptosis (222, 223). Considerable evidence from in vitro, cell culture and ex vivo studies shows that poly (ADP–ribosyl)ation plays a critical role in the survival and maintenance of genomic stability of proliferating cells exposed to low or moderate levels of DNA damaging agents. This has been linked mechanistically with PARP-1 function in base-excision repair. In stark contrast, there is a risk of necrotic cell death due to severe NAD\(^+\) and ATP depletion when PARP –1 is acutely over-activated. The ADP-ribose polymer binds specific domains of the tumor suppressor protein p53 in vitro and is suggested to modulate p53 DNA binding (224). Recently, a poly (ADP ribose) – binding sequence motif was identified in several important DNA damage checkpoint and repair proteins (225).

Cleavage of PARP is one of the earliest detectable protein-degradation events following fragmentation of chromatin DNA (226). The activation of PARP is well considered to play an important role in pathophysiological conditions like inflammation (227). Previous study had suggested the role of PARP in neutrophil infiltration that plays an important pathogenetic role in colitis (210). PARP is known to increase the expression of intercellular adhesion molecules, modulate post-adhesion event and affect endothelial integrity (141, 228-231).
Figure: 3.9
The oxidative and nitrosative injury pathways are triggered by oxygen – and nitrogen – centered oxidants and free radicals, which act in parallel or in synergy with PARP-mediated pathways of cell injury (232).
3.6 Defense mechanisms against free radicals

Anti-oxidants are a diverse group of molecules with many functions (233). They modulate the redox status in biological systems and help in removing toxic oxidants before they damage biological molecules. Primary anti-oxidant defenses can be considered to be those which prevent radical formation (155, 234). Secondary defenses are those which remove, or inactivate, formed reactive oxygen species, while tertiary defenses operate to remove and repair oxidatively damaged molecules and are particularly important in DNA integrity. In order to protect itself against damage, the mitochondria contain antioxidant systems. One such is glutathione. Between 10 – 15% of the total intracellular glutathione is located in the mitochondria. Mitochondria do not have the enzymes necessary for GSH synthesis and hence mitochondria import cytosolic GSH. Since catalase is compartmentalized in the peroxisome, mitochondrial GSH, in the presence of GSH peroxidase, is the only defense against toxic oxygen intermediates. Reduced glutathione (GSH) has unusually high affinity for N$_2$O$_3$, and is likely to play a critical role in inhibiting the toxicity of NO produced during times of enhanced NO production (156). GSH can readily repair the TyrO$^\cdot$ back to tyrosine through its one electron reduction (183). It has been shown that supplementation of mitochondrial GSH with glutathione monoethylester protects hepatocytes against oxidative stress, even when cytosolic GSH is depleted (235). Another antioxidant system operational in the mitochondria is the enzyme manganese superoxide dismutase (Mn SOD) which is present in the mitochondrial matrix. This enzyme promotes dismutation of superoxide (O$_2^-$) into hydrogen peroxide (H$_2$O$_2$).
3.6.1 Cellular anti-oxidant defenses

Oxygen metabolism occurs within cells and anti-oxidant enzymes deal speedily and specifically with reactive oxidants or reductants. These secondary enzymatic antioxidant systems are important to quickly remove reactive oxygen species.

i) Superoxide dismutase (SOD)

Superoxide dismutase promotes rapid dismutation of superoxide (O$_2^-$) into hydrogen peroxide (H$_2$O$_2$) and oxygen.

\[ 2 \text{O}_2^- + 2 \text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \]

Three forms of superoxide dismutase are found in the body: copper-zinc SOD in the cytoplasm, manganese SOD in mitochondria and extracellular SOD, the major form in the extracellular compartment (236). The Cu/Zn SOD is unaffected by oxidative stress, but the Mn$^{2+}$ form is an inducible enzyme.

ii) Catalase

H$_2$O$_2$ produced by dismutation of O$_2^-$ or generated by H$_2$O$_2$ generating oxidases (e.g. D-amino acid oxidase) is removed by two systems. One of these involves catalase. This is a tetrameric heme enzyme of 240–KDa that catalyzes the dismutation of H$_2$O$_2$ and water.

\[ 2 \text{H}_2\text{O}_2 \rightarrow \text{O}_2 + 2 \text{H}_2\text{O} \]

Though, catalase is considered to be active only at high H$_2$O$_2$ concentrations and serves principally as a backup for the glutathione systems.
iii) Glutathione peroxidase

The other cellular mechanism involved in dealing with hydrogen peroxide is the glutathione dependent anti-oxidant system. Glutathione peroxidase is a 22 – KDa enzyme present in almost all tissues. It catalyzes the reduction of H₂O₂ in the presence of reduced glutathione (GSH).

\[
2 \text{GSH} + \text{H}_2\text{O}_2 \rightarrow \text{GSSG} + 2 \text{H}_2\text{O}
\]

This is a major pathway of H₂O₂ metabolism in many cells and one which also catalyses reduction of other peroxides (237). Thus it plays an important role in protecting the membrane lipids against oxidation. Lipid peroxides, which are formed during peroxidation of lipids containing unsaturated fatty acids, are reduced by a specific phospholipid hydroperoxide, glutathione peroxidase (238), which is a 18 – KDa protein that can reduce both H₂O₂ and lipid hydroperoxides to the corresponding hydroxides.

iv) Glutathione reductase

This is a widely distributed 22-KDa flavoprotein, which catalyses the formation of reduced glutathione (GSH) from the oxidized form (GSSG). The presence of this enzyme in the cell helps to maintain a high GSH: GSSG ratio. GSSG, mixed disulphide between GSH and \( \gamma \)-glutamyl cysteine and between GSH and coenzyme – A can act as substrates for this enzyme (239).

\[
\text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow 2 \text{GSH} + \text{NADP}^+
\]

v) Glutathione-S-transferase

Conjugation of GSH with electrophilic compounds is catalyzed by glutathione S-transferase. This liver enzyme is a dimeric protein containing four types of subunits (240).
vi) Glutathione

Glutathione, a tripeptide made of glutamate, cysteine and glycine (L-glutamyl-L-cysteinylglycine), is an important anti-oxidant molecule. Reduced glutathione (GSH), along with glutathione peroxidase, has been shown to be involved in detoxification of free radicals generated during oxidative stress (241). This alters the thiol redox status and specific mechanisms are present to restore the thiol redox status. When there is excess formation of GSSG, it can also form mixed disulphides with proteins (242).
Figure: 3.10.

Free radicals and other reactants are enzymatically removed from cells by a series of antioxidative enzymes (180).
3.6.2 Membrane anti-oxidant defenses

Structural integrity plays an important role in protection of membranes from oxidative damage and this requires an exact ratio of cholesterol and phospholipids with specific fatty acid chains (243). Most of the antioxidants are excluded from the hydrophobic interior of membranes. This includes low molecular weight reductants such as glutathione (GSH) and ascorbate, and enzymatic antioxidants such as peroxiredoxins (183). Within the hydrophobic lipid interior of membranes, the fat soluble vitamin E (alpha – tocopherol) and carotenes are effective anti-oxidant when structurally incorporated in the membrane (244), where it neutralizes the lipophilic radicals formed. Hence lipid soluble antioxidants are extremely important to prevent oxidative stress in membranes, preventing the formation of lipid peroxidation products such as lipid peroxides and carbonyls, which are biologically reactive and may be used as signal molecules by the body.
Glutathione metabolism: Intracellular GSH cycles between reduced (GSH) and oxidized (GSSG) forms. GSH is oxidized by the action of GSH peroxidase, GSH – dependent transhydrogenases or by direct interaction with free radicals. GSSG is formed when glutathione transhydrogenase (glutaredoxin reductase) uses GSH to reduce the disulfide bond in glutaredoxin, the downstream action of which leads to the formation of deoxyribonucleotides (via the activation of ribonucleotide reductase) (245). GSSG is converted back to its reduced form by GSH reductase, an NADPH – dependent reaction (246).
3.7 Melatonin

Melatonin (N-acetyl-5-methoxytryptamine) is a natural occurring compound with well-known antioxidant properties. Melatonin is ubiquitously distributed and because of its small size and amphiphilic nature, it is able to reach easily all cellular and sub cellular compartments. Melatonin is derived from tryptophan and is produced in the pineal gland. It is secreted into the blood and cerebrospinal fluid as a neurohormone to exert regulatory roles on seasonal and circadian rhythms (247, 248). Melatonin is also produced in extra pineal organs and tissues such as the gastrointestinal tract, retina, skin, immune and hematopoietic cells, some reproductive organs, and endocrine glands (249-251). Melatonin acts as intracrine, autocrine, and/or paracrine factor in key homeostatic functions such as the control of energy metabolism, physiological growth, differentiation, and responsiveness to stress stimuli (252). Thus, melatonin is used as a non-conventional drug and as a dietary supplement for stress, ageing and immuno modulation (253). Melatonin is therapeutically used to treat circadian rhythm sleep disorders and depression, seasonal affective disorder, migraines, and cluster headache. Melatonin acts through G-protein coupled (GPC) high affinity melatonin receptors MT1, MT2 and the quinone reductase 2 enzyme family MT3 (254-256). Recently high affinity nuclear receptors that belong to the RZR/ROR nuclear hormone receptor family were also identified (31). MT1 receptors are present in retina, kidneys, and brain in particular in the SCN (suprachiasmatic nucleus). This receptor has role in the transmission of the circadian effects of melatonin, reproduction and on peripheral vasoconstriction. MT2 receptors are present in retina and brain, but not in SCN. MT2 receptors play a key role in the physiology of retina and in the regulation of body temperature (257). Melatonin acts by receptor dependent and receptor independent mechanisms. Melatonin does not undergo redox cycling, thus acting as suicidal or terminal antioxidant (32).
Figure: 3.12 Structure of melatonin
3.7.1 Pharmacodynamics

90% of melatonin secreted by the pineal gland is metabolized by liver. Melatonin is first hydroxylated by hepatic cytochrome P450 to 6-hydroxy melatonin that acts as hydroxyl radical scavenger (258-260), and is excreted in urine after conjugation with sulfate and glucuronic acid (261-263). The half life of melatonin in humans ranges from 10 to 60 minutes (264-266). Melatonin reacts with reactive oxygen species to form N$^1$-Acetyl-N$^2$-formyl-5-methoxykynuramine (AFMK) and N-acetyl-5-methoxykynuramine (AMK) that are also scavengers of free radicals resulting in a cascade of protective reactions (267-270). In retina, melatonin is first deacylated to 5-methoxytryptamine and then subsequently metabolized via the same pathway as indoleamines and catecholamines (271).

3.7.2 Biological functions

Melatonin acts as a regulator of circadian rhythms (272) in a hormone-like fashion by affecting target cells and by modulating other functions depending on the photoperiod including regulation of photoperiodic oscillations of the immune/ inflammatory response (272, 273). Melatonin contributes to the control of inflammatory tissue by finely regulating the leukocyte function and number (274). Melatonin acts as paracrine or autocrine regulator in leukocyte communication independent of the pineal gland. Melatonin binds to calmodulin that leads to the activation of phospholipase A2 and lipoxygenase (275). Melatonin was shown to have induced T-cell proliferation and up-regulated the pro-inflammatory cytokines (274, 276). Thus, melatonin acts as a pharmacological modulator of the inflammatory response.

In retina, melatonin controls rhythmic processes such as retinomotor movements (277), dopamine synthesis, release, and metabolism (278-280), disc shedding and phagocytosis...
It also modulates the glycine currents of retinal ganglion cells (283) and increases photoreceptor susceptibility to light – induced damage (284).

3.7.3 Therapeutic effects
Melatonin is therapeutically used in the treatment of circadian rhythm disorders (285), Alzheimer’s disease by preventing the aggregation of amyloid beta protein and by preventing the hyper phosphorylation of the tau protein (286, 287). It is also used to decrease delirium (288) and migraine (289).

3.7.4 Antioxidant functions
Melatonin is ubiquitously present and being a small molecule and its amphiphilic nature can diffuse easily across membranes to all cellular and sub-cellular compartments. Melatonin’s intracellular concentration is more in mitochondria (290) and scavenges the mitochondrial reactive oxygen species (291-293), thus preserving the stability, integrity and function of mitochondrial membranes (294-296). Melatonin has been shown to interact with lipid bilayers (29) and stabilize the mitochondrial inner membranes that may improve electron transport chain (ETC) activity. Melatonin is known to neutralize different reactive oxygen species and reactive nitrogen species (31, 297). Melatonin can act as cell redox modulator thus influencing the balance of redox systems such as that of GSH/GSSG and glutaredoxins or that of NADPH/NADP⁺ to provide transcriptional and post-translational effects with regulatory function on antioxidant and prooxidant pathways (257).
Figure: 3.13

Actions of melatonin at the mitochondrial level increase its efficiency as an antioxidant by reducing free radical generation and increasing ATP production (180).