Encephalopathy of various aetiology constitute a major part of childhood illnesses. Infection of the Central Nervous System (CNS) is the most common cause of fever associated with signs and symptoms of CNS disease in children.\textsuperscript{62} Although many organisms can cause CNS infection, specific pathogens are identifiable and are influenced by the age & immune status of the host. In general viral infections of CNS are more common in children than fungal and parasitic infections.

Regardless of aetiology most patients with CNS infection have similar clinical manifestations. Common symptoms include fever, headache, nausea, vomiting, anorexia, restlessness, altered state of consciousness and irritability, whereas common signs include photophobia, neck rigidity, obtundation, stupor, coma, seizures & focal neurologic deficits. The severity & constellation of signs are determined by the specific pathogens, the host & the area of the CNS affected.

Infection of the CNS may be diffuse or focal. Meningitis and encephalitis are examples of diffuse infections. Meningitis implies
primary involvement of the meninges, whereas encephalitis indicates brain parenchymal involvement. Because these anatomic boundaries are often not distinct, many patients have evidence of both meningeal & parenchymal involvement and should be considered to have meningoencephalitis.

Bacterial meningitis is one of the most potentially serious infection occurring in infants & older children. This infection is associated with a high rate of acute complications & risk of long term morbidity. The incidence of bacterial meningitis is so high in children that it should be included in the differential diagnosis of all children with altered mental status and other evidence of neurologic dysfunction. The causes of bacterial meningitis in neonatal period include group B and D streptococci, gram negative enteric bacilli and Listeria monocytogenes. The most common causative organism of bacterial meningitis in children between 2 months to 12 years include streptococcus pneumonia & H. influenzae type b. Risk of Tubercular meningitis is invariably present in children. Risk of developing Tubercular- meningitis following miliary tuberculosis is maximum in first 3 months of acquiring primary complex due to haematogenous spread of
Tuberculous organisms.\textsuperscript{6}

A major risk factor for meningitis is the lack of immunity to specific pathogenic organisms associated with young age. Additional risk factors include recent colonization with pathogenic bacteria, close contacts with individuals having infective disease e.g. Tuberculosis, overcrowding, poverty. The mode of transmission is probably person to person contact through respiratory tract secretions or droplets. Specific host defense defects due to altered immunoglobulin production in response to encapsulated pathogens may be responsible for increased risk of bacterial meningitis. Congenital or acquired CSF leak across a mucocutaneous barrier such as cranial or midline facial defects, chronic suppurative otitis media or CSF leakage through a rupture of the meninges due to basal skull fracture is associated with an increased risk of pneumococcal meningitis.

\textbf{Pathogenesis:-}

The development of bacterial meningitis progresses through four interconnected phases: (i) bacterial invasion of the host with subsequent infection of the CNS (ii) bacterial multiplication and
induction of inflammation in the subarachnoid and ventricular space. (iii) Progression of inflammation with associated pathophysiologic alterations, and (iv) development of neuronal damage.

Bacterial meningitis most commonly results from haematogenous dissemination of microorganisms from a distant site of infection. Bacteremia usually precedes meningitis or occurs concomitantly. Bacterial survival in the blood stream is enhanced by large bacterial capsules that interfere with opsonic phagocytosis and are associated with increased virulence. Bacteria gain entry into the CSF through the choroid plexus of the lateral ventricles and the meninges and then circulate to the extra cerebral CSF and subarachnoid space. Bacteria rapidly multiply because the CSF concentrations of complement and antibody are inadequate to control bacterial proliferation, chemotactic factors then incite a local inflammatory response characterized by polymorphonuclear cell infiltration. The presence of bacterial cell wall polysaccharide (endotoxin) of gram negative bacteria stimulates a marked inflammatory response, with local production of Tumour Necrosis Factor, Interleukin -1, Prostaglandin E &
other inflammatory mediators. The subsequent inflammatory response is characterised by neutrophilic infiltration, increased vascular permeability, alterations of blood brain barrier and vascular thrombosis.

It is likely that ROS play an important role in the pathophysiology of bacterial meningitis. In infant rats with experimental meningitis generation of ROS was localized to the cells of the subarachnoid and ventricular inflammation and to cerebral vasculature. Activated granulocytes releasing ROS and other harmful products to the cerebral vasculature traversing the subarachnoid space are not without consequences. At the level of vessels, inflammatory involvement leads to vasospasms and thrombosis with subsequent focal perfusion deficits of the brain. At that level of the microvasculature, the most important consequence of inflammation is disruption of the BBB. Inhibition of the biologic effect of ROS by radical scavengers in experimental meningitis prevents BBB breakdown, caused attenuation of lipid peroxidation and ischaemia in the brain, and protected neurons from injury.\textsuperscript{11, 58}
The sources of the ROS operative during meningitis include granulocytes at the site of inflammation and activated glial cells, which represent a source of oxidative stress within the brain parenchyma in the direct vicinity of neurons. Cerebral ischaemia, likely to be of critical importance in meningitis, also involves the production of ROS. Consequently scavengers of ROS have consistently shown beneficial effects in various models of cerebral ischaemia. Furthermore, the neurotoxic effect of excitatory amino acids (EAA), mediators of neuronal injury during cerebral ischaemia and other forms of brain damage, including meningitis have been shown to involve ROS. Many of the processes that are important in meningitis, including vascular endothelial cell activation, cerebral inflammation, ischaemia/reperfusion injury, neuronal apoptosis and excitotoxic neuronal injury, are linked to the generation of ROS. Production of proinflammatory cytokines is controlled by the tumour necrosis factor TNF-B, a factor that is also modulated by ROS. ROS may contribute to brain damage not only directly, but also by upregulating proinflammatory cytokines or by activating matrix metalloproteinases.28,35
Pathology:

A meningeal purulent exudate of varying thickness may be distributed around the cerebral veins, various sinuses, convexity of the brain and cerebellum, basal cisterns, spinal cord. Ventriculitis with bacteria and inflammatory cells in ventricular fluid may be present more often in neonates. Vascular & parenchymal cerebral changes are characterized by polymorphonuclear infiltrates extending to the subintimal region of small arteries and veins, vasculitis, thrombosis leading to occlusion of major venous sinuses, necrotizing arteritis producing subarachnoid hemorrhage.62

Cerebral infarction resulting from vascular occlusion due to inflammation, vasospasm and thrombosis is frequent sequelae following meningitis. Inflammation of spinal nerves and roots produces meningeal signs. Inflammation of the cranial nerves produces cranial neuropathies of optic, oculomotor, facial and auditory nerves. Increased intracranial pressure is due to cell death (cytotoxic cerebral edema); cytokine induced increased capillary vascular permeability, vasogenic cerebral edema and possibly increased hydrostatic pressure (interstitial cerebral
edema). Hydrocephalus can occur as an acute complication of bacterial meningitis. Communicating hydrocephalus is due to adhesive thickening of arachnoid villi around cisterns at the base of brain, while obstructive hydrocephalus develops after fibrosis and gliosis of the aqueduct of Sylvius.

Damage to cerebral cortex may be due to vascular occlusion, hypoxia, bacterial invasion toxic encephalopathy (due to bacterial toxin), elevated intracranial pressure, ventriculitis. Damage to cerebral cortex in the form of infarction, necrosis, cerebritis and toxic encephalopathy leads to elevated intracranial pressure. Ventriculitis results in the clinical manifestation of impaired consciousness, seizures, cranial nerve deficits, motor and sensory deficits and later psychomotor retardation. These pathologic factors result in the clinical manifestations of bacterial meningitis.

**Clinical Manifestations:**

The signs and symptoms of meningitis are related to the nonspecific features associated with a systemic infection and to manifestations of meningeal irritation. Nonspecific findings include fever, anorexia and poor feeding, headache, symptoms of upper respiratory tract infection, myalgias, arthralgia, tachycardia
and hypotension. Meningeal irritation is manifested as nuchal
rigidity, back pain, positive Kerning’s sign (flexion of the hip upto
90 degrees followed by extension of the leg causes pain) and
positive Brudzinki’s sign ( involuntary flexion of the both knees
and hips after passive flexion of the neck in supine state).
Increased intracranial pressure is suggested by headache, emesis,
bulging fontanel, Oculomotor or Abducens nerve paralysis.
Hypertension with bradycardia and irregular respiration may also
be associated with decorticate or decelerate posturing along with
stupor or coma due to increased intracranial pressure. At time
increased intracranial pressure is associated with papilledema.
Focal neurologic signs are usually due to vascular occlusion.
Seizures are due either to cerebritis or infarction. Alteration of
mental status may be due to increased intracranial pressure,
cerebritis or hypotension.62

Diagnosis:-

The diagnosis of acute pyogenic meningitis is confirmed by
analysis of CSF which reveals microorganisms on Gram stain and
culture , a neutrophilic pleocytosis, elevated protein and reduced
glucose concentration. Raised CSF protein levels are due in part to
increased vascular permeability of the blood brain barrier and loss of albumin rich fluid from capillaries and veins traversing subdural space. Reduced CSF glucose is due to decreased glucose transport by the cerebral tissue.

**Treatment:-**

The therapeutic approach to patients with presumed bacterial meningitis depends on the nature of initial manifestations of the illness. Increased intracranial pressure should also be treated simultaneously. Therapy for uncomplicated bacterial meningitis should be completed within 10 to 14 days. In addition, the child should also be given supportive care and anticonvulsant drugs if necessary.

In 1999 Uysal G. et al assessed the role of CSF nitric oxide (NO) in childhood bacterial meningitis. 18 children with bacterial meningitis and 18 normal as controls were included in the study. They found no correlation between CSF nitrate/nitrite levels and CSF white blood cell count ($r=0.22$), protein ($r=0.26$), tumour necrosis factor alpha (TNF) levels ($r=0.046$). However, moderate negative correlation between CSF nitrate/nitrite and glucose levels ($r=-0.46$) was noticed.
In 2000 Tsukahara H. et al studied the involvement of reactive oxygen species in bacterial meningitis and measured the concentration of 8-hydrxy-2-deoxyguanosine (8-OHDG), a biomarker of oxidative stress in the CSF samples from 63 children with and without meningitis. This study showed increased concentration of 8-OHDG in children with bacterial meningitis than that in control subjects. Findings suggested the presence of enhanced oxidative stress in the central nervous system of children with bacterial meningitis.

**Viral meningoencephalitis:**

Viral meningoencephalitis is an acute inflammatory process involving the meninges and brain tissue. Aetiology includes Enteroviruses, Arboviruses, Herpes, Measles & Mumps viruses.

Neurologic damage is caused by direct invasion and destruction of neural tissues by actively multiplying viruses or by host reaction to viral antigens. Tissue sections of the brain are characterized by meningeal congestion and mononuclear infiltration with perivascular cuffs of lymphocytes & plasma cells. Perivascular tissue necrosis along with myelin breakdown is also
seen. A marked degree of myelination with preservation of neurons & their axons is hallmark of post-infectious encephalitis.

The diagnosis of viral encephalitis is usually supported by examination of CSF which usually shows pleocytosis with mild mononuclear predominance and absence of microorganisms on Gram stain and routine bacterial culture.

Treatment of viral meningoencephalitis is usually supportive. Most children recover completely, although the prognosis depends on the severity of clinical illness, specific cause & age of the child.

**Tuberculous meningitis:**

Tuberculosis of the central nervous system is the most serious complication in children and is fatal without prompt and appropriate treatment. Tubercular meningitis develops as a metastatic caseous lesion in the cerebral cortex. Tuberculous meningitis develops during haematogenous dissemination of tuberculous bacilli from primary complex. In the cerebral cortex gelatinous exudates infiltrates the corticomeningeal blood vessels producing inflammation, obstruction and subsequent infarction of
cerebral cortex. Maximum involvement occurs in the brain stem accounting for dysfunction of cranial nerves III, VI, & VII. The exudate is also responsible for development of communicating hydrocephalus.

TBM is mainly caused by mycobacterium tuberculosis of human type. Small tuberculous foci reach the meninges or CNS at the time of primary infection via haematogenous route. Reactivation of such foci occurs depending on various factors including the immunologic status of the patients concomitant disease and virulence of organism. Enlargement of the lesion may result in a space occupying lesion (SOL) with surrounding oedema or it may rupture into the subarachnoid space causing meningitis.  

Pathology:

The pathological hallmark of the disease is proliferative arachnoiditis which is seen predominantly at the base of the brain extending from pons to optic nerves. Vasculitis with subsequent thrombosis and infarction may develop in the vessels traversing the thick exudates or else develop due to direct bacterial invasion.
The thick basal exudates forms a constricting collar around brain stem and obstructs the CSF flow leading to internal hydrocephalus.

Tuberculous encephalopathy is characterised pathologically by diffuse oedema and sometimes perivascular myelin loss and vasculitis of capillaries and small vessels.

**Clinical Manifestations:**

The clinical manifestations of TBM are extremely variable. In TBM it is customary to assess the severity of disease and to stage patients clinically.45

The combination of vasculitis, infarction, cerebral edema and hydrocephalus is responsible for the clinical manifestations of tuberculous meningitis which can be divided into three stages. The 1st stage is characterized by nonspecific symptoms such as fever, headache, irritability, drowsiness & malaise. The 2nd stage is associated with vomiting, nuchal rigidity, positive Kernig or Brudzinki signs, hypertonia, cranial nerve palsies and other focal neurologic signs. The 3rd stage is marked by coma, hemiplegia or paraplegia, decerebrate posturing, deteriorations of vital signs & eventually death. The prognosis of tubercular meningitis
correlates more closely with clinical stage of illness at the time treatment is initiated. The prognosis for infants is generally worse than older children. Diagnosis of Tubercular meningitis is done on basis of CSF leucocyte count varying from 10 to 500 cells per cmm with lymphocytic predominance accompanied by decrease in CSF glucose < 40mg/dl and markedly elevated protein level upto 1 gram /dl.

In management of tuberculous meningitis combination of drugs are used in view of rapid cure & prevention of the emergence of secondary drug resistance during therapy. The choice of regimen depends on extent of tuberculosis disease, the host and likelihood of drug resistance .Usually CNS tuberculosis is treated by combination of Isoniazid and Rifampicin supplemented in first two months by pyrazinamide & streptomycin. In addition to antituberculous medications, supportive drugs like cerebral decongestant and anticonvulsant are also given for reducing complications. In addition child also needs supportive care & adequate nutrition.

In 2000 Ray G.et al investigated the possible role of free radicals and antioxidants in childhood meningitis. 60 children
suffering from acute bacterial meningitis (ABM) or tuberculous meningitis (TBM) were studied. The production of superoxide anions (O\textsuperscript{-2}) hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}) and malondialdehyde (MDA) and activities of xanthine oxidase (XO), superoxide dismutase (SOD) and glutathione peroxidase (GPx) were monitored in the study groups and findings were compared with those of 20 age-matched controls. Children with ABM and TBM who died showed significant increase in the production of O\textsuperscript{-2} and MDA and in the activities of SOD and CPK compared with survivors. The rate of production of oxidants and MDA and the activities of XO, SOD and CPK were of much higher magnitude in decreased ABM and in TBM survivors than total TBM and survivors respectively. The alteration observed in 20 children who died (14 from ABM, 6 from TBM) revealed elevated levels of oxidants, antioxidants and toxicity makers which suggest the possibility that natural or synthetic antioxidants might prevent disease progression and tissue damage in childhood meningitis.

In the study on oxidative stress and antioxidants in Tubercular meningitis by Rao et al\textsuperscript{102}, erythrocytic lipid peroxidation, & antioxidant enzymes viz. glutathione reductase, glutathione peroxides, superoxide dismutase & plasma
Review of Literature

antioxidants viz. Ceruloplasmin, vit. A,E & C have been determined in 19 pts. of Tubercular meningitis and 50 normals. Treated patient were considered for follow-up. In the study it was found that, lipid peroxidation, & plasma ceruloplasmin of TBM patients were significantly higher whereas erythrocytic glutathione reductase, & plasma antioxidant vitamins A,E, & C were significant lower than those of the controls. In the follow up patients glutathione reductase and catalase levels were signifantly high compared to pretreated condition. This study indicated that the low blood antioxidant status of TB patient got improved after treatment suggesting the role of free radicals in TBM.

**Hypoxic Ischaemic Encephalopathy**

Hypoxic Ischaemic Encephalopathy in the perinatal period is characterized by neuropathological & clinical features that constitute a major portion of neonatal neurology.\(^{131}\)

Anoxia is a term used to indicate the consequences of complete lack of oxygen.

Hypoxia refers to decreased arterial concentration of oxygen.

Ischaemia refers to blood flow to cells or organs that is insufficient to maintain their normal function.
BASIC ASPECTS OF HYPOXIC ISCHAEMIC ENCEPHALOPATHY

Perinatal Asphyxia

Initially

Redistribution of Cardiac output

Blood Pressure

Hypercapnia
Hypoxemia
Acidosis other

Continuing

Loss of Vascular Autoregulation

Cerebral blood flow

Blood pressure

Haemorrhage

Cerebral blood flow

BRAIN INJURY
Hypoxic ischaemic encephalopathy is an important cause of permanent damage to CNS tissues that may result in neonatal death or manifest later as cerebral palsy or developmental delay.

Foetal hypoxia may be caused by various disorders in the mother including:

1. Inadequate oxygenation of maternal blood from hypoventilation during anesthesia or respiratory failure.
2. Low maternal blood pressure from acute blood loss or spinal anesthesia.
3. Premature separation of placenta.
4. Placental insufficiency.

After birth hypoxia may be caused by

1. Failure of oxygenation as a result of severe forms of congenital heart disease or severe pulmonary disease.
2. Severe anemia secondary to severe hemorrhage of hemolytic disease
3. Shock

Pathophysiology:

Animal studies suggest that hypoxia will not cause lethal brain injury without ischaemia. After an episode of hypoxia &
ischaemia anaerobic metabolism occurs which generates increased amounts of lactate & inorganic phosphate. Toxic amino acids particularly glutamate accumulate in the damaged tissue. Increased amounts of intracellular solute & calcium may result in tissue swelling of cerebral edema. There is also increased production of free radicals & nitric oxide in these tissues. The initial circulation response of the foetus is increased shunting through ductus venosus, ductus arteriosus and foramen ovale with transient maintenance of perfusion of the brain, heart & adrenals in preference to the lungs, liver, kidney & intestine.

The pathology of hypoxia and ischaemia is dependant on the affected organ & severity of injury. Early congestion, fluid leak from increased capillary permeability & endothelial cell swelling may then lead to signs of coagulation necrosis and cell death. Prolonged intrauterine hypoxia may result in inadequate perfusion of periventricular white matter results in turn in Periventricular Leukomalacia.

The combination of chronic foetal hypoxia and acute hypoxic ischaemic injury after birth results in gestational age specific neuropathology. Term infants demonstrate neuronal necrosis of
the cortex and parasaggital ischaemic injury. Preterm infants demonstrate periventricular leukomalacia, status marmoratus of basal ganglia & intra ventricular hemorrhage. Term infants often have focal or cortical infarcts that clinically cause focal seizures or neurodeficits.

The prognosis of HIE correlates to timing & severity of insult and depends on whether metabolic & contributory complications are treated. Severe encephalopathy characterized by flaccid tone, absent occulocephalic reflexes & refractoriness to treatment is associated with a poor outcome.

**Biochemical Aspects:**

The primary disturbance to neural tissue in Hypoxic Ischaemic Encephalopathy is a deficit in oxygen supply. Perinatal brain can be deprived of oxygen by two major pathogenic mechanisms i.e. hypoxemia which is diminished amount of oxygen in the blood supply or ischaemia which is diminished amount of blood perfusing the brain. As a result of asphyxia which refers to an impairment in the exchange of respiratory gases i.e. Oxygen and carbon dioxide, hypercapnia occurs which leads to a number
of metabolic and physiological effects e.g. acidosis and increase in cerebral blood flow.

As a result of initial dissociation of impaired brain function & energy metabolism, the occurrence of cerebral depression after hypoxia is characteristic. This led Lowry & coworkers to put the following hypothesis “Brain depression in severe hypoxia represents the exaggerated response of a protective mechanism. i.e. Nervous system is put to rest in a state of coma because it reduces oxygen expenditure. Following mechanisms are proposed to underlie the initial impairment in brain function.

1. Regional alterations in high energy compounds.
2. Accumulation of extracellular potassium.
3. Intracellular acidosis
4. Altered Neurotransmitters

In the brain ATP generated by glycolysis & citric acid cycle is transported from the mitochondria via a specific carrier and is utilized in brain for two major purposes: Transport & synthetic processes. Synthetic processes are particularly important in developing brain and involve neurotransmitters: structural and functional.
Review of Literature

The impact of hypoxemia, ischaemia and asphyxia on perinatal brain is modified by the state of maturation of the brain.

In hypoxic ischaemic encephalopathy the incidence of periventricular- intraventricular hemorrhage relates to four major factors which include.

1) Presence of the subependymal germinal matrix.
2) The distinctive state of arterial development in the cerebrum of premature brain.
3) Apparently tenuous capillary integrity in the periventricular region and
4) An impairment of vascular autoregulation.

Thus the brain is more susceptible to damage from chemical injury. It has a high proportion of polyunsaturated fatty acid chains forming myelin. Hence brain is at great risk of damage with exposure to chemical breakdown products called free radicals.

The brain also has special vulnerability to toxic damage due to the following factors.

1. Impaired energy metabolism and /or reduced oxidative metabolism increases risk of nerve cell damage.
2. Brain cells are unable to regenerate and damaged cell loss is usually permanent.
3. High metabolic rate of brain cells render them more susceptible.

Brain ischaemia leads to reduced oxygen and blood supply which can produce increased free radicals, death of brain cells and neurodegenerative disease. Increased free radicals cause increased risk of neurodegenerative diseases. Accelerated neurodegenerative diseases occur from increased oxidative stress.

Increased lipid peroxide is an indicator of brain and cellular membrane lipid damage. Free radicals damage cellular & other body membranes resulting in reduced polyunsaturated lipids making membranes less flexible and make them permeable to undesired amount of substances. This leads to further damage and death of brain cells and energy generating mitochondria.

Free radicals also attack proteins, including body enzymes, along with DNA, and RNA producing more damaged DNA products such as 8-oxo-2 – deoxyguanosine , a marker of DNA damage.

Thus hypoxic – ischaemic brain damage in an evolving process that begins during the insult and extends in to recovery.

Following severe perinatal asphyxia, the newborn infant is
affected by multi-organ dysfunction. While other organs may recover, the brain is often permanently injured by a pathophysiologic process that progresses over many days. The clinical encephalopathy peaks in severity after 3-4 days and the neurological sequel are directly related to the severity of the encephalopathy. What is both disturbing and exciting to the clinician is the fact that the evolving encephalopathy reflects progressive brain injury and that appropriate management can be protective in animal studies even if administered hours after reperfusion.85

CELLULAR AND MOLECULAR EVENTS

Reperfusion phase:40

During the reperfusion phase there is a return of oxygenated blood to previously ischaemic brain. Thus the microvasculature carries the brunt of reperfusion as free radicals are generated by the return of oxygen to the tissue. Activated neutrophils adhere to vascular endothelial cells, which are often swollen and the lumens narrowed.
There is a brief period of hyperemia followed by a few hours of reduced blood flow. The pathophysiology of no-reflow is multifactorial. It results from mechanical obstruction of the microcirculation, and includes such vascular factors as endothelial blebs, compression by swollen glial cells, blood factors like viscosity changes due to polycythemia, erythrocyte sludging, platelet aggregation, and general cardiovascular factors such as post ischaemic hypotension. The treatment of no reflow consists of maintaining adequate post ischaemic blood pressure.

Following cerebral ischaemia and a brief period of hyperemia, a phenomenon of delayed post ischaemic hypoperfusion can also occur. Post ischaemic hypoperfusion is a functional disturbance due to increased arteriolar tone. Delayed post ischaemic hypoperfusion may contribute to secondary delayed tissue injury. The increased vascular tone is due to failure of endothelium mediated vasodilatation, possibly an abnormality of the synthesis of nitric oxide. Other contributors are adhesion of polymorphonuclear leucocytes and platelets.
Lipid peroxidation and antioxidant status in encephalopathy in children
Molecular mechanisms of neutrophil adhesion and microvascular injury during reperfusion:\textsuperscript{83}

Inflammatory mediators (for example C\textsubscript{5}a, thrombin, histamine and reactive oxygen species) are released from damaged brain tissue and cause the activation of blood vessel endothelia. The activated endothelium expresses adhesion molecules to attract and then adhere to any circulating neutrophils. The first adhesion molecule to be expressed onto the endothelial cell surface is P-selectin. P-selectin, expressed rapidly on the endothelial surface, mediates the tethering of flowing leucocytes to the blood vessel wall by binding to selectin ligands on leucocytes. These interactions cause leucocytes to “roll” along the vascular endothelium in the direction of blood flow. Rolling is necessarily first step before firm adhesion can occur.

The vascular surface is activated by additional inflammatory stimuli (superoxide, the cytokines IL -1 and TNF \( \alpha \)) that stimulate the formation of ICAM -1 (intercellular adhesion molecule -1 ) and E- selectin. These adhesion molecules require mRNA transcription and protein synthesis and usually take 3 to 6 hours to be expressed. The next step in the inflammatory adhesion cascade
requires the activation of leucocyte, $\beta_2$ integrin molecules. Chemoattractants (PAF, IL-8 chemokines) induce a conformational change in the leucocyte integrin that allow them to bind to the endothelial cell ligands (ICAM-1). The appearance of ICAM-1 on the post capillary venule luminal membrane arrests granulocyte “rolling” and secures adhesion of the neutrophils to the micro vascular endothelium.

Cerebral hypoxia–ischaemia in the PD7 rat enhances rapid expression of brain inflammatory cytokines (IL6, IL-1B ) and the expression of inflammatory cell response to injury that includes neutrophils lymphocytes during the first 12 -24 hours of reperfusion. Interestingly, it was found that hypoxic-ischaemic brain damage in the immature rat could be reduced with neutrophil depletion with antineutrophil serum. If the antineutrophil serum was administered immediately after hypoxia-ischaemia (it takes 8 hours to make the rats neutropenic) the protective effect was lost. This suggests that the neutrophils exert their influence in producing ischaemic brain injury either during ischaemia or shortly afterward in early reperfusion, when
one might expect no reflow or delayed post ischaemic hypoperfusion.

Immediately following reperfusion, cytotoxic edema, which developed during ischaemia rapidly improves in those regions of the brain not permanently injured by the ischaemia. Unfortunately, improvement is only transient as cellular energy failure starts the biochemical cascade described below and after a few hours the brain swells from a combination of cytotoxic and vasogenic edema.

**Latent Phase: -**

The latent phase is characterized clinically by absence of seizures (pre seizures) and reduction in early cytotoxic edema. Cerebral blood flow often falls below normal following transient hyperemia. Cerebral impedance improves rapidly and then stabilizes, EEG activity is suppressed but begins to recover in this phase.

During this interval of relative neurophysiological suppression, there are many biochemical events occurring in the parenchyma and microvessels that contribute to injury.
**Pathways to cell death: Cell Injury Cycle**

Hypoxia-ischaemia results in depletion of ATP and the reduction of resting membrane potentials in neurons and glia (Primary energy failure). Potassium leaks out of cells and depolarize neurons leading to a massive release of glutamate (excitotoxicity). Acting via NMDA receptors Glutamate permits the intracellular influx of calcium, which triggers a number of potentially harmful enzymes including cyclooxygenase, lipoxygenase, proteases and nitric oxide (NO) synthase.

**Nitric oxide**: NO combines with superoxide anion to form the powerful oxidant peroxynitrite. Peroxynitrite damages proteins, lipids and DNA.

DNA fragments trigger a repair process by the nuclear enzyme poly ADP ribose polymerase (PARP).

Massive activation of PARP leads to ADP ribosylation and depletion of NAD+. NAD/NADH is vital cofactor for glycolysis and the electron transport chain. When PARP is overactive ATP is consumed in an effort to resynthesize NAD+. NO• may also directly interfere with cellular respiration and deplete cellular ATP via
Lipid peroxidation and antioxidant status in encephalopathy in children
direct inhibition of enzymes in the glycolytic pathway, Kreb’s cycle and electron transport chain. The combined NO/Peroxynitrite mediated cellular energy production leads to cell death by energy depletion (Persistent or Delayed Energy Failure). For surviving neurons, depletion of energy can lead to further loss of membrane potential and amplification of excitotoxicity in another self–perpetuating energy depleting cycle.

**Apoptosis and Delayed cell Death:**

In severe hypoxic ischaemic injury necrosis is the predominant modality of cell death but in less severe injury apoptosis seems to prevail.

Caspases (cystein aspartate- specific proteinases) are a family of 14 proteases that are activated by regulated proteolysis of proenzymes. Upstream caspases activate downstream “executionar” caspases. Active caspases have numerous target proteins including nuclear proteins, cytoskeletal proteins and cytosolic proteins. Activation of caspase leads to cleavage of the inhibitor of caspase–activated–DNAse which triggers the activation of caspase activated DNAse and subsequent DNA fragmentation.
Different caspase cascades not necessarily mediate apoptosis. The intrinsic pathway involves cytochrome-C released from mitochondria promoting the activation of caspase-9 through Apaf-1 and then caspase-3 activation. The consequences of mitochondrial injury after cerebral ischaemia and reperfusion are numerous. They include metabolic failure, oxidative stress, impaired Ca\(^{++}\) buffering and opening of the mitochondrial permeability pore with the release of apoptotic factors such as cytochrome-C and apoptosis inducing factor (AIF). In the immature rat mitochondrial dysfunction is characterized by early glucose hyperutilization in areas of the cerebral cortex followed by a secondary phase with low glucose utilization and infarction. Thereafter a secondary drop in mitochondrial respiration occurred reaching a minimum at 24hrs. of reperfusion. This secondary loss of respiratory function was accompanied by increased caspase-3 like activity and loss of cell integrity.

The extrinsic pathway to caspase activation leads to activation of cell-surface death receptors, including tumor necrosis factor receptor, leading to caspase-8 activation that in turn cleaves and activates downstream caspases. Caspases 8
cleaves a cytosolic substrate protein that translocates to mitochondria thereby transducing death receptor action at the cell surface to the mitochondrion. Activation of Fas is induced by the binding of Fas ligand, a member of the TNF–cytokine family. Fas is expressed on activated T killer and natural killer cells.

**Inflammation:**

Cerebral hypoxia-ischaemia enhances rapid expression of brain inflammatory cytokines (IL6, IL-1B) and an inflammatory cell response to injury that includes neutrophils, lymphocytes, and microglia.

Strong epidemiologic evidence indicates that the babies who are at greater risk of developing an adverse outcome are those born to mothers who have chorioamnionitis. There are elevated concentrations of the cytokines, tumor necrosis factor and interleukins 1 β (IL-1β) and IL -6 in the amniotic fluid and umbilical cord plasma of foetuses and prematurely born infants who sustain periventricular leukomalacia. Both tumor necrosis factor and IL -6 are expressed in areas of PVL in premature infants who expire with such lesion. Thus, infection and
ischaemia share the cytokine pathway as upstream modulators of brain injury. In a recent report, Nelson et al, showed that children with cerebral palsy had elevated inflammatory cytokines in their cord blood. It is possible that the foetal inflammatory response made the brain more susceptible to asphyxiating insults.

In human infants the neuropathology of PVL is diverse and includes diffuse astrogliosis, characteristic loss of oligodendrocytes, and deficiencies in myelination. Cell culture experiments have shown that immature oligodendrocytes are particularly vulnerable to free radical mediated apoptotic cell death. In areas of white matter injury, microglia stains positive for inflammatory cytokines TNF-α and IL-6. Activated microglia is capable of secreting a vast array of neurotoxins, including free radicals. Finally, to test the role of intrauterine infection as a cause of foetal brain injury, Yoon et al, developed a model in which foetal rabbits were exposed to intrauterine infection. Some rabbits developed areas of necrosis in the white matter.

There is evidence that blood supply to the white matter is precarious because there are relatively fewer blood vessels to that area, especially in the 28 week premature infant who is most
susceptible to PVL. Impaired auto regulation also places the white matter at increased risk of damage. Thus, there are two main contributors to white matter damage in the preterm. The first relates to elevated levels of inflammatory cytokines, and the second to vascular factors leading to ischaemia. These two apparently separate mechanisms may have common components. One possible link could be the role of inflammatory cytokines in activating neutrophil adhesion to blood vessels. Under low flow states, neutrophil plugging might contribute to cerebral ischaemia and periventricular leukomalacia.  

Clinical Grading:  

I) Mild Encephalopathy (Stage I):  

In newborns with a mild encephalopathy the symptoms are maximum during the first 24 hrs and then progressively diminish. The characteristic feature is jitteriness, a hyperalert state in which there are prolonged periods of wakefulness, irritability and excessive responsiveness to stimulation. The typical response to stimulation is a low frequency, high amplitude shaking of the limbs and jaw jitteriness is commonly associated with a low threshold, but it can occur in the absence of any apparent
external manipulation which may be mistaken for seizure.

Muscle tone is normal. Mild head lag is demonstrable with the traction response but the head becomes erect with the body as the child achieves the sitting position. Spontaneous movement and strength are normal in the limbs. Deep tendon reflexes are normoactive or complete and repetitive extension and flexion movements are generated by stimuli of very low threshold.

State of sympathetic overactivity characterised by tachycardia, a tendency for dilation of the pupils and decreased bronchial and salivary secretions is seen. This state may be caused by an increased release of epinephrine secondary to the stress of anoxia. The high levels of circulating epinephrine may also account for a transitory hypoglycemia encountered in one third of stressed newborns. Seizures are not an expected feature of the mild encephalopathy. The occurrence of seizures suggests concurrent hypoglycemia or the presence of a pre-existing condition. The EEG is normal but may show lack of background variability.

Symptoms progressively diminish and disappear in the first week, with the exception that some degree of over responsiveness
may persist for indefinite periods of time. The possibility exists that jitteriness in newborns may evolve into hyperactivity in children in speculative newborns who have sustained a mild encephalopathy but are not at the risk for major neurological handicaps.

**II) Moderate Encephalopathy (stage- II):**

Newborn with moderate encephalopathy are lethargic or obtunded for at least the first 12 hrs. In the usual course of events, the period between 48 and 72 hrs. after birth is a critical interval in which the encephalopathy either worsens or improves. In those newborns who improve spontaneously, tone increases and arousal is more readily accomplished and is associated with jitteriness. In others there is either lack of improvement or progressive deterioration caused by oedema. Other factors that may add to the state of decreased consciousness are hyponatremia secondary to inappropriate secretion of antidiuretic hormone and hyperammonemia due to hypoxic liver damage. The presence of seizure prolongation of the obtunded state or a progression to stupor is associated with a worsening prognosis.

The EEG is always abnormal and demonstrates lack of background variability.
At the end of 3\textsuperscript{rd} day some children show signs of recovery. Others deteriorate further in stupor coma and the remainder continues in the obtunded state for more than 5 days. Recovery from the encephalopathy is characterized by increased levels of consciousness, establishment of awake sleep cycle and a transitory state of increased jitteriness. The Moro reflex becomes more complete and easier to elicit, seizures cease and the EEG becomes normal.

\textbf{III) Severe Encephalopathy (Stage- III)}

Newborns with severe encephalopathy are stuporous or comatose immediately after birth, respiration is irregular or periodic and mechanical ventilation is necessary to sustain life. Apnea and seizures begin during the first 12hrs. and progress to tonic and multifocal clonic pattern before the end of the first day.

The newborn lies motionless with legs extended and fully abducted and the arms remain in any position in grasp reflex. The Moro reflex, tonic neck reflex and deep tendon reflexes are usually absent. These features of severe motor dysfunction may be due in part to ischaemic necrosis in watershed areas of the spinal cord.

Pupillary reflexes and dolls eye movements are normal but
occulomotor palsies may be present. Normal sucking and swallowing are depressed or absent but intermittent sucking and chewing movements may be present as seizure manifestations.

Between 12 and 24 hrs., some improvement in the state of consciousness may be noted. Stimulation at this stage provokes a jittery response. Most children remain stuporous. Seizures increase in frequency and severity, sometimes progressing to status epilepticus. EEG reveals a burst suppression pattern and CT demonstrates areas of hypodensity representing cerebral infarctions.

Overall deterioration in the child’s condition with brainstem dysfunction as a prominent feature occurs within 24 to 72 hrs. postpartum and includes coma, loss of pupillary and oculovestibular reflexes and respiratory arrest.

Survivors may remain in a stuporous state for weeks although the frequency of seizures progressively declines while the level of consciousness slowly improves. Development of the most survivors is delayed. Some ultimately die during infancy and the remainders have severe neurological handicaps.

Hypoxic-ischaemic /reperfusion (HI/R) injury remains one of
the most important neurologic complications in the newborn. Injury to the immature nervous system has potential repercussion in motor, cognitive, and behavioral functions that span the infants lifetime.

Mechanisms of brain injury in the newborn differ in many ways from those in the mature brain. One critical difference in the newborn is the overlap between processes essential for normal brain development and those mediating cellular injury.

Cerebral hypoxic ischaemic encephalopathy (HIE) results when the decrease in cerebral perfusion exceeds the ability of the brain to increase the extraction of oxygen from the blood thereby uncoupling cerebral blood flow and oxidative metabolism.

In a study on oxidants and antioxidants in hypoxic-ischaemic encephalopathy carried out by A.N. Suryakar et al, the role of serum lipid peroxide and serum nitric oxide as oxidants and erythrocytic superoxide dismutase and serum vitamin E as antioxidant were determined in 50 neonates with Hypoxic ischaemic encephalopathy as against 25 healthy neonates as controls. In this study the levels of serum lipid-peroxide, serum
nitric oxide & erythrocytic superoxide dismutase were significantly elevated in both group of neonate with HIE than those of controls (P<0.001), where as the serum vitamin E levels were significantly decreased in both groups of HIE patients than those of controls (P<0.001). Thus from the study it can be concluded that alterations in the status of oxidants & antioxidant indicate a pivotal role of free radicals in the development of HIE.

In 1999 Saroj Kumar singh et. al\textsuperscript{112} compared the activities of key antioxidant enzymes and the level of malondialdehyde (MDA) in neonates with hypoxic ischaemic encephalopathy and controls, 15 term born neonates (gestational age 37-41 wks) with birth asphyxia and HIE surviving for 24 hrs or more were included in the study. 19 normal term newborns served as controls. The level of superoxide dismutase (SOD) and catalase were significantly high. There was no significant difference in level of GPX in HIE cases as compared to controls except in HIE stage. The increased level of MDA indicates that the upregulation of SOD and catalase was not able to prevent lipid peroxidation by oxygen free radicals. Hence study shows that oxygen free radicals may play a significant role in the pathogenesis of HIE\textsuperscript{108}. 

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In 1999 Ebru Ergenekon et al found that HIE remained one of the most important neurologic complications in the newborn period. To investigate nitric oxide (NO) involvement in asphyxiated newborns, serum and CSF values of NO levels in 17 neonates with HIE were determined. Infants at or above 37 wks of gestation were classified into mild (n=5), moderate (n=6) and severe (n=6) HIE according to Sarnat and Sarnat. The CSF NO levels and serum NO levels were significantly higher in moderate and severe HIE groups as compared to the mild HIE group.\textsuperscript{32}

In 1999 Ikeda. T. et al examined the role of oxidative stress in asphyxia induced prenatal brain damage. Near term foetal lambs were subjected to umbilical cord occlusion for approximately 60 min until foetal arterial pH diminished to less than -20meq/l. The levels of superoxide, hydrogen peroxide, glutathione (GSH) and thiobarbiturate reactive substances (TBARs) within brain gray and white matter were determined. These white matter changes were accompanied by significant increases in hydrogen peroxide and TBARs levels as compared to those in grey matter. A highly significant rise in the levels of TBARs was again noted in the parietal and frontal white matter. SOD levels were higher in the
frontal and parietal white matter, basal ganglia and cerebellum. These results suggested that the developing telencephalic white matter appeared to be most vulnerable to the effects of intrauterine foetal asphyxia and that oxidative stress might be a major contributing factor in the pathogenesis of perinatal HIE.

In 2000 Yuan et al studied the levels of nitric oxide (NO\(^\cdot\)) in the HIE patients. Among the 33 asphyxiated term neonates plasma NO\(^\cdot\) levels in 25 neonates with HIE of various stage were assessed. Out of 28 neonates 13 had stage-I HIE and 5 had stage –III HIE. The nitrite/nitrate concentration has been a good indicator for NO\(^\cdot\) production. The plasma NO\(^\cdot\) levels were significantly higher in the newborn infants, with HIE stage-III than those in the neonates with HIE stage –I and II. NO\(^\cdot\) levels were significantly increased in neonates with brain damage and adverse out come as compared with those in patients with normal neuroimaging and normal outcome.\(^{138}\)

In a study on free radical injury and Blood –Brain Barrier permeability in hypoxic ischaemic encephalopathy done by Ashok Kumar et al\(^8\) it was found that plasma malondialdehyde and nitrate/nitrite levels were significantly higher in infants with
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hypoxic ischaemic encephalopathy compared with control subjects. Although there was a progressive increment in plasma levels of malondialdehyde with increasing severity of hypoxic ischaemic encephalopathy, the differences were not statistically significant. Plasma nitrate/nitrite levels were almost similar in all stages of hypoxic-ischaemic encephalopathy. Plasma albumin levels were comparable in infants with hypoxic ischaemic encephalopathy and control subjects, whereas cerebrospinal fluid albumin levels were significantly higher in infants with hypoxic ischaemic encephalopathy. Significant correlation was observed between plasma malondialdehyde and nitrate/nitrite levels with blood brain barrier permeability. Hence it was concluded that increased plasma levels of malondialdehyde and nitrates /nitrites are found to be associated with hypoxic ischaemic encephalopathy, indicating the possible role of free radical injury in its causation. Increased blood–brain barrier permeability may be another contributory factor to the progression of the disease.

Neuronal Events:

In recent years, the excitotoxic theory of neuronal death has become contrary to our understanding of HI/R and its
repercussion have become a major target for neuroprotective strategies. During HIE glutamate and other excitatory neurotransmitters are released and their reuptake inhibited, with progressive energy failure. ATP dependent exocytosis is inhibited and subsequent glutamate release occurs by a reversal of normal glutamate uptake mechanisms. Finally as cells die and disintegrate glutamate release occurs by membrane leakage. Excitotoxicity results when toxic levels of glutamate cross to activate special receptors that in turn, mediate an overwhelming influx of calcium into the post synaptic neurons.

Inhibition of free radical toxicity: Free radical injury may in theory be limited by boosting the availability of scavengers e.g. Catalase and superoxide dismutase (SOD) inhibiting lipid peroxidation chain reactions or by inactivating mechanisms that amplify free radical toxicity (e.g. Iron chelators). The newborn particularly the premature infant has a reactive deficiency of SOD. Free radicals are produced by a number of mechanisms and have different toxicities. Less toxic free radicals (e.g. Superoxide and NO) can be potentiated to highly reactive radicals by e.g. a transition metal like iron to form hydroxyl radical or by combining
to form the peroxynitrite radical.\textsuperscript{83}

Free radical scavengers occurring endogenously include SOD, catalase and glutathione. The therapeutic potential of exogenous SOD is limited by a short circulating half life and an inability to penetrate lipid membranes (including the blood- brain barrier) thus limiting the protective, effect to the endothelial surface\textsuperscript{79}. These limitations are reduced markedly by conjugating SOD to polyethylene glycol (PEG) or by entrapping it in liposomes. A novel group of free- radical scavenger the PBN (N-tert- butyl-alpha-phenynitrone) spin traps have shown a promising decrease in infarct size in animal models of focal ischaemia but not against selective neuronal necrosis. The primary protective effect of PBN spin traps may be at the level of microcirculation. The therapeutic window might be several hours post strokes.

Chain breaking agents are incorporated into lipid membranes where they block the spreading chain reaction of peroxidation. Vitamin E (α tocopherol) is an endogenous chain breaker. The neuroprotective efficacy of exogenous vitamin E appears confined to preinsult treatment though this is controversial.
Hypoxic–ischaemic Brain injury in the newborn:

Energy failure –

When cerebral oxygen and substrate supply is progressively reduced below critical threshold for reversible cell dysfunction an irreversible cell death occurs. Synaptic inactivation is an initial, reversible adaptive response to HIE and precedes any significant decline in cerebral high-energy phosphates. Further decrease in perfusion causes progressive energy failure eventually leading to irreversible injury. Cell death becomes inevitable when the available energy no longer is capable of sustaining the membrane pumps that are critical for maintaining normal ion gradients. ATP initially is converted to adenosine a vasodilator and inhibitor of glutamate release. Adenosine in turn is converted to hypoxathine an important precursor of free radical generation.

Nitric oxide synthases (NO) is a widely distributed enzyme with multiple and complex biologic functions HI/R has emerged as powerful stimulus for NOS activation and an activator of NOS encoding genes. NOS converts D arginine to nitric oxide (NOS) a weak radical that has become implicated as a potent mediator of excitotoxic injury. Three structurally distinct NOS isoforms with
specific cellular origins and different mechanisms of action have been identified. These isoforms are neuronal (nNOS) endothelial (eNOS) and inducible (iNOS). NOS also is produced by glial cells. During normal NO homeostasis, fluctuations of cytoplasmic calcium activate a constitutive form of NOS present in endothelial and neuronal cells. In this manner NOS mediates tightly regulated and rapidly responsive biologic function.\textsuperscript{37}

**Reactive free Radical:**

Use of oxygen as metabolic fuel during evolution allowed aerobic organisms to have an attractive harvest of energy rich phosphate however a significant fraction of energy rich phosphate by the organism is incompletely reduced which is shown to be toxic. Such partially reduced forms of oxygen & some of the derivatives such as superoxide anion, hydroxylradicals etc. are highly reactive prooxidants that are collectively referred to as reactive oxygen species (ROS). The toxicity of oxygen could be attributed to free radicals in the formation of oxygen free radicals capable of independent existence containing one or more unpaired electron in their valence shell.

The presence of free radicals in biological materials was
discovered more than 50 years ago. Soon thereafter, Denham Harman hypothesized that oxygen radicals may be formed as by-products of enzymic reactions in vivo. In 1956, he described free radicals as a Pandora’s Box of evils that may account for gross cellular damage, mutagenesis, cancer and last but not the least, the degenerative process of biological ageing.

The science of free radicals in living organisms entered a second era after McCord and Fridovich discovered the enzyme Superoxide Dismutase (SOD) and, finally, convinced most colleagues that free radicals are important in biology. Numerous researchers were now inspired to investigate oxidative damage inflicted by radicals upon DNA, proteins, lipids, and other components of the cell.

A third era began with the first reports describing advantageous biological effects of free radicals. Mittal and Murard provided suggestive evidence that the superoxide anion (O$_2^-$), through its derivative, the hydroxyl radical, stimulates the activation of guanylate cyclase and formation of the “second messenger” cGMP. Similar effects were reported for the superoxide derivative hydrogen peroxide. Ignarro and Kadowitz and Moncada
and colleagues discovered independently the role of nitric oxide (NO') as a regulatory molecule in the control of smooth muscle relaxation and in the inhibition of platelet adhesion. Roth and Droge found that inactivated T cells the superoxide anion or low micromolar concentrations of hydrogen peroxide increase the production of the T-cell growth factor interleukin-2, an immunologically important T-cell protein. Keyse and Tyrrell showed that hydrogen peroxide induces the expression of the heme oxygenase (HO-1) gene. Storz and colleagues reported the induction of various genes in bacteria by hydrogen peroxide, and Schreck and Baeuerle reported the activation of the transcription factor nuclear factor kB (NF-kB) by hydrogen peroxide in mammalian cells.

At the beginning of the 21st century, there is now a large evidence showing that living organisms have not only adapted to an unfriendly coexistence with free radicals but have, in fact, developed mechanisms for the advantageous use of free radicals. Important physiological functions that involve free radicals or their derivatives include regulation of vascular tone, sensing of oxygen tension and regulation of functions that are controlled by oxygen
concentration, enhancement of signal transduction from various membrane receptors including the antigen receptor of lymphocytes and oxidative stress responses that ensure the maintenance of redox homeostasis (Table 1).

**Table 1.- Important physiological functions that involve free radicals or their derivatives.**

<table>
<thead>
<tr>
<th>Type of Radical</th>
<th>Source of Radical</th>
<th>Physiological Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitric oxide (NO(^{\cdot}))</td>
<td>Nitric oxide synthase</td>
<td>Smooth muscle relaxation (control of vascular tone) and various other cGMP-dependent functions.</td>
</tr>
</tbody>
</table>
| Superoxide (O\(^{2-}\)) and related ROS | NAD(P)H oxidase | .Control of ventilation.  
..Control of erythropoietin production and other hypoxia-inducible functions  
..Smooth muscle relaxation.  
..Signal transduction from various membrane receptors/enhancement of immunological functions. |
| Superoxide (O\(^{2-}\)) and related ROS | Any source | Oxidative stress responses and the maintenance of redox homeostasis. |

The field of redox regulation is also receiving growing attention from clinical colleagues in view of the role that oxidative stress has been found to play in numerous disease conditions.
These pathological conditions demonstrate the biological relevance of redox regulation. The delicate balance between the advantageous and detrimental effects of free radicals is clearly an important aspect of life. The science of biological “redox regulation” is a rapidly growing field of research that has an impact on diverse disciplines including physiology, cell biology and clinical medicine.

**Reactive Oxygen Species (ROS):**

Oxygen exists in air as a molecule (O₂) known as dioxygen or molecular oxygen. It was first isolated and characterized between 1772 and 1774 by the individual skills of Priestley, Lavoisier and Scheele. Dioxygen, also referred to as oxygen, appeared in significant amounts on the surface of Earth -2.5 X 10⁹ years ago, and geological evidence suggests that it was created by the photosynthetic activity of microorganisms (blue-green algae). The slow and steady rise in atmospheric oxygen concentration was accompanied by the formation of the ozone layer in the stratosphere. Both oxygen and the ozone layer were critical filters against the intense solar ultraviolet light reaching the surface of the Earth. The universe exists predominantly of hydrogen and
helium, with Earth as a unique center of oxidation in an otherwise reducing universe.\textsuperscript{22,83}

The percentage of oxygen in dry air is now 21\%, making it, after nitrogen (78\%), the second most abundant element in the atmosphere. However, the amount in the air is negligible when compared with the oxygen present as part of the water molecule in oceans, lakes and rivers and as part of mineral reservoirs in Earth’s crust, where it is by far the most abundant element. When Earth’s atmosphere changed from a highly reducing state to its present oxygen-rich state, anaerobic life forms ceased to exist or retreated to places where oxygen was excluded. The slow change from anaerobic to aerobic life necessitated the evolution of specialized antioxidants to protect against the toxic properties of oxygen. Aerobic life uses oxygen to oxidize (burn) carbon- and hydrogen-rich substrates (foods) to obtain the chemical and heat energy essential for life.

Unfortunately, when we oxidize molecules with oxygen, the oxygen molecule itself becomes reduced and forms intermediates, two of which are free radicals,
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\[ O_2 + e^- + H^+ \rightarrow HO_2^- \] (hydroperoxyl radical) (1a)

\[ HO_2^- \rightarrow H^+ + O_2^- \] (superoxide radical) (1b)

\[ O_2^- + 2H^+ + e^- \rightarrow H_2O_2 \] (hydrogen peroxide) (2)

\[ H_2O_2 + e^- \rightarrow OH^- + \cdot OH \] (hydroxyl radical) (3)

\[ \cdot OH + e^- + H^+ \rightarrow H_2O \] (4)

Under normal conditions, oxygen is a stable, odorless, tasteless, and colourless gas of limited solubility in water. This limited solubility is vital for the aquatic life and essential for normal respiratory functions in humans. When compared with the other elements, oxygen has the third highest electron affinity and should be considerably more reactive than it is observed to be. Its reactivity is masked because oxygen contains two unpaired electrons with the same spin quantum number (parallel spin) and only when this spin restriction is overcome can the true reactivity of oxygen be expressed.

When oxygen is reduced by the stepwise addition of electrons (Eqs. 1-4), two free radicals (HO_2^-, \cdot OH) are formed, together with H_2O_2. At pH 7.4, the hydroperoxyl radical (HO_2^-), with a pKa of 4.8, dissociates to give the superoxide anion radical.
A free radical is any chemical species capable of independent existence and containing one or more unpaired electrons. This definition is broad and does not specify exactly where the unpaired electron is. It is preferred because it allows us to classify most of the transition metal ions as free radicals and thus better understand the close interrelation between oxygen and reactive metal ions.

**Superoxide Anion Radical**

Superoxide (O$_2^-$) is formed when one electron enters one of the orbitals of oxygen. The chemistry of superoxide differs greatly depending on its solution environment. In aqueous solution O$_2^-$ is a weak oxidizing agent able to oxidize molecules such as ascorbic acid and thiols.

Superoxide (O$_2^-$) is produced by the addition of a single electron to oxygen and several mechanisms exist by which superoxide can be produced *in vivo*. Several molecules, including adrenaline, flavine nucleotides, thiol compounds, and glucose, can oxidise in the presence of oxygen to produce superoxide, and these
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Reactions are greatly accelerated by the presence of transition metals such as iron or copper. The electron transport chain in the inner mitochondrial membrane performs the reduction of oxygen to water. During this process free radical intermediates are generated, which are generally tightly bound to the components of the transport chain. However, there is a constant leak of a few electrons into the mitochondrial matrix and this results in the formation of superoxide. The activity of several other enzymes, such as cytochrome p450 oxidase in the liver and enzymes involved in the synthesis of adrenal hormones, also results in the leakage of a few electrons into the surrounding cytoplasm and hence superoxide formation. There might also be a continuous, production of superoxide by the vascular endothelium to neutralize nitric oxide, introduction of superoxide by other cells to regulate cell growth and differentiation and the production of superoxide by phagocytic cells during the respiratory burst.

Superoxide rapidly disappears in aqueous solution because of its dismutation reaction in which hydrogen peroxide and oxygen are formed.

\[ O_2^- + O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \]
The copper-zinc superoxide dismutase enzyme discovered by McCord and Fridovich in 1968 greatly accelerates the above reaction. Several different forms of the enzyme are now known to exist in plant, microbial and mammalian cells, differing in structure and transition metal ions at their active centers, but catalyzing the same chemical reaction shown in Eq. 6. The protonated form of $\text{O}_2^-$, the hydroperoxyl radical (HO$_2^-$), is both a more powerful oxidant and a more powerful reductant than O$_2$ , but little HO$_2^-$ is present at pH 7.4.

$\text{O}_2$ attacks unsaturated lipids and breaches the integrity of membranes. When the membranes are free of lipid hydroperoxides then both O$_2$ and H$_2$O$_2$ are required and singlet oxygen appears to be the proximal attacking species. When the membrane contains some lipid hydroperoxide the O$_2$ is itself sufficient and seems to generate an alkoxy radical by reacting with the lipid hydroperoxide. It appears likely that attack on membranes is one of the reasons for the cytotoxicity of O$_2$.

In Escherichia coli the manganese –SOD is depressed by O$_2$. This enzyme is not made in the absence of oxygen and in aerobic conditions any change which results in enhanced production of O$_2$
calls for an increased synthesis of this enzyme. Increased levels of SOD however achieved correlation with greater resistance towards oxygen toxicity.

It is generally true that respiring cells contain more SOD than non respiring cells. Among obligate anaerobes there is correlation between SOD content and tolerance towards oxygen.

**Hydrogen Peroxide**

Any system producing superoxide will, as a result of the dismutation reaction, also produce H$_2$O$_2$. Many enzymes such as urate oxidase, glucose oxidase and D-amino acid oxidase produce H$_2$O$_2$ directly by the transfer of two electrons to oxygen. H$_2$O$_2$ is a weak oxidant and a weak reducing agent that is relatively stable in the absence of transition metal ions. The molecule has an uncharged covalent structure it readily mixes with water and is treated as a water molecule by the body, rapidly diffusing across cell membranes. The redox properties of H$_2$O$_2$ and its ability to form highly reactive free radicals in the presence of transition metal ions have necessitated the evolution of body defenses against it. Unwanted H$_2$O$_2$ is removed from cells by the action of
catalase, glutathione peroxidase (selenium containing) and certain other peroxidases.

Hydrogen peroxide is not a free radical itself, but is usually included under the general heading of Reactive Oxygen Species (ROS). It is a weak oxidising agent that might directly damage proteins and enzymes containing reactive thiol groups. However, its most vital property is the ability to cross cell membranes freely, which superoxide generally cannot do. Therefore, hydrogen peroxide formed in one location might diffuse a considerable distance before decomposing to yield the highly reactive hydroxyl radical, which is likely to mediate most of the toxic effects ascribed to hydrogen peroxide. Therefore, hydrogen peroxide acts as a conduit to transmit free radical induced damage across cell compartments and between cells.

In the presence of hydrogen peroxide, myeloperoxidase will generate hypochlorous acid and singlet oxygen, a reaction that plays an important role in the killing of bacteria by phagocytes. The hydroxyl radical (OH’) or a closely related species, is probably the final mediator of most free radical induced tissue damage.
All of the reactive oxygen species exert most of their pathological effects by giving rise to hydroxyl radical formation. The reason for this is that the hydroxyl radical reacts, with extremely high rate constants, with almost every type of molecule found in living cells including sugars, amino acids, lipids, and nucleotides.

Although hydroxyl radical formation can occur in several ways, by far the most important mechanism in vivo is likely to be the transition metal catalysed decomposition of superoxide and hydrogen peroxid. All elements in the first row of the d-block of the periodic table are classified as transition metals. In general, they contain one or more unpaired electrons and are therefore themselves radicals when in the elemental state. However, their key property from the point of view of free radical biology is their variable valency, which allows them to undergo reactions involving the transfer of a single electron. The most important transition metals in human disease are iron and copper. These elements play a key role in the production of hydroxyl radicals in vivo. Hydrogen peroxide can react with iron II (or copper I) to generate the hydroxyl radical, a reaction first described by Fenton in 1894:
Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^-

This reaction can occur in vivo, but the situation is complicated by the fact that superoxide (the major source of hydrogen peroxide in vivo) will normally also be present. Superoxide and hydrogen peroxide can react together directly to produce the hydroxyl radical, but the rate constant for this reaction in aqueous solution is virtually zero. However, if transition metal ions are present a reaction sequence is established that can proceed at a rapid rate:

Fe^{3+} + O_2 \rightarrow Fe^{2+} + O_2

Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^-

Net result:

O^2^- + H_2O_2 \rightarrow OH^- + OH^- + O_2

The net result of the reaction sequence illustrated above is known as the Haber-Weiss reaction. Although most iron and copper in the body are sequestered in forms that are not available to catalyse this reaction sequence, it is still of importance as a mechanism for the formation of the hydroxyl radical in vivo. The actual reactions, however, may be somewhat more complex than
those described above and it is possible that other reactive intermediates such as the ferryl and perferryl radicals might also be formed.

**Hydroxyl Radicals**

The hydroxyl radical (‘OH) is a major product arising from the high-energy ionization of water (radiolysis)

\[
\text{H}_2\text{O} \rightarrow \text{OH} + \text{H}^+ + \text{e}_\text{aq} \rightarrow \text{H}_2\text{O}_2 \quad (7)
\]

(aq, aqueous)

Most of our definitive knowledge about the hydroxyl radical derives from studies by radiation chemists who, by convention, show the unpaired electron on the hydrogen atom, i.e., OH’. This is incorrect because the unpaired electron is on the oxygen atom. The ‘OH radical is an extremely aggressive oxidant that can attack most biological molecules at an almost diffusion controlled rate. During the 1890s, the Cambridge chemist H.J.H. Fenton described a reaction between iron salts and H\textsubscript{2}O\textsubscript{2} that caused oxidative damage to organic molecules such as tartaric acid. The Fenton reaction is widely represented as in Eqs. 8-10.

\[
\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}’ + \text{OH}^- \quad (8)
\]
Fe^{3+} + H_2O_2 → Fe^{2+} + HO_2^- + H^+  (9)

Overall reaction

Ironsalt + 2H_2O_2 → 2H_2O + O_2  (10)

Eqs. 8 and 9 are gross oversimplifications of the chemistry involved, particularly when such reactions are considered in biological systems, where oxo-iron species are also likely intermediates formed along with 'OH. At physiological pH (7.4), ferrous ions (Fe^{2+}), in the presence of oxygen and phosphate ions (PO_4^{2-}), exist only transiently before auto-oxidizing to the ferric state (Fe^{3+}). In the process of changing from the ferrous to the ferric state, an electron is transferred from iron to oxygen to make superoxide as shown in Eq. 11.

Fe^{2+} + O_2 ↔ Fe^{2+}O_2 ↔ Fe^{3+}O_2^- ↔ Fe^{3+} + O_2  (11)

The intermediates Fe^{2+}O_2 and Fe^{3+}O_2^- are known as the perferryl ion, in which iron is said to have an oxidation state of V.

Thus ROS includes not only the oxygen radicals but also some non radical derivatives of oxygen. These are shown below.
Reactive oxygen Species

<table>
<thead>
<tr>
<th>Radicals</th>
<th>Non- Radicals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>Hydroxyl</td>
<td>Hypochlorous acid</td>
</tr>
<tr>
<td>Peroxyl</td>
<td>Ozone</td>
</tr>
<tr>
<td>Alkoxyl</td>
<td>Singlet oxygen</td>
</tr>
<tr>
<td>Hydroperoxyl</td>
<td>Peroxynitrite</td>
</tr>
</tbody>
</table>

A relatively small number of free radicals such as the superoxide anion & the hydroxyl radicals were recognized as a minor byproduct of oxidative phosphorylation. Approximately 2% of the oxygen reduced by the mitochondrion forms superoxide or the dismutation product, hydrogen peroxide superoxide. Superoxide & peroxyl react with metal ions to promote additional radical generation with the release of particularly reactive hydroxyl radicals. Hydroxyl radicals reacts at nearly diffusion limited rates with any component of the cell including lipids, DNA, lipoproteins etc. The results of these free radical injuries are loss of integrity, enzymatic function & genomic stability.

Consequently to minimize the damaging effects of ROS, aerobic organisms evolve both nonenzymatic & enzymatic
defenses. Superoxide dismutase (SOD) converts superoxide to hydrogen peroxide which is subsequently reduced to water by catalase or otherwise decomposed by glutathione dependent peroxidase. Small molecule reducing agents such as glutathione there by shelter the intracellular environment against ROS. In synergy with the aqueous defense mechanisms (e.g vitamin C, Uric acid) lipid phase antioxidants (e.g. Tocopherols, carotenoides) exist naturally to scavenge radical intermediates.

Over the past decade, the menagerie of ROS has been extended to include reactive nitrogen species (RNS) derived from nitric oxide reaction with superoxide or peroxide specific defense mechanisms evolved to counteract RNS stress will probably be identified in coming years.

Thus ROS normally exist in all aerobic cells in balance with biochemical antioxidants. Oxidative stress occurs when this critical balance is disrupted because of oxidant excess ROS anti reliant depletion or both. To counteract the oxidant effects & to restore balance cells must reset important homeostatic parameters. This frequently leads to the activation or silencing of genetic encoding regulatory transcription factors, antioxidant
defense enzymes & structural proteins.

Because hydroxyl radical reacts at its site of formation usually leaving behind a legacy in three form of propogating free radical chain reactions (i.e. the unpaired electron is located on oxygen). Superoxide $O_2^\cdot$ is made by adding one electron to the oxygen molecules. It is generally poorly reactive.

Some superoxide is made by “Accidents of chemistry” in that many molecules in the body react directly with oxygen to make superoxide e.g. catecholamines, tetrahydrofolates & some constituents of mitochondrial & other electron transports chains. Such superoxide generation is unavoidable In addition some superoxide is made deliberately for instance activated phagocytes generate superoxide as part of mechanism by which foreign organisms are killed during chronic inflammation. This normal protective mechanism may become damaging. Neutrophils, monocytes, macrophages, eosinophils generate large amounts of oxygen free radicals.
Functions of free Radical –

The generation of free radical by polymorphonuclear cell provides protection from infection or foreign body inclusion. Polymorphonuclear cells are normally found in the blood circulation in an active cell membrane. Enzymes such as NADPH oxidase also exist in an inactive form. In this setting polymorphonuclear cell may encircle or even ingest bacteria but they are incapable of damaging or killing them. Exposures to immune globulin coated bacteria immune complexes, complement or leukotriene, however activate the enzyme NADPH oxidase. This activation initiates a respiratory burst of the cell membrane to produce superoxide. Activation of NADPH oxidase occurs at pH 7 to 7.5 & therefore is effective in infection sites with low oxygen tension. The pH within the vacuoles of polymorphonuclear cell initially rises, plateaues & then quickly becomes acidic. Apparently, most of the killing action by superoxide occurs during the elevated pH phase, because, neutrophils produce much less superoxide at acidic pH than at neutral pH26.

Once the polymorphonuclear cell encodes the particle granules released by the cell generate ROS within seconds to
destroy the bacterial membrane. The actual killing of bacteria or neutralization depends on increased pH & the presence of superoxide or reduced components $\text{H}_2\text{O}_2$ hydroxyl ions of HOCl as well as number of bactericidal peptides. Subsequent events working through G protein, phospholipase C & inositol 1, 4, 5 triophosphate produces an increase in cytosolic calcium & bacterial death or neutralization.

**Formation of Free Radicals**

The most important radicals which may be involved in disease processes are species which may be derived from molecular oxygen & certain oxides of nitrogen. The unpaired electrons of the radical nature of a species are conventionally indicated by writing it with a heavy superscript dot.

The processes by which free radicals can be formed are illustrated below.

1. Radical formation by the loss of a single electron from a non radical

   $X\rightarrow \text{-}\rightarrow \text{-} \rightarrow \text{-} \rightarrow e \rightarrow+ X^\cdot+ \text{+}$

2. Radical formation by the gain of a single electron from a non radical.
Y + e⁻ → Y⁻

3. Radical formation by hemolytic fission:

Radicals can be formed when a covalent bond is broken. The energy required to dissociate the covalent bond can be provided by heat, electromagnetic radiation or other means.

A:B → A⁻ + B⁺

Generally free radicals are highly reactive species & tend either to lose an electron thereby acting as reducing agents or to gain an electron acting as an oxidizing agent. If a radical reacts with a non-radical another free radical must be produced. This characteristic feature enables free radical to participate in chain reactions.

Some of the reaction which involves oxygen derived free radicals give rise to compounds which are not themselves free radicals.

They are nevertheless reactive & for this reason, oxygen derived free radicals & related non radical compounds are referred to as Reactive oxygen species (ROS)

In 1954 Rebecca Gershman & Daniel L Gilbert proposed that many of the damaging effects of oxygen could be attributed to the
Tissue damage by radicles

Modification of amino acids in apoproteins in LDL
Peroxidation of lipids in LDL
Dialdehydes

Macrophages engulf modified LDL
Antibodies raised against modified proteins
Modification of amino acids in proteins
Peroxidation of lipids in membranes
Dialdehydes

Strand breaks and modification of bases in DNA

Lipid peroxidation and antioxidant status in encephalopathy in children
formation of oxygen free radicals superoxide theory of oxygen toxicity which states that formation of the superoxide theory of oxygen superoxide radical in vivo plays a major role in the toxic effects of oxygen.

Properties of free radicals –

Free radicals broadly have the following properties

- High reactivity with a consequent extremely short life span.
- Self –perpetuating (autocatalytic) & diverse chemical reactivity
- Low chemical specificity
- Generated both in vivo & in vitro

Why Radicals are made in Human Body –

We are exposed to electromagnetic radiation from the environment, both natural & from man made sources. Low wavelength electromagnetic radiation (e.g. gamma rays) can split water in the body to generate hydroxyl radical OH⁻.

Oxidative stress has long been considered as an accident of aerobic metabolism a characteristic process of free radical production & non-specific tissue damage which is a fundamentally
unregulated aside from the normal phalanx of antioxidant defense mechanisms. In recent years shift has been occurring where in certain ROS & RNS have become appreciated as signaling molecules whose production may be regulated as a part of routine cellular signal transduction.

Only recently it has been recognized that ROS are widely used as messengers to propagate pro-inflammatory or growth stimulatory signals. Various agents proceeding through a common pathway involving ROS generation can activate necrotic factor KB (NF KB) & AP both of which have key roles in cell proliferation differentiation & morphogenesis. ROS can arrest cell division & the cell cycle progression. ROS can play different or even opposing roles during different cellular processes. Consequently the steady state level of ROS within cells is crucial. This level determines mechanisms & the subtle modulation roles played by cellular antioxidants.

Incordinate or aberrant generation of ROS is widely incriminated in the pathogenesis of tissue injury. This ubiquitous involvement of ROS in disease process reflects the pathobiologic effects of ROS. Action of ROS affects cellular vitality & it
determines cellular growth & proliferation, tissue repair & regeneration, inflammatory & immune processes, & regulation of various haemodynamic; haemostatic & vascular systems. The biomedical literature is full of claims that free radicals & other ROS are involved in different human diseases They have been implicated in over 100 disorders ranging from rheumatoid arthritis & hemorrhagic shock through cardiomyopathy & cystic fibrosis to gastrointestinal ischaemia.

**Biologically important radical –**

Superoxide radical – If a single electron is added to the ground state oxygen molecule the product is superoxide radical $O_2\cdot$:

$$O_2 + \text{One electron} \rightarrow O_2\cdot$$

Sources – Some $O_2$ is produced deliberately in vivo e.g. by activated phagocytic aldehyde oxidase tryptophan dioxygenase & even nitric oxide synthase are reported to produce $O_2$ xanthine dehydrogenase is an enzyme which transfers electron from the substrates on to NAD rather than $O_2$. In injured tissues a dehydrogenase – oxidase conversion can occur & $O_2$ acts as the
electron acceptor for this xanthine oxidase leading to the production of $O_2^-$.

Several biologically important molecules such as glyceraldehyde FMN H2, FADH2, the hormones adrenaline, the neurotransmitter dopamine tetrahydropteridines & thiol compounds undergo auto oxidation reactions in the presence are greatly accelerated by the addition of transition metal ions. These heme proteins are also known to release $O_2$.

Mitochondrial electron transport chain is probably the most important source of $O_2$ in vivo & at physiological levels of $O_2$ it has been suggested that about 1-3 % of the Oxygen reduced in mitochondria may form $O_2$. Isolated subcellular fractions containing endoplasmatic reticulum have shown to produce $O_2$ & also $H_2O_2$. These ROS largely varies from the cytochrome P-450 system. The membrane surrounding the cell nucleus also contains an electron transport chain that can leak electrons to give $O_2$ & this may be of special importance in vivo because the ROS that it generates are close to the nuclear DNA.
Transition metals Cu, Fe & Mn

Transition metal ions are considered as free radicals since most of their biological effects whether beneficial or deleterious involve their ability to accept & donate single electrons.

Transition metal ions mainly act as catalysts of free radical reactions. The variable oxidation number of transition metals helps them to be effective catalysts of reactions involving oxidation & reduction & they are used for this purpose at the active sites of many enzymes. The single electron transfers promoted by metals can overcome the spin restriction. The potential danger is that unless their availability is carefully controlled transition metals will catalyse unwanted free radical reactions. Transition metals catalyzes auto oxidations of several biologically important compounds which are then capable of reducing O$_2$. O$_2$ mixtures of thiols, Catecholamine’ ascorbate etc. with iron or copper ions will often cause free radical damage to biomolecules. It has been shown that in the presence of free transition metal-ions ascorbic acid function as a pro-oxidant & lipids by ionizing radiation. DNA damage especially double strand breaks is considered to be an
important damaging event especially as double strand breaks cannot easily be repaired by the cell.

**Peroxyl & alkoxy radical**s –

In general, peroxyl (RO₂) & alkoxy (RO⁻) radical are good oxidizing agent. Alkoxy radicals formed in biological system often undergo rapid molecular rearrangement to other molecular species H₂O⁻ protonated O⁻₂ can be regarded as the simplest peroxyl radical.

Attack of OH⁻ upon organic compounds often generates these carbon centered radicals. Most organic peroxides (ROOH) are stable at room temperature but on addition of transition metal ions they can form alkoxy & peroxyl radicals.

\[
\text{ROOH} + \text{Fe (III)} \rightarrow \text{RO}_2^- + \text{Fe}^{2+} + \text{H}^+
\]

\[
\text{ROOH} + \text{Fe}^{2+} \rightarrow \text{RO}^- \text{OH} + \text{Fe (III)}
\]

These reactions account for much of the stimulation of lipid peroxidation by transition metal ions in biological systems.

Sulphur radicals – In vivo thiols (Specially reduced glutathione GSH) are often regarded as antioxidant agents since
they protect protein –SH groups against oxidation & can scavenge oxygen radicals & some other reactive species such as hypochlorous acid & peroxynitrous acids.

**The Fenton reaction:**

Fenton chemistry is a prime example of damaging free radical catalysed by transition metals. A mixture of H₂O₂ with Fe²⁺ salt oxidizes many different organic molecules. It probably involves several oxidizing species the best characterized being hydroxyl radical.

Fe²⁺ + H₂O₂ ----> intermediate complexes ----> Fe (III) + OH⁻ + OH⁻

Traces of Fe(III) might be able to react further with H₂O₂ although this is slower than the reaction of H₂O₂ with Fe²⁺ at physiological PH & very much depends on the ligand to iron.

Fe(III) + H₂O₂ ----> intermediate complexes Fe²⁺ + O₂ ++ 2H⁺

Thus this simple fenton mixture of Fe²⁺ & H₂O₂ which almost certainly forms in biological systems under certain circumstances can provoke a whole series of radical reaction. The overall sum of these unless some other reagent is added is an iron catalysed decomposition of H₂O₂.
**Hydroxyl radical (OH⁻):**

Hydroxyl radical can be generated in biologically relevant systems by multiple reactions one is by Fenton chemistry as described above. UV induced hemolytic fission of the O-O bond in H$_2$O$_2$ makes OH⁻ since the major constituent of living cells is water exposure to high energy radiation such as Drays will result in OH⁻ production. Hydroxyl radicals are also generated by reaction of hypochlorous acid with superoxide.

Hydroxyl radicals are responsible for a large part of the damage done to cellular DNA proteins.

Thiyl radicals are formed when thiols react with many carbon centered radicals.

\[ \text{RSH} + \text{-----→C ------→CH} + \text{RS} \]

& with several oxygen radicals including

\[ \text{OH}⁻, \text{Ro}⁻, \text{RO}²⁻, \text{O}²⁻ \]

\[ \text{RSH} + \text{OH} \rightarrow \text{RS} + \text{H}_2\text{O} \]

\[ \text{RSH} + \text{R}_2\text{O} \rightarrow \text{RS} + \text{ROOH} \]

Thiyl radicals are also generated by reaction of thiols with transition metal ions & by the hemolytic fission of disulphides, including disulphide bridges in protein.
Reduction of molecular Oxygen:

End products of GSH oxidation by Oxygen radicals under aerobic condition includes GSSG sulphanic acid (GSOH) & sulphonic acid (GSO₃H) Oxidizing thiyls generate a whole series of potentially cytotoxic oxygen sulphur & oxysulphur radicals. Thiyls may also be involved in the oxidation of low density lipoproteins during atherosclerosis, indeed high plasma levels of the thiol homocysteine is a risk factor for this process.

Oxygen toxicity and free Radical Injury:

O₂ is both essential to human life and toxic to human life. We are dependent on O₂ for oxidation reactions in the pathways triphosphate (ATP) generation, detoxification and biosynthesis. However, when O₂ accepts single electrons, it is transformed into highly reactive oxygen radicals that damage cellular lipids, proteins, and DNA damage by reactive oxygen radicals contributes to cellular death and degeneration in a wide range of diseases.

Radicals are compounds that contain a single electron, usually in an outer orbit. Oxygen is a biradical, a molecule that has two unpaired electrons in separate orbitals. Through a
number of enzymatic and nonenzymatic processes that routinely occur in cell. $O_2$ accepts single electrons to form reactive oxygen species (ROS). ROS are highly reactive oxygen radicals, or compounds that are readily converted in cells to these reactive radicals. The ROS formed by reduction of $O_2$ are the radical superoxide ($O_2^-$) the nonradical hydrogen peroxide ($H_2O_2$) and the hydroxyl (OH')

ROS may be generated nonenzymatically, or enzymatically as accidental byproducts or major products of reaction. Superoxide may be generated nonenzymatically from metal-containing enzymes (e.g. cytochrome P450, Xanthine oxidase, and NADPH oxidase). The highly toxic hydroxyl radical is formed nonenzymatically from superoxide in the presence of $Fe^{3+}$ or $Cu^{+}$ by the Fenton reaction, and from hydrogen peroxide in the Haber–Weiss reaction.

Oxygen radicals and their derivatives can be deadly to cells. The hydroxyl radical cause oxidative damage to proteins and DNA, It also forms lipid peroxides and malondialdehyde from membrane lipids containing polyunsaturated fatty acids. Free radical damage is the direct cause of a disease state (e.g. tissue damage initiated
by exposure to ionizing radiation. In neurodegenerative disease, such as Parkinson’s disease, or in ischaemia–reperfusion injury, ROS may perpetuate the cellular damage caused by another process.

Oxygen radicals are joined in their destructive damage by the free radical nitric oxide (NO) and the reactive oxygen species hypochlorous acid (HOCl).

B) Characteristics of Reactive Oxygen Species

Reactive oxygen species (ROS) are oxygen containing compounds that are highly reactive free radicals, or compounds readily converted to these oxygen free radicals in the cell. The major oxygen metabolites produced by one-electron reduction of oxygen (superoxide, hydrogen peroxide, and the hydroxyl radical) are classified as ROS.

Reactive free radicals extract electrons (usually as hydrogen atoms) from other compounds to complete their own orbital, thereby initiating free radical chain reactions. The hydroxyl radical is probably the most potent of the ROS. It initiates chain reactions that form lipid peroxides and organic radicals and adds directly to
compound. The superoxide anion is also highly reactive, but has limited lipid solubility and cannot diffuse further. However, it can generate the more reactive hydroxyl and hydroperoxyl radicals by reacting nonenzymatically with hydrogen peroxide in the Haber-Weiss reaction.

Hydrogen peroxide, although not actually a radical, is a weak oxidizing agent that is classified as an ROS because it can generate the hydroxyl radical (OH\(^{-}\)). Transition metals Such as Fe\(^{2+}\) or Cu\(^{+}\), catalyze formation of the hydroxyl radical from hydrogen peroxide in the nonenzymatic Fenton reaction.

Oxygen radicals produce cellular dysfunction by reacting with lipid, proteins, carbohydrates, and DNA to extract electrons. Evidence of free radical damage has been described in over 100 disease states. In some of these diseases free radical damage is the primary cause of the disease while in others, it enhances complications of the disease.

**Lipid Peroxidation:**

Lipid peroxidation is a process of oxidative deterioration of polyunsaturated fatty acids (PUFA). In the process of oxidation of
target PUFA(LH) generates a fatty acid radical (L) This radical takes up oxygen forming fatty acid peroxyl radical. These peroxyl radicals are propagatory of chain reaction. They can oxidize further PUFA (LH) molecules & initiate new chains producing lipid hydroperoxides (LOOH) & a new fatty acid radical(L).

**Chemistry of Lipid Peroxidation**: 53:

Fats and oils oxidize with characteristic changes in texture, colour, taste and odor. This process, known as rancidity, was chemically defined in the 1940s as an autoxidative free-radical chain reaction. The free-radical oxidation of polyunsaturated fatty acids in biological systems is known as lipid peroxidation. The detection and measurement of lipid peroxidation is the evidence most frequently cited to support the involvement of free-radical reactions in toxicology and disease. Many techniques are available to measure the progress of oxidation, but none is applicable to all circumstances.

First-chain initiation of a peroxidation sequence in a membrane or polyunsaturated fatty acid results from the attack by any species with sufficient reactivity to abstract a hydrogen atom (H⁺) from a methylene (-CH₂-) group. Because a hydrogen
atom contains only one electron, abstraction leaves behind an unpaired electron on the carbon, -CH-. The presence of a double bond in the fatty acid weakens the C-H bonds on the carbon atom adjacent to the double bond and thus facilitates H⁺ removal. Hence, the polyunsaturated fatty-acid side chains of membrane lipids are particularly sensitive to peroxidation.

The carbon-centered radical undergoes a molecular rearrangement to form a conjugated diene which then combines with oxygen to form a peroxyl radical, itself able to abstract a hydrogen atom from another fatty acid and thus start a chain reaction. Peroxidation continues to use up the substrate unless a chain-breaking antioxidant such as vitamin E is added to terminate the chain reaction. Lipid peroxidation, like all chain reactions, has three stages: initiation, propagation and termination. Unfortunately, the term “initiation” is often loosely applied in the literature to refer to any reaction that increases the observed overall rate of lipid peroxidation, whereas initiation strictly means the initial hydrogen atom abstraction, i.e. first chain initiation. Species that can abstract the first hydrogen atom include the hydroxyl (‘OH) radical, alkoxy radical (RO’), peroxyl
radical (ROO•) and possibly HO2• but not H2O2 or O2•. The end products of the chain reaction are a variety of hydroperoxides and cyclic peroxides. A major problem encountered when studying lipid peroxidation mechanistic is that all commercial samples of polyunsaturated fatty acids contain varying amounts of contaminating peroxides.

Lipid peroxides are fairly stable molecules at physiological temperatures, but their decomposition is catalyzed by transition metals and metal complexes. For example, all redox-active iron complexes present in vivo that participate in the Fenton reaction can also promote lipid peroxide decomposition. Molecules such as hemoglobin and the cytochromes, however, can also facilitate peroxide decomposition, although they do not directly catalyze Fenton chemistry. However, heme proteins can release chelatable iron that can participate in Fenton chemistry. Ferritin and hemosiderin are effective at stimulating lipid peroxidation and catalase is weakly effective under certain circumstances, an action that has sometimes caused problems in attempts to use catalase as a probe for the role of H2O2 in peroxidizing lipid systems. By contrast, iron correctly bound to the two high-affinity iron binding
sites of transferrin or lactoferrin does not seem to promote peroxide decomposition. Reduced metal complexes [e.g., iron(II) or copper(I)] react with lipid peroxides (LOOH) to give alkoxy radicals.

\[ \text{LOOH} + \text{Mn}^+ \rightarrow \text{LO}^- + \text{M}^{n+1} + \text{OH}^- \]

Whereas oxidized metal complexes [i.e., iron(III) or copper(II)] react more slowly to produce peroxyl and alkoxy radicals. Both alkoxy and peroxyl radicals stimulate the chain reaction of lipid peroxidation by abstracting further hydrogen atoms. Much of the alleged initiation of lipid peroxidation reported when metal chelators are added to lipid systems in vitro is real decomposition of preformed lipid peroxides by the metal complexes and hydrogen atom abstraction by the resulting LO and LOO radicals.

Some ions of metals with a fixed oxidation number can affect the rate of peroxidation; e.g., Ca$^{2+}$, Al$^{3+}$ and Pb$^{2+}$ ions can accelerate peroxidation stimulated by iron salts under certain circumstances. This may be the result of changes in membrane structure and has important implications for environmental toxicology.
Rawis and Van Santen have suggested that singlet $O_2^+$ is formed during the complex degradation reactions of lipid peroxidation and that this species might contribute to the chain reaction by causing more initiation.

**Types of Biological Lipid Peroxidation**

**Nonenzymic Lipid Peroxidation:**

Any free radical (R’) with sufficient energy to abstract a hydrogen atom from a methylene carbon of an unsaturated fatty acid (LH) can initiate a chain reaction in bulk lipid. The free-radical chain reaction of lipid peroxidation propagates until two free radicals combine each other to terminate the chain.

$$\text{LO}_2^- + \text{LO}_2^- \rightarrow \text{LOOL} + \text{O}_2$$

$$\text{LO}_2^- + \text{L}^- \rightarrow \text{LOOL}$$

LOOL is cyclic peroxide.

In nonenzymic lipid peroxidation, the peroxyl radicals survive long enough to be able to move to new fatty acid molecules, given that they can readily be intercepted and scavenged by a variety of chemically different antioxidants. Lipid peroxides formed during the chain reaction (LOOH) are a complex mixture of isomers.
**Enzymic Peroxidation**

Enzymic peroxidations refer to the generation of lipid peroxides at the active center of an enzyme. The hydroperoxides and endoperoxides produced are stereospecific and have important biological functions. Cyclooxygenase and lipo-oxygenase fulfill this definition. Free radicals are probably important intermediates in the reaction but are localized to the active sites of the proteins. During formation of endoperoxides by cyclooxygenase, a powerful oxidant is generated that is amenable to scavenging by some antioxidants.

**Consequences of Lipid Peroxidation in Biological Material**

Extensive lipid peroxidation in biological membranes causes loss of fluidity, falls in membrane potential, increased permeability to H+ and other ions, and eventual rupture leading to release of cell and organelle contents. Some end products of peroxide fragmentation are also cytotoxic. Not all lipid oxidation processes are harmful, and peroxidation products may play useful roles in the arachidonic acid cascade and in the wound response of plant tissues. The production of lipid peroxides and their fragmentation
to carbonyl compounds in injured plant tissues may help the plant by killing bacteria or fungal spores entering the damaged site.

**How Important Is Lipid Peroxidation in Radical-Induced Damage?**

A wide variety of techniques have been used to show that lipid peroxidation increases in many disease states and in tissues poisoned by a variety of toxins. Behind many of these reports is the unspoken assumption that the disease or toxin causes increased lipid peroxidation, which is then responsible for the toxicity. However, it was established many years ago that disrupted tissues undergo lipid peroxidation more quickly than healthy ones; e.g., lipid peroxides accumulate in a brain homogenate much more quickly than they do in an isolated intact brain. Reasons for this increased peroxidizability of damaged tissues include inactivation of some antioxidants, leakage of antioxidants from the cell, and the release of metal ions (especially iron and copper) from storage sites and from metalloproteins hydrolyzed by enzymes released from damaged lysosomes. Hence the series of events -
Disease or toxin → cell death or damage → increased lipid peroxidation

The peroxidation precedes or accompanies the cell damage and that prevention of peroxidation by antioxidants prevents the cell damage. Measurement of lipid peroxidation may therefore be an excellent marker of tissue damage.

\[
\begin{align*}
LH + R & \rightarrow L^\cdot + RH \\
L^\cdot + O_2 & \rightarrow LOO^\cdot \\
LOO + LH & \rightarrow LOO^\cdot + L^\cdot \\
LOOH & \rightarrow LO^\cdot, LOO Aldehydes \\
LH & \rightarrow \text{Target PUFA} \\
R & \rightarrow \text{Initiating radical} \\
L & \rightarrow \text{Fatty acid radical} \\
LOO & \rightarrow \text{Fatty acid peroxyl radical} \\
L & \rightarrow \text{New Fatty acid radical} \\
LOOH & \rightarrow \text{Lipid hydroperoxide}
\end{align*}
\]

The breakdown of lipid hydroperoxide involves transition metal ion catalysis yielding lipid peroxyl & lipid alkoxy radicals. A
large number of toxic byproducts are formed during lipid peroxidation. These have effects at a site away from their site of generation. Hence they behave as toxic second messengers. Membrane lipids are particularly susceptible for lipid peroxidation. Since membranes form the basis of many cellular organelles like mitochondrial membrane the damage caused by lipid peroxidation is highly detrimental to the functioning of the cells & its survival.

Thus lipid peroxidation reactions are important because they can directly damage the structure of the membrane & indirectly damage other cell component by production of reactive aldehydes.

Lipid peroxidation can be formed by several mechanisms. Severe damage to the structural components of the cell is followed by autooxidation of unsaturated fatty acids on the other hand enzymes such as lipooxygenase & cyclooxygenase catalyses the peroxidation of arachidonic acid leading to formation of thromboxane and prostaglandins. The free radicals formed are highly reactive & can attack DNA or protein in the cell. The cell membranes of all cells are potential sites of attacks by the free radicals. Since PUFA are present as constituent of phospholipids in these membranes.
Presence of polyunsaturated fatty acids (PUFA's) in the phospholipids of the bilayer of biological membranes is the basis of their critical feature of fluidity. Since LP affects the components that impart these properties, it affects the biophysical properties of membranes. LP decreases the membranes & decreases electrical resistance. Also cross linking of membrane components restricts mobility of membrane proteins. Peroxidative attack on PUFAS of a biological membrane will compromise one of its most important functions, its ability to act as barrier. Hence LP causes lysosomes to have a decreased latency i.e. they become fragile or simply “Leaky”. Similarly the leakage of cytosolic enzymes from whole cells e.g. Peroxidative attack on the plasma membrane of hepatocytes causes extensive damage such that molecules as large as enzymes are able to leak out.

**Chain reaction of lipid peroxidation:**

Lipid peroxidation is a chain reaction. It is not simply that OH· can be formed near PUFA & attack it to end the reaction. It is an autocatalysis process. The autooxidation of membrane lipid occurs in three steps.
Initiation phase-

During this phase, the primary event is the production of R or a carbon centered radical by the interaction of a PUFA with a free radical which has sufficient reactivity to extract a hydrogen atom from a reactive methylene group of PUFA.

Propagation Phase-

The carbon centered radical (R) rapidly reacts with molecular oxygen forming peroxyl radical can attack membrane proteins but they are also capable of abstracting hydrogen from adjacent fatty acid side chain in a membrane & to propagate the chain reaction of lipid peroxidation hence a single initiation event.

Oxidation of proteins & nucleic acids

1) Proteins – They are less susceptible than lipids to free radical attack as the propagation of chain reaction is less likely.

L - Fatty acid with carbon centered free radical

LH- Fatty acid

LOO - Lipid peroxide

LOOH – Lipid hydroperoxide
Lipid peroxidation and antioxidant status in encephalopathy in children

THE MECHANISM OF RELEASE OF ARACHIDONIC ACID

Oxidative Stress

Excess ROS / RNS & low antioxidant defence

Damage to biomolecules (Lipid, DNA, Protein)

Lipid peroxidation
(Damage to membrane ion channel, ion transporters)

DNA damage
(Strand breakage Base modification)

Protein damage
(Damage to Receptor, enzyme ion channel Raised Intracellular Ca$^{2+}$)

Raised intracellular Ca$^{2+}$

Cellular damage with release of more radicals

Cell death & tissue damage

ENCEPHALOATHY
Termination phase-

The reaction would process unchecked till a peroxyl radical reacts with another peroxyl radical to form inactive products. If the free radical attack is of high intensity then it may lead to

1) DNA strand breaks or
2) Mutation

Mechanism of brain injury by ROS-

ROS induced oxidative damage can be divided into 2 categories

1) That which occurs at high oxidative challenge &
2) That which occurs at low oxidative challenge.

1) At high oxidative challenge –

Those events which occur with high oxidative challenge are mediated through hydroxyl radical. They include.

A) Lipid peroxidation-
   Leading to cell membrane injury &
B) Protein SH oxidation leading to altered enzyme activity.
C) High oxidative challenge may also lead to damage to DNA strards which in turn may lead to mutation & cancer.
2) **At low oxidative challenge** –

At low oxidative challenge release of endogenous mediators like arachidonic acid metabolites may occur.

**Nitric oxide** ;\(^{27,28,29,101}\)

Nitric oxide (NO') and nitrogen dioxide (NO\(_2\)') contain odd numbers of electrons and are therefore free radicals, whereas nitrous oxide (N\(_2\)O) does not. NO\(_2\)' is a dense brown poisonous gas and a powerful oxidizing agent. NO', on the other hand, is a colourless gas and a weak reducing agent.

It was first recognized as a distinct gas in 1772 by Joseph Priestley, who prepared an iron complex of it. Biological interest in NO' has centered on the observation that the vascular endothelium and other cells in the body produce small amounts of the gas from the amino acid L-arginine. At present NO' is indistinguishable from the vasodilator endothelium derived relaxing factor, which can react with another endogenous free radical, superoxide, to produce a reactive intermediate, peroxynitrite (ONOO'). ONOO' is a powerful oxidant, able to damage many biological molecules, and can decompose at acid pH
to release small amounts of hydroxyl radicals independent of metal catalysis.

\[ \text{ONOO}^- + H^+ \rightarrow \cdot \text{OH} + \text{NO}_2 \]

Nitric oxide is a weak radical produced from L arginine via the enzyme Nitric oxide synthases (NOS) for years. Nitric oxide was just one of the polluting gas produced in car exhausts. But then surprisingly it was found to serve as a chemical signal in many different parts of the body.

NO is a simple heterodiatomic molecule with broad & diverse effects in human biology that have been recognized only recently.

**Synthesis of nitric oxide**

The synthesis of NO occurs in response to a stimulator binding to a receptor on some cells (e.g. Endothelial cells) or to a nerve impulse in neurons. Nitric oxide is synthesized from amino acid L- arginine by a family of enzymes nitric oxide synthase (NOS). Nitric oxide formed in this reaction is free radical in nature having a single unpaired electron.

i) Endothelial NOS (e NOS) Seen in endothelial cells & myocardium.
ii) Neuronal NOS (n NOS) seen in central & peripheral neuron

iii) Inducible NOS (i NOS) is found in macrophages & monocytes

Nitric oxide (NO) is derived from the conversion of L-arginine to L-citrulline by nitric oxide synthase (NOS). There are two physiologically distinct forms of NOS. A constitutive isoform (cNOS) is basally expressed and releases NO transiently after activation by increased intracellular calcium concentrations. In the brain, this isoform is found in vascular endothelial cells (eNOS) and in some neurons (nNOS). A calcium-independent form of NOS is regulated by gene expression. The presence of this inducible NOS(iNOS) has been demonstrated in macrophages, neutrophils, vascular smooth muscle cells, and in subpopulations of glial cells after induction by cytokines and bacterial products. Once expressed iNOS continuously produces high levels of NO that can act as a microbicidal agents or cause cytotoxicity. NO plays a critical role in the regulation of vascular tone and organ blood flow. In the CNS NO produced by each of the three NOS isoforms (eNOS, nNOS, iNOS) has been linked to the regulation of
cerebral blood flow during various physiologic and pathologic states.

CSF concentration of the stable NO metabolite, nitrite, are increased in animal models of bacterial meningitis and in patients with the disease. iNOS is a likely source of NO produced in meningitis, because the enzyme is strongly unregulated in rats with meningitis, albeit almost exclusively in inflammatory and perivascular cells in the subarachnoid space. The role of NO produced during disease states is generally assessed by the effect of NOS inhibitors. NOS inhibitors have produced both beneficial and detrimental effects on neuronal injury in various paradigms of brain damage and studies in meningitis have also yielded conflicting information. Results from several models of meningitis indicate that NO may have harmful effects by contributing to CSF pleocytosis, alteration, of the BBB permeability, intracranial hypertension, brain edema and alteration of cerebral metabolism.

**Chemical properties of nitric oxide**: 

Nitric oxide is a free radical species as it contain an odd number of electrons 15 of which 7 are located in the other shell.
This results in the presence of the last electron in a TT anti-bonding molecular orbital & on over all bond order between the nitrogen & oxygen atoms of 2.5

Nitric oxide has proved to be a ubiquitous signal transduction molecule & mediator of tissue physiology & pathology because of its chemical properties which include relatively low reactivity for free radical species.

Nitric oxide readily crosses biological membranes & can concentrate in hydrophobic compartments being able to participate in chemical reactions both in aqueous & lipid phases. However although being a free radical NO\(^\cdot\) does not readily react with most of organic molecules, which are neither a strong oxidant nor a strong reductant. The one electron reduction of NO\(^\cdot\) to nitroxyl anion (NO\(^{−}\)) occurs with a standard one electron redox potential of + 0.3 8v. However NO\(^\cdot\) rapidly reacts with both organic & oxygen centered radicals to yield a variety of reactive intermediates & with transition metal centers e.g. iron & copper. Nitric oxide can also be oxidized under aerobic conditions to form nitrogen dioxide (NO\(_2\)) an oxidant & nitrating agent.
**Review of Literature**

**Pro-oxidant properties of nitric oxide:**

**A Nitric oxide derived oxidant**

Although under physiological concentrations NO• predominantly elicits antioxidant actions, excess NO• formation observed under different pathological conditions owing to either excessive activation of constitutive nitric oxide synthase (NOS) or expression of inducible nitric oxide synthase (iNOS) can result in the formation of NO• derived oxidant that cause biological damage. It is important emphasize that NO• itself is not an efficient oxidant & its pro-oxidant activity depends in the formation of more reactive intermediates such as NO₂ or peroxynitrite by its reaction with molecular oxygen or O₂• respectively.

The formation of NO₂ from NO• is alternatively slow process in biology because the concentration of NO• is typically in the nummular or submicromolar range & the peculiar mechanism of reaction with molecular oxygen which involves a molecular reaction being second order in NO• (k = 6.2 x10⁻⁵ M⁻² s⁻¹). This reaction may be relevant in the interior of biological membranes where NO• & oxygen can concentrate several folds Nitrogen dioxide can be also formed secondary to the decomposition & target molecule...
reaction of peroxynitrite & heme peroxides dependent oxygen of nitrite.

Nitrogen dioxide is a strong one-electron oxidant \( (E^0 = +0.99 \text{ V}) \) it crosses membranes readily & at low concentration efficiently initiates free radical oxidation reactions, whereas at higher concentration it will further react with most organic radical at near diffusion limited rates to form nitrogen derivatives \((\text{RNO}_2)\).

Nitric oxide reacts at diffusion limited reacts with \( \text{O}_2^- \) to form peroxynitrite \( (L \sim 10^{10} \text{ M}^{-1} \text{s}^{-1}) \) a reactive nitrogen species with strong oxidizing properties. In contrasts to extremely reactive biological oxidants such as the hydroxyl radical \((\text{OH}^-)\) for which the high rate of reaction with most molecules \( (> 10^9 \text{ M}^{-1} \text{s}^{-1}) \) determines that it will react nonspecifically & close to its site of formation of peroxynitrite.

Peroxynitrites reactions are typically much slower & more selective Peroxynitrites biological half-life can be estimated in the range of 10-100ms therefore at a tissue level it could potentially diffuse some distance before enacting target molecule reactions. Mechanisms for membrane diffusion of both the anionic
propagated from of peroxynitrite have been recently revealed.

Peroxynitrite anion (ONOO⁻) is in rapid prolongations equilibrium with peroxynitrous acid (ONOOH) (PK a=6.8) & both species have unique reactivities towards bimolecules relevant biological reactions of peroxynitrite being catalyzed or mediated through transition metal centers or carbon dioxide.

\[
\text{ONOO}^- + \text{H}^+ \rightarrow \text{ONOOH}
\]

\[
\text{ONOOH} + \text{RH} \rightarrow \text{R} + \text{H}_2\text{O} + \text{NO}_2
\]

\[
\text{R} + \text{NO}_2 \rightarrow \text{RNO}_2
\]

Recently it has been shown that different peroxides including myeloperoxidase & eosinophil peroxidase promotes nitration reactions by peroxidase – hydrogen peroxidase dependent oxidation of nitrite presumably through NO₂ formation.

In the central nervous system nitric oxide is a neurotransmitter that underspins several functions including the formation of memory. The synthesis of nitric oxide by vascular endothelium is responsible for the vasodilator tone that is essential for the regulation of blood pressure & also it prevent platelet aggregation. These actions are all mediated by the
activation of soluble guanylate cyclase & consequent increase in the concentration of cyclic Guanosine monophosphate (cGMP) in target cell.

Nitric oxide may play a part in tissue damage for it may be cytostatic & cytotoxic not only for invading micro organisms but also for the cell that produce it & for neighbouring cells. Nitric oxide interacts with oxygen derived radical to generate molecules that could enhance its cytotoxicity. The superoxide & nitric oxide combines to form peroxynitrite. At physiological pH peroxynitrite damage protein directly & decomposes into toxic products that include nitric dioxide gas (NO\textsuperscript{2-}) and hydroxyl radical (OH\textsuperscript{-})

As it has cytotoxic & cytostatic effects & is generated by macrophages it is likely to have role in nonspecific cell immunity. Macrophages generate nitric oxide in response to lipopolysaccharide moiety or interferon.

The toxicity of NO\textsuperscript{-} is primarily ascribed to its near diffusion limited irreversible reaction with superoxide radical to produce potent oxidant peroxynitrite (OO NO\textsuperscript{-}) Since increased oxidant production of superoxide anion often occurs in concert with high
output of NO\(^*\) production, during inflammation & injury the formation of peroxynitrite is promoted in tissues.

The excessive production of peroxynitrite can damage normal tissue. Peroxynitrite can mediate oxidation nitration reaction leading to impaired function, toxicity & alteration in signaling pathways. Protection against peroxynitrite is important for defense of normal tissue. Peroxynitrite superoxidase & nitric oxide can mediate oxidation nitration reaction leading to impaired function, nitration reaction, leading to impaired function toxicity & alteration in signaling pathways. Protection against peroxynitrite especially is important for defense of normal tissue especially during inflammation.

Levels of product of the L-arginine nitric oxide pathway such as nitrite & possibly L-citrus line in biological fluids may become clinical markers for the monitoring of certain pathologic condition & the progression of their treatment.

Studies of cerebral blood flow during meningitis support a critical albeit complex role of NO. In a rat model limited to the first 6 hours of meningitis, scavenging of \(O_2\) by superoxide dismutase and of hydrogen peroxide catalase and inhibition of NO formation
by a nonselective competitive NOS inhibitor all reduced the hyperemia observed early in the disease. In infant rats with group B streptococcal meningitis found that inhibition of iNOS with the relatively selective inhibitor aminoguanidine was detrimental and significantly increased the amount of ischaemia in the brain and the extent of subsequent neuronal injury. Taken together, these studies suggest an important but dynamic role of NO at the level of the cerebral vasculature during meningitis. Early on, this potent vasodilator is responsible for the hyperemia induced by the subarachnoid space inflammation later as cerebral blood flow tends to progressively decline under the influence of vasoconstrictive events such as ROS-induced vascular inflammation NO produced in the vasculature has some protective effect against ischaemia. Its inhibition further pushes the balance between vasoconstrictive and vasodilative elements toward the vasoconstrictive side, with a subsequent increase in cerebral ischaemia and neuronal injury.

Current interest is focussing on the role of peroxynitrite in causing neuronal injury in meningitis\textsuperscript{68}. Peroxynitrite results from the reaction of superoxide and NO and is highly cytotoxic at least
in part by interfering with mitochondrial function and leading to cellular energy depletions. Based on the presence of NOS-positive neurons, which can be activated by increased glutamate stimulation to produce NO, and on the evidence that superoxide is being produced during meningitis, this pathway is an attractive candidate as a late event in the cascade leading to neuronal injury. Preliminary result support the hypothesis that peroxynitrite is produced in the brain parenchyma during meningitis and that the inhibition of its effect on mitochondria is neuroprotective.

**Effects on nitric oxide -**

Nitric oxide activates guanylate cyclase & increases the concentration of CGMP in vascular smooth muscle, neural & ganglial cells.

**Vascular effects :**

The endothelial L- arginine / NO$^*$ pathway is a physiological vasodilator mechanism which influences peripheral vascular resistance & systemic blood pressure. It contributes to homeostasis in several regional vascular beds including the cerebral, pulmonary & coronary circulation.
Platelets & leucocytes:

NO· potently inhibits adhesion & aggregation of platelets, neutrophil, leucocytes & monocytes. The high concentration of haemoglobin in blood probably presents endothelium derived NO· from effective platelets function under normal conditions. Reaction of peroxynitrite exhibit increased platelet aggregation leading towards vascular complications.

Host defense & cytotoxicity –

Cytotoxic &/ or cytostatic effects of NO· are important in non–spectic host defense against numerous pathogens including bacteria, fungi, protozoa, metazoan, parasites & tumor cells. The antiviral effect of interferon is accounted for by induction of NOS. Neutrophils as well as monocytes produce NO· after induction of NOS & some stimuli to NO· biosynthesis also cause neutrophil superoxide anion (O₂) production NO· reacts with O₂ to yield cytotoxic, peroxynitrite anion (ONOO') which can destroy invading organisms but if produced in excess can also damage the host. Other mechanisms of NO· induced cell damage include nitrosylation of nucleic acids & combination with heme containing enzymes including those involved in cell respiration.
Peroxynitrite-

Peroxynitrite is formed by the combination of superoxide with NO·

\[ \text{NO}^\cdot + \text{O}_2 \rightarrow \text{ONOO}^\cdot \]

The above reaction is biologically significant for two reasons. First NO· & O₂ can antagonize each others biological actions. In vivo excess O₂ production in or close to vascular endothelium can cause vasoconstriction which has been suggested to be one factor causing hypertension. Similarly if an injury system is O₂ dependent NO· can protect by reforming O₂. NO· can inhibit lipid peroxidation in some systems by removing chain propagating RO₂ radicals. A second consideration is a consequence of peroxynitrite generation. Addition of ON OO· to cells, tissues or body fluids will lead to its rapid protonation followed by ONOOH dependent depletion of SH- groups & other antioxidants, oxidation of lipids, DNA strand breakage nitration & deamination of DNA bases & formation of aromatic aminoacids residues in proteins. Nitration of tyrosine residues could conceivably lead to enzyme inactivation & interference within signal transduction other protein targets of attack by ONOOH may include glutathione transferase manganese
SOD. Structural proteins such as actin & neurofilament L-prostacyclin synthases & the copper transport protein ceruloplasmin which is attacked by ON OOH to base release of copper ions. Oxidation reaction of peroxynitrite include DNA damage leading to base modification & mutation as well as single & double strand breaks.

Peroxynitrite also cause one or two electron oxidation of sulfahydryls leading to thiol radical formation. Protein tyrosine nitration by peroxynitrite may interfere with phosphorylation dephosphorylation signaling pathway or other proteins function.

At the level of whole organism the reactive chemistry of peroxynitrite can be considered beneficial because of its cytotoxicity to bacteria However excessive production of peroxynitrite can damage normal tissues also. Nitric oxide is therefore likely to have multifaceted role in inflammatory vasodilation & formation of oedema through modulation of sensory nerve ending & leucocyte activity to tissue injury.

In 1996 Kornelisse, R.F. et .al determined the role of NO in bacterial meningitis. Concentrations in serum, CSF, or both of the precursor (L-arginine and degradation products of NO
(nitrate/nitrite) and tumor necrosis factor (TNF-alpha) were measured in 35 patients and 30 controls. CSF nitrate levels were significantly elevated. CSF NO/nitrite levels were significantly elevated in patients. NO/nitrite levels decreased over time. CSF level of NO/nitrite correlated with those or TNF-alpha (r=0.55) and glucose (r=0.43). CSF levels of L-arginine were lowered in patients than in controls. Enhanced NO production might have contributed to anaerobic glycolysis and neurologic damage in bacterial meningitis.

In 1998 Tsukahara H et al examined the role of nitric oxide (NO) in childhood meningitis by measuring the concentration of NO\textsubscript{2} (a stable metabolite of NO) in serial samples of CSF from 11 children with septic and 7 with aseptic meningitis and 26 control patients without meningitis. The mean concentration of NO\textsubscript{2} in the samples obtained during the early stages of septic meningitis but not aseptic meningitis, was significantly higher than in control samples. It indicates that NO production was enhanced in the CSF compartment of children with septic meningitis and supported the hypothesis that NO was involved in the patho-physiology of septic meningitis.
Biologically Important Non Radicals:

Hydrogen Peroxide:

Hydrogen peroxide is toxic to most cells at levels in the 10-100 M range. Several enzymes found in vivo can generate H$_2$O$_2$ including xanthine, urate & D amino acid oxidises. In addition, any biological system that generates O$_2$ will also produce H$_2$O$_2$ by dismutation.

Despite its poor reactivity, H$_2$O$_2$ can be cytotoxic. Some cellular damage by H$_2$O$_2$ is direct, e.g., attacking glyceraldehyde 3-phosphate dehydrogenase. Yet addition of H$_2$O$_2$ to cells frequently lead to lipid, DNA & protein oxidation that cannot be mediated by H$_2$O$_2$ alone. H$_2$O$_2$ can cross cell membrane rapidly & once inside can probably react with iron & possibly copper ions to form much more damage done to DNA in H$_2$O$_2$ treated cells. In addition, the conversion of H$_2$O$_2$ to OH$^-$ can be achieved by ultraviolet light. H$_2$O$_2$ can degrade certain heme proteins to release iron.

In 2002, Levitina E.V. et al carried out a complex prospective clinical and biochemical study in 252 children with prenatal encephalopathy during the first year of their life. The lipid...
Lipid peroxidation and antioxidant status in encephalopathy in children

peroxidation process, antioxidant system lysosomal were studied. Activation of the lipid peroxidation process and lysosomal enzymes, as well as decrease in antioxidant defense level were observed, Membrane destabilization processes in the children with perinatal encephalopathy correlated with a character of hypoxic influence, a degree of severity and clinical manifestations of pathology and disease stage. Clinical and biochemical efficacy of antioxidant mexidol (0.1-0.2 ml/kg/of body weight intravenously for 10 days) and inhibitor of lysosomal enzymes centrically (1000 U/kg intravenously for 3-5 days) in the infants with perinatal central nervous system damages was claimed.

**Hypochlorous Acid:**

Hypochlorous acid, HOCl, is a powerful oxidant, formed in the body by activated neutrophils. The heme-containing enzyme myeloperoxidase in the phagocyte cytoplasm can catalyze the formation of HOCl from H₂O₂ and chloride ions.

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

Candeias et al suggested that HOCl may give rise to hydroxyl radicals by an iron-independent reaction and an iron-dependent reaction
HOCl +O$_2$→ ·OH + Cl$^-$ +O$_2$

HOCl+Fe$^{2+}$ → ·OH +Cl$^-$+Fe$^{3+}$

The hydroxyl radical is the most powerful oxidant formed in biological systems and can readily attack any biological molecule. Hydroxyl radicals can attack polyunsaturated fatty acids to initiate lipid peroxidation.

In 1990, Karmazsin L et al, observed that an autooxidation of bovine-brain homogenate proved to be good modal to determine the antioxidant capacity of sera. It was measured in parallel with the level of ceruloplasim and apotransferrin in sera of 35 healthy children and 20 premature babies. Antioxidant capacity was very low in premature babies. Lipid –peroxidation of bovine- brain homogenate could be inhibited in vitro by exogenous antioxidant supplementation.

In 1996 Lackmann et al found that oxygen radical injury could be a common pathogenic mechanism in several neonatal diseases. To test the hypothesis that overload with ionic iron due to decreased concentrations of iron-oxidizing and iron-binding proteins induces free radical damage in premature asphyxiated newborns suffering periventricular hemorrhage (PVH) blood
plasma of newborn with PVH (n=7) was compared with that of controls (n=10) within the first 12hrs of life. It was observed that decreased transferring saturation in those newborns who later developed PVH. These finding support the theory that iron-catalysed lipid peroxidation of the brain during reoxygenation after periventricular asphyxia may be involved in the pathogenesis of PVH.

In 1997 Ramaekers V.T. et al noticed that the malondialdehyde (MDA) in plasma is regarded as an indicator for increased lipid peroxidation. Measurement for MDA concentrations in plasma were compared among healthy children (n=31). Patients with neurological disorders or epileptic syndromes (n=15) and children with pontocerebellar structural defects (n=31) in group with various neurological disorders or epilepsy, the values were similar with the median value of MDA compared with healthy controls and neurological / epileptic group. The 31 children with pontocerebellar structural defects had significantly increased MDA values with a median value. These findings of increased plasma MDA in the majority of children having pontocerebellar structural defects with increased lipid
lipid peroxidation correlates with prenatal and postnatal pontocerebellar maldevelopment or degeneration.

In 1998, Van Bel F et al\textsuperscript{128} found that infants having free radical induced asphyxia reperfusion injury were given 40mg/kg allopurinol intravenously while 11 infants served as controls. Plasma non-protein bound iron, antioxidative capacity and MDA were measured before 4 hrs between 16 and 20 hrs and at the second and third days of age. Changes in cerebral blood volume and electrocortical brain activity were monitored between 4 and 8, 16 and 20, 58 and 62 and 104 and 110 hrs of age. Six controls and two allopurinol fed infants died after neurological deterioration. No toxic side effects of allopurinol were detected. Non-protein bound iron in the control group showed an initial rise but dropped at day 3. MDA remained stable in the allopurinol group, but increased in the control group at 8 to 16 hrs versus less than 4 hrs. During 4 to 8 hrs, cerebral blood volume of control showed a large drop than cerebral blood volume of allopurinol fed infant from baseline. This study suggested a beneficial effect of allopurinol treatment on free radical formation, cerebral blood volume and electrical brain activity, without toxic side effects.
PRO-OXIDANT : ANTIOXIDANT BALANCE

- Food / drugs
- aerobic
- Metabolism
- Environment
- Radiation
- activated phagocytes
- Reactive Oxygen Species
- Oxidative Stress
- Anti Oxidants
- Endogenous
  dietary source
Antioxidants as Markers of Oxidative Stress:22,75,80,89,99

The term antioxidant is frequently used in the scientific literature but is rarely defined with a strong implication that it refers to chemicals with chain breaking properties such as vitamin E (α-tocopherol) and vitamin C (ascorbic acid). An antioxidant can be defined as any substance that, when present at low concentrations, considerably delays or inhibits oxidation of the substrate 22,75.

Antioxidants can act at several different stages in an oxidative sequence, as illustrated by considering lipid oxidation occurring in cell membranes of lipid-rich products. Antioxidants can act by: (a) removing oxygen or decreasing local oxygen concentrations; (b) removing catalytic metal ions; (c) removing key reactive oxygen species such as superoxide and hydrogen peroxide; (d) scavenging initiating free radicals such as hydroxyl, alkoxy, and peroxy species; (e) breaking the chain of an initiated sequence; or (f) quenching or scavenging singlet oxygen.

Antioxidants inhibiting peroxidation by the mechanisms listed in a, b, d, and f can be called preventive antioxidants. Those
acting by mechanisms are also preventive, but because they are enzymes (e.g., catalase, SOD, and glutathione peroxidase) they are not consumed by the reaction. Chain-breaking antioxidants, singlet oxygen quenchers and metal chelators are consumed while performing their protective functions.

**Cellular Antioxidants:**

Cells have formidable defenses against oxidative damage, many of which may not be readily recognized as antioxidants. For example, antioxidant protection can operate at several different levels within cells by (a) preventing radical formation; (b) intercepting radicals when formed; (c) repairing oxidative damage caused by radicals; (d) increasing the elimination of damaged molecules; and (e) not repairing excessively damaged molecules to minimize the introduction of mutations.

Oxygen is metabolized inside cells where antioxidants evolved to deal speedily and specifically (enzymically) with reduced intermediates of oxygen (Table 2). Enzymes such as the SODs rapidly promote the dismutation of superoxide into hydrogen peroxide and oxygen at a rate considerably faster than it occurs uncatalyzed.
H$_2$O$_2$, a product of the dismutation reaction, can be destroyed by two enzymes, catalase and glutathione peroxidase (GSHPx) [a selenium-containing enzyme requiring glutathione (GSH)].

\[
\begin{align*}
\text{catalase} \\
2 \text{H}_2\text{O}_2 & \rightarrow \text{O}_2 + 2\text{H}_2\text{O} \\
2\text{GSH} & \\
\text{H}_2\text{O}_2 & \rightarrow \text{GSSG} + 2\text{H}_2\text{O} \\
\text{GSHPx} & \\
\end{align*}
\]

(GSSG, oxidized glutathione.)

During normal oxygen metabolism, these enzymes eliminate toxic reduction intermediates of oxygen inside the cell, allowing a small pool of low-molecular mass iron safely to exist for synthesizing DNA and iron-containing proteins, as well as signaling functions. Prevention of radical formation inside cells must have evolved to restrict oxygen toxicity. An example is cytochrome oxidase, the terminal oxidase of the mitochondrial electron transport chain, which, although functioning catalytically, does not release reactive oxygen intermediates from its active center.
Table 2 – Intracellular antioxidants.

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Superoxide dismutase (Cu., Zn, Mn)</td>
</tr>
<tr>
<td>2.</td>
<td>Catalase; contains 4 NADPH molecules (Fe)</td>
</tr>
<tr>
<td>3.</td>
<td>Glutathione peroxidase (Se)</td>
</tr>
</tbody>
</table>

Membrane Antioxidants:

Within the hydrophobic lipid interior of membranes, lipophilic radicals are formed that are different from those seen in the intracellular aqueous milieu. Lipophilic radicals require different types of antioxidants for their removal (Table 3). Vitamin E (α-tocopherol) which is a fat-soluble vitamin, is a poor antioxidant outside a membrane bilayer but is very effective when incorporated into the membrane. An important part of membrane stability and protection is how the membrane is assembled from its lipid components. This structural organization requires that the correct ratios of phospholipids to cholesterol are present and...
that the correct types of phospholipids and their fatty acids are attached.

**Table 3 - Membrane antioxidants**

<table>
<thead>
<tr>
<th></th>
<th>Vitamin E</th>
<th>Lipid soluble, chain – breaking antioxidant</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.</td>
<td>B Carotene</td>
<td>Lipid –soluble radical scavenger and singlet oxygen quencher</td>
</tr>
<tr>
<td>3.</td>
<td>Coenzyme Q</td>
<td>May act as an antioxidant in addition to its major role in energy metabolism</td>
</tr>
</tbody>
</table>

**Extracellular Antioxidants:**

Glutathione peroxidases and SODs have been identified as distinct glycosylated extracellular proteins. By allowing the limited survival of reactive oxygen species such as superoxide and H$_2$O$_2$ in extracellular fluids, the body can utilize these molecules and others such as NO' as messenger, signal or trigger molecules. A key feature of this proposal is that superoxide and H$_2$O$_2$ do not meet with reactive extracellular iron or copper and that extracellular antioxidant protection has evolved to keep iron and copper in poorly or nonreactive forms (Table 4).
The iron transport protein transferrin is usually one-third loaded with iron and keeps the concentration of free iron in plasma effectively nil. Iron bound to transferrin will not participate in radical reactions, and the available iron-binding capacity gives it a powerful antioxidant property towards iron-stimulated radical reactions. Hemoglobin, myoglobin, and heme compounds can accelerate lipid peroxidation. However, plasma contains proteins such as haptoglobins and hemopexin to bind and conserve hemoglobin and heme iron, respectively, and at the same time to greatly diminish their ability to accelerate lipid peroxidation.

The major copper-containing protein of human plasma is ceruloplasmin, unique for its intense azure blue coloration. Apart from its known acute-phase reactant properties, its biological functions remain an enigma. However, it is pointed out that the ferroxidase activity of the protein makes a major contribution to extracellular antioxidant protection against iron driven free-radical reactions.
### Table 4 – Extracellular antioxidants

<p>| | | |</p>
<table>
<thead>
<tr>
<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Transferrin</td>
<td>Binds ferric ions (2 per mole of protein)</td>
</tr>
<tr>
<td>2.</td>
<td>Lactoferrin</td>
<td>Binds ferric ions at lower pH (2 per mole of protein)</td>
</tr>
<tr>
<td>3.</td>
<td>Haptoglobins</td>
<td>Bind hemoglobin</td>
</tr>
<tr>
<td>4.</td>
<td>Hemopexin</td>
<td>Binds heme</td>
</tr>
<tr>
<td>5.</td>
<td>Albumin</td>
<td>Binds copper, heme, and scavenges HOCl</td>
</tr>
<tr>
<td>6.</td>
<td>Ceruloplasmin</td>
<td>Ferroxidase activity – stoichiometric O$_2^-$ Scavenging binds ions (nonspecific) utilizes H$_2$O$_2$ for reoxidation of coppers</td>
</tr>
<tr>
<td>7.</td>
<td>EC-SOD</td>
<td>Removes O$_2$ catalytically</td>
</tr>
<tr>
<td>8.</td>
<td>EC-GSHPx</td>
<td>Removes H$_2$O$_2$ and hydroperoxides Catalytically. Little GSH available in plasma</td>
</tr>
<tr>
<td>9.</td>
<td>Bilirubin</td>
<td>Scavenges peroxyl radicals</td>
</tr>
<tr>
<td>10.</td>
<td>Urate</td>
<td>Radical scavenger and metal binder</td>
</tr>
<tr>
<td>11.</td>
<td>Ascorbic acid</td>
<td>OH$^-$ radical scavenger</td>
</tr>
</tbody>
</table>
The antioxidant enzymes:

Catalase:

Catalase is a common enzyme found in nearly all living organisms which are exposed to oxygen, where it functions to catalyze the decomposition of hydrogen peroxide to water and oxygen. One molecule of catalase can convert millions of molecules of hydrogen peroxide to water & oxygen per second.

Catalase was first noticed as a substance in 1818 when Louis Jacques Thenenzd who discovered $\text{H}_2\text{O}_2$ (Hydrogen peroxide) suggested that its breakdown is caused by a substance.

In 1900, Oscar Loew was the first to give it the name catalase and found its presence in many plants and animals.

In 1937, Catalase from Beef liver was crystallized by James B. Sumner & the 3-D structure was revealed in 1981

Catalase was the first antioxidant enzyme to be characterized and catalyses the two stage conversion of hydrogen peroxide to water and oxygen:

$$\text{Catalase} - \text{Fe(III)} + \text{H}_2\text{O}_2 \rightarrow K_1\text{compound I} + \text{H}_2\text{O}_2$$
Compound I + H₂O₂ → K₂ catalase–Fe(III) + H₂O₂ + O₂

H₂O₂ + H₂O₂ → 2 H₂O + O₂

Thus Hydrogen peroxide is a harmful byproduct of many normal metabolic processes, to prevent damage by H₂O₂ catalase is frequently used by cells to rapidly catalyze, the decomposition of hydrogen peroxide into less reactive gaseous oxygen and water molecules.

Catalase consists of four protein subunits each containing a haem group and a molecule of NADPH. The rate constant for the reactions described above is extremely high (10⁷ M/sec), implying that it is virtually impossible to saturate the enzyme in vivo. Catalase is largely located within cells in peroxisomes, which also contain most of the enzymes capable of generating hydrogen peroxide. The amount of catalase in cytoplasm and other subcellular compartments remains unclear, because peroxisomes are easily ruptured during the manipulation of cells. The greatest activity is present in liver and erythrocytes but some catalase is found in all tissues.
Structure of serum CATALASE

Crystallographic structure of the human SOD1 enzyme (rainbow colored N-terminus = blue, C-terminus = red) complexed with copper (blue-green sphere) and zinc (grey spheres).
Glutathione peroxidase and glutathione reductase:

Glutathione peroxidase catalyze the oxidation of glutathione at the expense of a hydroperoxide, which might be hydrogen peroxide or another species such as a lipid hydroperoxide.

$$\text{ROOH} + 2\text{GSH} \rightarrow \text{GSSG} + \text{H}_2\text{O} + \text{ROH}$$

Other peroxides, including lipid hydroperoxides, can also act as substrates for these enzymes, which might therefore play a role in repairing damage resulting from lipid peroxidation. Glutathione peroxidases require selenium at the active site and deficiency might occur in the presence of severe selenium deficiency.

Superoxide dismutase :\(^{46,78,79,137}\)

The superoxide dismutases catalyse the dismutation of superoxide to hydrogen peroxide:

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

The hydrogen peroxide must then be removed by catalase or glutathione peroxidase, as described above. There are three important forms of superoxide dismutase in mammalian tissues, each with a specific subcellular location and different tissue distribution.
1. Copper zinc superoxide dismutase (CuZn-SOD): CuZnSOD is found in the cytoplasm and organelles of virtually all mammalian cells. It has a molecular mass of approximately 32000 kDa and has two protein subunits, each containing a catalytically active copper and zinc atom.

2. Manganese superoxide dismutase (MnSOD): MnSOD is found in the mitochondria of almost all cells and has a molecular mass of 40000 kDa (125). It consists of four protein subunits, each probably containing a single manganese atom. The amino acid sequence of MnSOD is entirely dissimilar to that of CuZnSOD and it is not inhibited by cyanide, allowing MnSOD activity to be distinguished from that of CuZnSOD in mixtures of the two enzymes.

3. Extracellular superoxide dismutase (ECSOD): EC-SOD was described by Marklund in 1982 and is a secretory copper and zinc containing SOD distinct from the CuZnSOD described above. EC-SOD is synthesised by only a few cell types, including fibroblasts and endothelial cells, and is expressed on the cell surface where it is bound to heparan sulphates. EC-SOD is the major SOD detectable in extracellular fluids.
and is released into the circulation from the surface of vascular endothelium following the injection of heparin. EC-SOD might play a role in the regulation of vascular tone, because endothelial derived relaxing factor (nitric oxide or a closely related compound) is neutralised in the plasma by superoxide.

**The chain breaking antioxidants:**

Whenever a free radical interacts with another molecule, secondary radicals may be generated that can then react with other targets to produce yet more radical species. The classic example of such a chain reaction is lipid peroxidation, and the reaction will continue to propagate until two radicals combine to form a stable product or the radicals are neutralised by a chain breaking antioxidant.

Chain breaking antioxidants are small molecules that can receive an electron from a radical or donate an electron to a radical with the formation of stable byproducts. In general, the charge associated with the presence of an unpaired electron becomes dissociated over the scavenger and the resulting product
will not readily accept an electron from or donate an electron to another molecule, preventing the further propagation of the chain reaction. Such antioxidants can be conveniently divided into aqueous phase and lipid phase antioxidants.

**Lipid phase chain breaking antioxidants:**

These antioxidants scavenge radicals in membranes and lipoprotein particles and are crucial in preventing lipid peroxidation. The most important lipid phase antioxidant is probably vitamin E. Vitamin E occurs in nature in eight different forms, which differ greatly in their degree of biological activity. The tocopherols (α, β, γ and δ) have a chromanol ring and a phytol tail, and differ in the number and position of the methyl groups on the ring. The tocotrienols (α, β, γ and δ) are structurally similar but have unsaturated tails. Both classes of compounds are lipid soluble and have pronounced antioxidant properties. They react more rapidly than polyunsaturated fatty acids with peroxyl radicals and hence act to break the chain reaction of lipid peroxidation.
In addition to its antioxidant role, vitamin E might also have a structural role in stabilizing membranes. Frank vitamin E deficiency is rare in humans, although it might cause haemolysis and might contribute to the peripheral neuropathy that occurs in abetalipoproteinaemia. The absorption, transport and regulation of plasma concentrations of vitamin E in humans has been reviewed by Kayden and Traber. In cell membranes and lipoproteins the essential antioxidant function of vitamin E is to trap peroxyl radicals and to break the chain reaction of lipid peroxidation. Vitamin E will not prevent the initial formation of carbon centred radicals in a lipid rich environment, but does minimise the formation of secondary radicals. α-tocopherol is the most potent antioxidant of the tocopherols and is also the most abundant in humans. It quickly reacts with a peroxyl radical to form a relatively stable tocopheroxyl radical, with the excess charge associated with the extra electron being dispersed across the chromanol ring. This resonance-stabilised radical might subsequently react in one of several ways.

α-Tocopherol might be regenerated by reaction at the aqueous interface with ascorbate or another aqueous phase chain
breaking antioxidant, such as reduced glutathione or urate. Alternatively, two α-tocopheryl radical might combine to form a stable dimer, or the radical may be completely oxidised to form tocopherol quinone.

The carotenoids are a group of lipid soluble antioxidants based around an isoprenoid carbon skeleton. The most important of these is β-carotene, although at least 20 others may be present in membranes and lipoproteins. They are particularly efficient scavengers of singlet oxygen, but can also trap peroxyl radicals at low oxygen pressure with efficacy at least as great as that of α-tocopherol. Because these conditions prevail in many biological tissues, the carotenoids might play a role in preventing in vivo lipid peroxidation. The other important role of certain carotenoids is as precursors of vitamin A (retinol). Vitamin A also has antioxidant properties, which do not, however, show any dependency on oxygen concentration.

The main function of vitamin E in organisms is to prevent the free radical mediated lipid peroxidation i.e. it functions as an antioxidant, this fact was noted very early by investigators that animals fed with oils rich in polyunsaturated fatty acids (which
are highly susceptible for perooxidation), such as cod liver oil, without vitamin E supplementation, developed signs of vitamins E deficiency. These signs were prevented by supplementing codliver oil with tocopherol. Furthermore peroxides were detected in adipose tissues of rats & chicks fed diets deficient in vitamin E, & rich in PUFA.

**Role of vitamin E in peroxidation:**

Vitamin E has been termed as lipid soluble chain breaking antioxidant although this is not the only means by which vitamin E prevents lipid peroxidation. Vitamin E both quenches and reacts with singlet oxygen and thus protects against the peroxidation initiated by this radical. It reacts with superoxide. Also the photon on the hydroxyl group of the chromane ring is readily donated by vitamin E to superoxide and consequently vitamin gets converted into a vitamin E radical. There are many sources of superoxide in vivo and hence this reaction may be of importance.

In vitro model also supports the capability of vitamin E to scavange superoxide radical. It has been found that the superoxide generated by xanthine-xanthine oxidase system oxidizing the tocopherol dispersed in aqueous media with
deoxycholate and the reaction can be inhibited by addition of superoxide dismutase. A water soluble model composed of tocopherol, 6 hydroxy 2578 tetramethyl 2 chroman carboxylic acid a compound where 16 –C isoprenoidonic at C-2 is replaced by COOH group also gets oxidized by xanthine–xanthine oxidase system. However many counteragents also have been put forward with respect to superoxide scavenging function of tocopherol. First the rate constant for reaction between Tocopherol and superoxide being 5.9 x 10.3 is so slow that this reaction is unlikely to be significant in biological system. Second at physiological pH the peroxidation of lysosomes prepared from dimethyl phosphatidylchlorine has shown to be considerably low in radiation induced generation of free radicals when format was induced. Format converts all the radicals into superoxide radical. Third the oxidation of water soluble model compound (mentioned above) may be due to small amount of hydrogen peroxide produced by xanthine–xanthine oxidase sysem. which can give rise to hydroxyl radical. The model compound also does not prevent the reduction of cytochromes by xanthine-xanthine oxidase system which is mediated by superoxide.
Since the discovery of vitamin E in 1992 by Evans and Bishop, its role in human health has been extensively investigated. Vitamin E refers to a group of eight naturally occurring compounds tocopherols & tocotrienols – tocopherol, especially the naturally occurring α-tocopherol has the highest biological activity.

Vitamin E is the major chain breaking antioxidant in body tissues and it is considered the first line of defense against lipid peroxidation protecting cell membranes of an early stage of free radical attack. Unchecked by an antioxidant highly unstable free radicals attack cell constituents particularly those containing polyunsaturated fatty acids, and can damage both the structure and function of cell membranes. Nucleic acids and electron dense regions of proteins also came under attack there is evidence to implicate free radicals in development of degenerative disease and conditions.

Vitamin E is nature’s most effective lipid soluble antioxidant protection of unsaturated fatty acids in cell membranes that are important for membrane function and structure. Increased vitamin E intake may enhance the immune response. Vitamin E
regulates platelet aggregation by inhibiting prostaglandin (thromboxane) production. It also has role in the regulation of protein kinase C (PKC) activation.

The most important fact concerned with α-tocopherol as antioxidant is that it can react with lipid peroxyl and alkoxy radical to form vitamin E radicals which are insufficiently reactive to abstract hydrogen atom from membrane lipids.

Vitamin E radical thus produced is highly stable because the unpaired electron on the oxygen atom can continuously get delocalized from it.

Animal models also provide evidence for the protective function of vitamin E against lipid peroxidation. When animals are made vitamin E deficient the peroxidation process is increased as judged by ethane evolution.

It has been proposed that the chromanol ring of tocopherol is located at the polar surface of membranes and phytol chain interacts with hydro- carbon side chains of membrane lipids. It is further proposed that tocopherol is adjacent to membrane bound enzymes that generate free radical. This localization in the close
vicinity of the surface of free radicals make it possible to scavenge the radical immediately because it is able to move very rapidly through the non polar portion of membrane. In addition to the primary defenses (scavenger enzymes and metal-ion sequestration) secondary defenses are also present. Attached to the hydrophobic structure of α-tocopherol is an ‘OH’ group whose hydrogen atom is easily removed hence peroxyl and alkoxyl radicals generated during lipid peroxidation preferentially combine with the antioxidant instead of with an adjacent fatty acid side chain which therefore terminate the chain reaction hence the term chain breaking antioxidant.

The main function of vitamin E in organisms is to prevent the free radical mediated lipid peroxidation. i.e. it functions as an antioxidant, this fact was noted very early by investigators that animals fed with oils rich in polyunsaturated fatty acids (which are highly susceptible for peroxidation), such as cod liver oil, without vitamin E supplementation, developed signs of vitamins E deficiency. These signs were prevented by supplementing cod liver oil with α-tocopherol. Furthermore peroxides were detected in adipose tissues of rats & chicks fed diets deficient in vitamin E, &
rich in PUFA. Finally it was observed that a variety of synthetic antioxidants such as ethoxiguin and diphenylphenylenediamine (DPPD) with structures quiet unrelated to tocopherol were found to prevent symptoms of vitamin deficiency.

**Vitamin E is the most important natural antioxidant**

Vitamin E appears to be the first line of defense against peroxidation of polyunsaturated fatty acids contained in cellular and subcellular membrane phospholipids. The phospholipids of mitochondria, endoplasmic reticulum and plasma membrane have affinity for tocopherol and the vitamin appears to concentrate at these sites. The tocopherol act as antioxidants breaking free radical chain reaction breaking antioxidant as a result of their ability to transfer a phenolic hydrogen to a peroxyl free radical of a peroxidized polyunsaturated fatty acid. The phenoxy free radical formed may react with vitamin E to regenerate tocopherol or it reacts with a further peroxy free radical so that the side chain are oxidized to the non free radical product.

Vitamin E has been termed as lipid soluble chain breaking antioxidant although this is not the only means by which vitamin E prevents lipid peroxidation. Vitamin E both quenches and reacts
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with singlet oxygen and thus protects against the peroxidation initiated by this radical. It reacts with superoxide also the photon on the hydroxyl group of the chromane ring is readily donated by vitamin E to superoxide and consequently vitamin gets converted into a vitamin E radical. There are many sources of superoxide in vivo and hence this reaction may be of importance.

**Vitamin E and Encephalopathy :**

The preterm infant has a greater requirement of vitamin E than the term infant because of lower tissue store of tocopherol at birth poor absorption of dietary fat and rapid postnatal growth

A recently investigated use of vitamin E is to decrease the risk of intra cranial haemorrhage (ICH) in infants born at gestational wks 32 or less. Free radicals generated during ischaemia and tissue perversion have been implicated in the injury to the capillary endothelium of the brain in sick preterm, making them vulnerable to haemorrhage. It was postulated that the free radical trapping of vitamin E would limit the extent of intracranial haemorrhage. A decrease in severity or incidence of ICH after administration of vitamin E in the early neonatal period is observed.
In 1984 Speer M.E. et al. analysed 134 infants enrolled on a protocol to evaluate the efficacy of intramuscular plus oral Vit E supplementation alone in the treatment of retrolental fibroplasias. All 134 infants received via nasogastric tube, 100 mg /kg/d of vit E daily for at least eight hours of life. 64 patients received in addition intramuscular vit E on days 1, 2, 4 and 6 of life and 70 patients received placebo injection in randomized double blind fashion. In the first week vit-E plasma levels were significantly higher in the 64 patients given intramuscular vit- E. The data suggested that vit-E a natural antioxidant might have played an important role in protecting the CNS microcirculation form the effects of hypoxic /ischaemic injury.

In 1987 Sinha S. et al. studied 231 babies born at less than or equal to 32 wks gestation was enrolled in a randomized controlled trial to assess the efficacy of vit-E in the prevention of periventricular haemorrhage. Daily supplementation with 3 days of life was associated with a rise in plasma vit –E concentration and reduction in hydrogen peroxide and haemolysis of RBC in vitro. Among babies without haemorrhage on entry to the trial (n=210) supplemented babies had a lower frequency of
intraventricular haemorrhage than controls and a lower combined frequency of intraventricular parenchymal haemorrhage on the final ultrasound brain scan this protective effect was observed in both inborn and referred babies but was stronger in the former. Supplementation had no effect on mortality but among survivors fewer supplemented babies than controls had intraventricular or parenchymal haemorrhage possibly. Vitamin E scavenges free radicals generated during ischaemic injury of the subparenchymal region and thereby limits tissue damage and the extent of periventricular haemorrhage on reperfusion.

**Aqueous phase chain breaking antioxidants:**

These antioxidants will directly scavenge radicals present in the aqueous compartment. Qualitatively the most important antioxidant of this type is vitamin C (ascorbate). In humans, ascorbate acts as an essential cofactor for several enzymes catalyzing hydroxylation reactions. In most cases, it provides electrons for enzymes that require prosthetic metal ions in a reduced form to achieve full enzymatic activity. Its best-known role is as a cofactor for prolyl and lysyl hydroxylases in the synthesis of collagen. However, in addition to these well-defined
actions, several other biochemical pathways depend upon the presence of ascorbate. In addition to its role as an enzyme cofactor, the other major function of ascorbate is as a key chain breaking antioxidant in the aqueous phase. Ascorbate has been shown to scavenge superoxide, hydrogen peroxide, the hydroxyl radical, hypochlorous acid, aqueous peroxyl radicals, and singlet oxygen. During its antioxidant action, ascorbate undergoes a two-electron reduction, initially to the semidehydroascorbyl radical and subsequently to dehydroascorbate. The semidehydroascorbyl radical is relatively stable owing to dispersion of the charge associated with the presence of a single electron over the three oxygen atoms, and it can be readily detected by electron spin resonance in body fluids in the presence of increased free radical production. Dehydroascorbate is relatively unstable and hydrolyses readily to diketogulonic acid, which is subsequently broken down to oxalic acid. Two mechanisms have been described by which dehydroascorbate can be reduced back to ascorbate; one is mediated by the selenoenzyme thioredoxin reductase and the other is a non-enzyme mediated reaction that uses reduced glutathione. Dehydroascorbate in plasma is probably rapidly taken up by red blood cells before recycling, so that very little, if
any, dehydroascorbate is present in plasma.

Apart from ascorbate, other antioxidants are also present in plasma in high concentrations. Uric acid efficiently scavenges radicals, being converted in the process to allantoin. Urate might be particularly important in providing protection against certain oxidising agents, such as ozone. Indeed, it has been suggested that the increase in life span that has occurred during human evolution might be partly explained by the protective action provided by uric acid in human plasma. Part of the antioxidant effect of urate might be attributable to the formation of stable non-reactive complexes with iron, but it is also a direct free radical scavenger. Albumin bound bilirubin is also an efficient radical scavenger.

**VITAMIN – C :**

Vitamin C is a water-soluble versatile vitamin. It plays an important role in human health & disease. Vitamin C has become the most controversial vitamin in recent years. Vitamin C is called ascorbic acid(AA) due to its anti ascorbutic properties. Ascorbic acid is a hexose’s derivative and closely resembles monosacchride in structure. The acidic property of vitamin c is due to the enolic
hydroxyl groups. It is a strong reducing agent. Ascorbic acid undergoes oxidation to form dehydroascorbic acid (DHA) which is biologically active. Oxidation of ascorbic acid is rapid in the presence of copper. Hence vitamin C becomes inactive if the food is prepared in copper vessels.

In 1928 Alber szent Gyorgyi isolated a six carbon reducing substance from Ox adrenals, Oranges and cabbage. In 1932 he and CC king showed this substance to be the antiscorbutic principle. Albert Szent Gyorgyi named it ascorbic acid and was awarded the Nobel prize in 1937.

Vitamin C (ascorbic acid, acrobat) is a six-carbon lactrone. Most animals synthesize it from glucose in the liver (mammals) or kidneys (birds and reptiles). Human species is unable to synthesize vitamin C. In human, the gene encoding this enzyme has extensive mutations so that there is no protein product for humans the inability to synthesize ascorbic acid makes this otherwise ubiquitous chemical as a vitamin. Other animals unable to synthesize vit. C usually obtain sufficient amount from their largely plant diet but similar to humans, will rapidly develop scurvy when fed on processed diets in captivity vitamin C is
synthesized by plants from several precursors and is abundant in leaves and in particular the chloroplast. It may play a role in photosynthesis, stress resistance and plant growth and development.

**Nonenzymatic functions of Vitamin C:**

Vitamin C may have nonenzymatic functions owing to its redox potential & free radical intermediate and may be an electron ion or in many intracellular or extracellular reactions. Intracellularly vitamin C might act as an antioxidant to regulate gene expression, mRNA translation or prevent oxidant damage to intracellular proteins. Extracellular Vitamin C might also be protective against oxidant & oxidant mediated damage.

Many studies have described that vitamin C prevents low density lipoprotein (LDL) oxidation in vitro. Although high LDL is a risk factor for atherosclerosis, it is atherogenic only when oxidized. It is possible that antioxidant inhibit LDL oxidation possibly by quenching aqueous free radicals. Ascorbic acid protects LDL from oxidation at concentrations above 50 ml/L.

Another potential protective mechanism is indirect, as Vitamin C can regenerate oxidized alphatocopherol, whether
Vitamin C has these effects in vivo is unknown.

Furthermore, the oxidant itself may not be present at sufficient concentrations for these reactions to occur clinically. Additional effects of extra cellular Vitamin C in arteriosclerosis could be due to its effects on adhesion of monocytes to endothelium or aggregation of platelets & leukocytes. Vitamin C may quench oxidants that leak from activated neutrophils or macrophages, which in turn may damage supporting tissues, such as collagen or surrounding fibroblasts.

Vitamin C may be the primary antioxidant in plasma for quenching aqueous peroxy radicals & lipid peroxidation products. It is preferentially oxidized before other antioxidants in plasma including uric acid tocopherols & bilirubin.

Vitamin C can quench reactive oxygen metabolites in the stomach or duodenum and prevent the formation N-Nitrous compounds that are mutagenic. In normal subjects, the concentration of vitamin C gastric juice is three times higher than that of plasma. These properties make it attractive for the prevention of gastric cancer. Ascorbic acid content is low in the
gastric juice of patients with atrophic gastritis & Helicobacter pylori infection a condition associated with gastric cancer.

**Tissue distribution of Vitamin C:**

Ascorbic acid is widely distributed in the human and animals and is concentrated in many organs. The highest concentrations are found in adrenal and pituitary glands at 30-400mg/1000g of tissue. Liver spleen, pancreas kidney, brain and lens contain 10-50mg/100g. Liver is the largest store of Vitamin C. The choroid plexus actively secretes ascorbate into the cerebrospinal fluid where it is taken up and concentrated by many parts of the brain.

Never the less, these studies indicate the wide range in ascorbic acid tissue concentration and its selective uptake by specific organs. The reason that many of these tissues concentrate Vitamin C is unknown; it is also concentrated by white blood cells. These have been studied extensively owing to their easy availability and a possible link between vitamin C and infection.

**Zinc**

The word Zinc in German means of unknown origin. Its discovery was accidental in fourth century AD while heating
certain type of earth bearing Zinc with copper was producing Brass. In India, it was first produced in 13th century AD. The Scientist Ebener recognized Zinc as discrete element. Zinc has an atomic number 30 & atomic weight 65%. It is bluish white in colour.

The presence of zinc in living organism & its role as an essential nutrient for plants & animals have been recognized ever since it was shown by Reulin to be necessary for growth of Aspergillus Niger. Occurrence of Zinc in biological matters was just described by lechartier & Belany. Presence of zinc in human liver was described by Racult & Breton in the same year (1877). Todd & Hart in 1940 discovered that zinc was an essential nutrient for Rat.


Winder et al (1959) & Valkar et al (1962) reported DNA Synthesis inhibited in Zinc deficiency. This suggests an impaired protein synthesis due to deficiency of zinc, which was confirmed by Somar & under wood (1969).
Zinc is a necessary nutrient, more than 200 zinc dependant enzymes are known many of which are in the liver. In these enzymes zinc may have a regular role or be required for structure or for catalytic activity. Zinc is not readily oxidized or reduced from its usual oxidation state of and is not involves in redox reactions.

Zinc has an important regular function in Fructose 1,6 diphosphatase and has structural catalytic and regular roles in DNA and RNA polymerases. Many of the transcription factors namely DNA finding proteins contain Zinc.

Along with copper zinc is required for activity of superoxide dismutase.

The zinc content of human blood is 8.8mg/dl of which 80-90% is within erythrocytes, mostly in carbonic anhydrase. Zinc binds to hemoglobin, increasing its oxygen affinity.

The total body content of Zinc in hypothetical 70kg man approximates 1.4-2.5gms. The highest concentrations occurs in eyes, hair, male reproductive organs, bone, skin, intermediate levels are present in liver, kidney and muscle. Zinc is present in
higher concentration in RBC’s than in Plasma.

Zinc from animal sources is generally better absorbed than that from plant products. This is partly due to the phytate content of plants. Phytate binding zinc in the intestinal lumen and rendering is unavailable.

Zinc is an essential micronutrient trace element required for growth and development of tissues, normal and enzymatic action on activity of cells.

The metabolic functions of Zinc are largely based on its presence in zinc metalloenzymes, over 70%, which have been identified in various living systems. Important zinc metalloenzymes in human include carbonic anhydrase, alkaline phosphatase, RNA and DNA, polymerizes, thymidine kinase carboxypeptidases and alcohol dehydrogenate a super oxide dismutase.

The Zinc atoms are an integral, firmly bound part of metalloprotein molecule and often are directly involved in the active catalytic site, they contribute also to the structural stability of metalloenzymes.
It has been emphasized that Zinc could act as a biological antioxidant by mechanism including the protection of sulphydryl groups against oxidation and inhibition of the free radical production.

**Interactions between chain breaking antioxidants:**

It is vital to remember that *in vivo* complex interactions between antioxidants are likely to occur. For instance, it is likely that ascorbate will recycle the tocopheryl radical at the aqueous-lipid interface, so regenerating tocopherol. This might be crucial in ensuring that tocopherol concentrations are maintained in lipoproteins and membranes. In a similar manner, glutathione can regenerate ascorbate from dehydroascorbate. A complex interplay is therefore likely to exist between antioxidants, making it difficult to predict how antioxidants will function in vivo. It therefore becomes meaningless to ask which antioxidant is most important: the answer will depend on the circumstances existing in a particular microenvironment at a specific time, and on the nature of the oxidant injury taking place. A second important property of chain breaking antioxidants is their ability to act as pro-oxidants. In certain circumstances, the presence of an antioxidant might
paradoxically lead to increased oxidative damage. For instance, it has been reported that the administration of vitamin C can sometimes lead to an increase in oxidative damage, particularly if iron is also administered. Similarly, it has been clearly shown in vitro that tocopherol might promote LDL oxidation in the absence of an aqueous phase antioxidant such as ascorbate. Whether these reactions are important in vivo is as yet unclear. However, the possibility that antioxidants may have prooxidant effects in vivo must be considered when designing and interpreting the results of clinical trials of antioxidant supplementation.

Reactive oxygen species are constantly produced during normal aerobic metabolism and are safely removed by a variety of biological antioxidants. Antioxidant protection is never 100% efficient; thus, mechanisms of repair are of key importance for survival. When pro-oxidants increase or antioxidants fail, it is referred as oxidative stress, a situation that leads to excessive molecular damage and tissue injury.
Superoxide dismutase, Catalase and Glutathione protect blood cells from oxidative stress and damage\textsuperscript{46}

Several powerful oxidants are produced during the course of metabolism in both blood cells and most other cells of the body. These include superoxide (\(O_2\)) Hydrogen peroxide (\(H_2O_2\)) peroxyl radicals (ROO\(^{•}\)) and Hydroxyl radicals (OH\(^{•}\)) The last is a particularly reactive molecule and can react with proteins, nucleic acids, lipids and other molecules to alter their structure and produce tissue damage.

Superoxide is formed in the red blood cells by the auto-oxidation of hemoglobin to methemoglobin (approximately 3 % of hemoglobin in human red blood cells has been calculated to auto-oxidize per day) in enzymes such as cytochrome p450 reductase and xanthine oxidase when stimulated by contact with bacteria neutrophils exhibit a respiratory burst and produce superoxide in reaction catalyzed by NADPH oxidase.

Superoxide spontaneously dismutases to form \(H_2O_2\) and \(O_2\) however the rate of this same reaction is speeded up tremendously by the action of the enzyme superoxide dismutase.
Hydrogen peroxide is subject to a number of fates. The enzyme catalase present in many types of cells converts it to H$_2$O and O$_2$. Neutrophils possess a unique enzyme, myeloperoxidase that uses H$_2$O$_2$ and adds to produce hypothalamus oxidase. This subject is discussed further below. The selenium containing enzyme glutathion peroxidase will also act on reduced glutathione (GSH) and H$_2$O$_2$ to produce oxidized glutathione (GSSG) and H$_2$O$_2$. This enzyme can also use other peroxides as substrates OH and OH$_{-}$ can be formed from H$_2$O$_2$ in a nonenzymatic reaction catalyzed by Fe$^{++}$ chemical compounds and reactions capable of generating potential toxic oxygen species can be referred to as prooxidants on the other hand, compounds and reactions disposing of these species scavenging them suppressing of their actions are antioxidants and include compounds such as NADPH, GSH, ascorbic acid and vitamin E.

In a normal cell there is an appropriate prooxidant antioxidant balance. However, this balance can be shifted towards the prooxidants when production of oxygen species is increased greatly or when levels of antioxidants are diminished (e.g. by inactivation of enzymes involved in disposal of oxygen species and
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by conditions that cause low levels of the antioxidants mentioned above). This state is called oxidative stress massive or prolonged.

Oxygen species are now thought to play an important role in many type of cellular injury (e.g. resulting from administration of various toxic chemicals or from ischaemia). Some of which can result in cell death. Indirect evidence supporting a role for these species in generating cell injury is provided if administration of an enzyme such as superoxide dismutase or catalase is found to protect against cell injury in the situation under study.

Free radicals of oxygen, having unpaired electron in the outermost cell (superoxide anion, hydroxyl , alkoxyl and peroxyl radicals ) and nonradical oxygen species (Hydrogen peroxide and singlet oxygen ) are highly reactive molecules. These can cause damage to DNA protein and cell membranes leading to oxidative stress to the cell. Free radicals have many physiological roles also e.g. Killing of bacteria in immunocytes during oxidative burst or acting as signaling molecules in many cells. Nitric oxide radicals act as a vasodilator and blood pressure regulator.
The damage can be prevented by antioxidants in the cell (glutathione, vitamin E, superoxide dismutase) or plasma (Bilirubin, uric acid, albumin, transferrin and ceruloplasmin).

Minerals such as selenium, iron, copper, and zinc, vitamins such as vitamins C, E and β carotene and unsaturated fats seem to play an important role in antioxidant systems. The exact role that they play in health and disease is the subject of interest in current research.