METABOLISM

Pathophysiology of Sickle cell anemia:

Sickle cell disease is the genetic blood disorder which is caused by abnormal hemoglobin. The abnormal hemoglobin leads to damaging and reforming red blood cells. Therefore the red blood cells break down causing anemia and because of its ability to transform or turn into sickle cell shaped cell and obstruct the blood vessels the patients will experience the recurrent manifestations of pains and multi-organ ischemic damage (Williamson et al 2007).

Sickle cell disease occurs when the person inherits two abnormal genes, one from each parent. The person is said to have the sickle cell disease. However, if the person inherits one sickle cell gene from one parent and inherits a normal gene from the other parent, then the person is said to be born with sickle cell trait (Muscari et al 2005). The disease is characterized by hemolytic anemia and by three types of crises: painful (vaso-occlusive), sequestration, and aplastic. Complications include splenic infarction and autosplenectomy, stroke, bone infarcts and aseptic necrosis of the femoral head, leg ulcers, priapism, pulmonary hypertension, and renal failure.

The pathogenesis of Sickle cell disease evolve from a central molecular event, the polymerization of sickle hemoglobin. The aggregation of Hb S into fibers depends on the molecules being deoxygenated, it is necessary to have
an understanding of structure – function relationship and assembly of normal Hemoglobin.

Human hemoglobin (Hb A) is globular protein with a diameter of approximately 5.5 nm and a molecule consists of two pairs of unlike globin polypeptide chains (α²β₂). A heme group, ferroprotoporphyrin IX is linked covalently at a specific site to each chain. When heme iron is in the reduced (ferrous) state, it can bind reversibly with gaseous ligands, such as oxygen or carbon mono oxide. In methemoglobin, oxidization of the heme iron atoms prevents binding to these ligands. Such modifications of the hemoglobin cause specific alteration in its color and absorption spectrum.

In developing human erythroid precursors, eight genes direct the synthesis of six structurally different globin polypeptide chains, designated α, β, γ, δ, ε and ζ. The α chain gene is duplicates in humans and localized on chromosomes 16. The β, γ, δ, ε and ζ are arranged in sequential order on chromosomes 11. Alpha chain contain 141 amino acid in linear sequence whereas β (as well as γ, δ, ε) have 146 residues. Approximately 80 percent of hemoglobin in its native state is in the form of an α helix.

Forms of hemoglobin: Hb A1 (α²β₂) found as 98% of adult Hb, Hb A2 (α²δ₂) 1.7-3.5 % of adult Hb, Hb F (α²γ₂) fetal Hb - usually converts to adult HbA 90 d post partum, hereditary persistence of fetal Hb about 2%. 
**Figure 1.** Hemoglobin structure (Source: themedicalbiochemistrypage.org)

**Fig. 1: Normal and sickle haemoglobin**

**Figure 1a.** Hemoglobin structure normal and sickle (Source: Gupta et al 2004)
Biochemical Basis of Sickle Cell Anemia:

Sickle cell anemia is due to the substitution of thymine for adenine in the glutamic acid DNA codon (GAG→GTG), which results, in turn, in substitution of β6 valine for glutamic acid (Jyoti Titus et al 2004). Hemoglobin exists in two conformations, designated the oxy (relaxed, R) and deoxy (tense, T) states. Deoxygenation of hemoglobin shifts this equilibrium toward the T conformation. Molecules of deoxyhemoglobin S have a strong tendency to aggregate, and such aggregation requires the substitution of valine for glutamic acid in the β6 position, since only those hemoglobin variants with this substitution (e.g., S and Harlem) undergo sickling (Harlan & Goldberg 2000). Electron micrographs of deoxygenated sickle hemoglobin show the presence of multiple microtubules consisting of hemoglobin molecules stacked on top of each other. The molecules do not lie directly over one another, so that a helical structure is formed. Fourteen strands of the fiber are organized into pairs, giving rise to a fiber that is 21 nm in diameter. (Steinberg et al 2013). The deoxygenated hemoglobin solution turns into a firm gel. The distorted sickled red cell is the visible end result of this molecular aggregation. Initially there is a rate-limiting nucleation process, a few molecules of sickle hemoglobin must aggregate, forming a “seed” on which aggregation of further molecules occurs rapidly. Thus, the sickling process is characterized by a long delay that is strongly dependent on
temperature and concentration (Burnette 2011). The delay is inversely proportional to approximately the thirtieth power of the hemoglobin concentration. This delay is quite important in protecting the patient from even more dire consequences than might otherwise be anticipated. Even though the oxygen concentration of venous blood is sufficiently low so that at equilibrium about 85 percent of the red cells would contain sickle hemoglobin polymer, kinetic data suggest that about 80 percent of cells are prevented from sickling during their round trip through the circulation because they reach the lungs and become reoxygenated before significant polymerization has occurred. When a cell sickles and unsickles repeatedly, the membrane is affected and the cell becomes irreversibly sickled, it remains so even when the oxygen pressure is increased (Huan Lei, George E Karniadakis 2013). These cells appear to be derived directly from reticulocytes but have a short intravascular life span, and the severity of the hemolytic process is directly related to the number of these cells in a patient’s circulation. However, the relationship between the number of irreversibly sickled cells and the number and severity of painful crises is an inverse one (E.M. Isoa 2009). A polymer forms and lengthens in helical fibres which, grouped together, stiffen, and induce the characteristic SS-RBC shape change, classically in the shape of a sickle. This process needs a certain time to be primed, the so-called “delay time”, which is inversely proportional to the
intracellular concentration of HbS.

Figure 2: Pathophysiology of sickle-cell disease: The roles of HbS polymerisation, hyperviscosity, vaso-occlusion, haemolysis, and endothelial dysfunction are shown. Deoxygenation causes HbS to polymerise, leading to sickled erythrocytes. Vaso-occlusion results from the interaction of sickled erythrocytes with leucocytes and the vascular endothelium. Vaso-occlusion then leads to infarction, haemolysis, and inflammation; inflammation enhances the expression of adhesion molecules, further increasing the tendency of sickled erythrocytes to adhere to the vascular endothelium and to worsen vaso-occlusion. Reperfusion of the ischaemic tissue generates free radicals and oxidative damage. The damaged erythrocytes release free haemoglobin into the plasma, which strongly bind to nitric oxide, causing functional nitric oxide deficiency and contributing to the development of vasculopathy. HbS=sickle haemoglobin. NO=nitric oxide. VCAM=vascular cell-adhesion molecule.
Membrane changes in Sickle cell

The primary defect in sickle cell disease is clearly in the hemoglobin, secondary alterations in red cell metabolism and membrane structure and function. Dysregulation of cation homeostasis resulting from the activation of some ion channels, such as the K-Cl co-transport system and the Ca-dependent K-channel (Gardos channel) in particular, leads to a loss of potassium and cellular dehydration occurs early in the sickling process (Brugnara 2000). Which, in turn, by increasing the intracellular Hb concentration, favours deoxy-HbS polymerization. Hb becomes denatured and hemichromes concentrate at the internal side of the membrane together with proteins of the cytoskeleton, in particular protein band 3. This process comes along with the loss of heme and with the liberation of Fe3+ which promotes the existence of an oxidizing micro environment. Calcium content of sickle cell membranes, particularly of those cells that are irreversibly sickled are increased, because the calcium pump is abnormal in sickle cell disease (Bogdanora et al 2013). The location of the excess calcium appears to be in endocytic vacuoles, so that from a functional point of view its location is extracellular. The normal asymmetry of membrane phospholipids is disrupted with the exposure of anionic phosphatidylserine at the cell surface. Anti-band 3 IgGs accumulate on the protein band 3 aggregates, inducing erythrophagocytosis by macrophages (Stuart et al 2004). All these
membrane changes give rise to the production of microparticles. Macrophages seem to ingest sickle cells more readily than normal cells, and this could be a result of excessive auto-oxidation of membrane components with the acquisition of immunoglobulins on the cell surface38 all these membrane changes give rise to the production of microparticles.

Figure 3: Membrane alterations in the sickle red blood cell. Formation of the deoxy-HbS polymer fibres triggers a whole series of changes of the red blood cell membrane. Ion channels are affected and their dysfunction is responsible for a cellular dehydration which, in a vicious circle, favours deoxy-HbS polymerization. (Source: Odièvre et al 2011)
Oxidative stress and sickle cell disease:

SCD is characterized by a lifelong continuous oxidative stress. Increased generation of free radicals may occur in sickle cells due to instability of hemoglobin S (HbS) results in generation of superoxide (\(O_2^-\)) and hydrogen peroxide (\(H_2O_2\)) (Jeney et al. 2002). A high production rate of reactive oxygen species (ROS) in SCD is caused by factors such as increased intravascular hemolysis, ischemiareperfusion injury, and chronic inflammation (Akhoue et al. 2007).

ROS are produced as the result of intracellular catabolism that requires oxygen as a terminal electron acceptor (oxidant). During this process ROS such as superoxide (\(O_2\)), hydrogen peroxide (\(H_2O_2\)) and hydroxyl radicals (\(OH^-\)) are produced as intermediates, even in healthy individuals (Droge et al. 2002).
Figure 4. Causes and pathophysiologic role of oxidative stress in haemolysis, coagulation, inflammation an endothelial activation and damage resulting in vaso-occlusive painful crises and ischemic organ damage in sickle cell disease.
Role of ROS in SCD:

A. What are free radical?

A free radical (FR) is a molecule or molecular fragment containing an unpaired electron in the valence shell (i.e. radical and capable of existing independently (i.e. free). In 1892 it was established that molecular oxygen has two unpaired electrons in its valence orbit, therefore it is biradical. However because of quantum mechanical restrictions $O_2$ is not extremely reactive (Sen 1995). The two unpaired electrons of oxygen are located in different antibonding orbital and have the same spin quantum number with parallel spins. This electronic arrangement provides the most stable state to the oxygen known as ground state of oxygen (Halliwell et al 1985)

Free radicals damage our body silently and invisibly. Everything in our body is at risk, proteins, lipids, hormones, Cells tissues, genetic code etc. Free radical damage leads to loss of energy, diseases, pain, aging and eventually death. FRs are scientifically proven to cause heart disease, cancer and a variety of degenerative diseases.

Broadly, free radicals may be classified as :-

1. According to type
   a. Inorganic radicals
   b. Organic radicals

2. According to reactivity
a. Reactive Oxygen species (ROS)

b. Transitional metal ions

c. Reactive hydrogen

d. Reactive nitrogen intermediates (nitric oxide & nitrogen dioxide)

Oxygen is a good oxidizing agent and its reduction yields following free radicals and non-radical molecule

<table>
<thead>
<tr>
<th>Free Radicals</th>
<th>Non Radicals</th>
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<tbody>
<tr>
<td>1. Superoxide anion radical (O$_2^-$)</td>
<td>1. Hydrogen peroxide</td>
</tr>
<tr>
<td>2. Hydroxyl radical (OH$^-$)</td>
<td>2. Singlet oxygen</td>
</tr>
<tr>
<td>3. Peroxyl radical (LOO$^.$)</td>
<td>3. Ozone</td>
</tr>
<tr>
<td>4 Hydroperoxyl radical (LOOH)</td>
<td>4. Oxides of nitrogen</td>
</tr>
<tr>
<td>5. Alkoxy radical (LO$^.$)</td>
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**Figure 5.** Balance of ROS and antioxidants. Oxidative stress is the imbalance between the production of ROS and antioxidants. The antioxidant properties of GPX, SOD, and catalase control the production of oxygen species. Abbreviations: GPX, glutathione peroxidase; GSH, reduced glutathione; GSSG, glutathione disulfide; H2O2, hydrogen peroxide; O2, superoxide; _OH, hydroxyl radical; ROS, reactive oxygen species; SOD, superoxide dismutase. (Source: Aslan et al 2000).

**Sources of ROS in SCD**

Source of reactive oxygen species in SCD may be viz.

Blood cell auto oxidation,

Cell free hemoglobin,

RBC Adhesion and Vaso-Occlusion,

Ischemia-reperfusion
**Blood Cell Auto-Oxidation**

The intracellular polymerization of HbS during deoxygenation is the primary pathogenetic event in SCD. Polymerization can transform a normal red blood cell (RBC) into a dense, inflexible blood cell. The rate of polymerization has been shown in vitro to be correlated with the concentration of HbS and with the cell-free heme released after autoxidation. The RBC reoxygenation phase is a major source of free radical production in SCD. During this period, normal RBCs can generate a significant amount of superoxide due to an electron transfer between the heme iron and oxygen. In the presence of oxygen, heme auto-oxidizes inducing methemoglobin and superoxide formation. Although both hemoglobin A (HbA) and HbS blood have a tendency to autoxidize into methemoglobin and superoxide. Unlike HbA, which can counter this reaction to form harmless byproducts, HbS can become overwhelmed by the continual source of superoxide and, via its dismutation, H$_2$O$_2$. The formation of H$_2$O$_2$, when exposed to methemoglobin, decomposes hemoglobin and releases iron. This iron can then react with remaining H$_2$O$_2$ to further produce _OH, the most reactive and harmful of the reactive species. Sickle cells ultimately generate about twofold greater quantities of superoxide, H$_2$O$_2$, and _OH than HbA (Aslan et al 2000)
Cell-free hemoglobin

Under physiological circumstances iron homeostasis is tightly regulated by complex mechanisms in order to avoid cellular injury. As a result of continuous intravascular hemolysis, sickle cell patients have highly increased plasma levels of cell-free hemoglobin. By inactivating nitric oxide (NO), cell-free ferrous hemoglobin reduces the NO bioavailability, limiting the important vasodilatative, anti-thrombotic and anti-inflammatory properties of this molecule. The hydrophobic heme also rapidly intercalates into the plasma membrane of endothelial cells where it releases its iron. This induces endothelial cell activation and damage by catalyzing non-enzymatic generation of ROS (McCord 2004, Papanikolaou et al 2005, Nagababu et al 2008).

**Figure 6.** Degradation of NO. NO is decreased in three ways: through the reaction with \( \text{O}_2^- \) forming ONOO\(_2\) and through its inhibition via the byproducts of hemolysis. Abbreviations: BH4, tetrahydrobiopterin; eNOS, endothelial nitric oxide synthase; NO, nitric oxide; \( \text{O}_2^- \), superoxide; ONOO\(_2\), peroxynitrite. [source: wileyonlinelibrary.com.]
**RBC Adhesion and Vaso-Occlusion**

As in SCD, decreased bioavailability of NO, which can reduce vasodilation, a variety of adhesion molecules expressed on sickled erythrocytes can also impair blood flow. In SCD, there is an increase in adherence to the vessel walls. Activation of vascular endothelial cells and circulating blood cells represent the continual inflammation seen in SCD. Upon activation, circulating white blood cells and platelets express adhesion glycoproteins. Consequently, endothelial dysfunction is modulated by the interaction between blood cells and platelets and the cellular and molecular components in the endothelium. In this context, blood cell adherence to the endothelium can be modulated by factors such as decreased NO bioavailability, hemolysis, ROS, and inflammation. This abnormal interaction involves adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), intracellular adhesion molecule-1 (ICAM-1), E-selectin, and P-selectin, which are overexpressed in SCD. Because of this overexpression, sickled RBCs are at least 2.5 times more likely to adhere to endothelial cells than normal RBCs (Solovey et al 2001, Kato et al 2005, Wood et al 2006).

**Ischemia-reperfusion**

Restoration of oxygen-rich blood flow after an episode of ischemia adds significantly to tissue damage, which is mediated by oxidants generated upon reperfusion and is referred to as ‘reperfusion injury’. Low oxygen tension due
to discontinuation of blood flow induces the generation of hypoxanthine and xanthine oxidase from adenosine triphosphate and xanthine dehydrogenase, respectively. After restoration of oxygen rich blood flow, xanthine oxidase generates superoxide while catalyzing the conversion of xanthine or hypoxanthine to uric acid. Catalyzed by iron, the superoxide radical is ultimately converted to the extremely powerful and damaging hydroxyl radical that is reactive with almost all biological substances (Szocs et al 2004). In SCD, high levels of xantine oxidase are released into the circulation after ischemia-reperfusion injury, especially of hepatocellular tissue, which after diffusing to the endothelium, enhances vascular ROS production and NO scavenging resulting in impaired vascular function (Aslan et al 2001).

Figure 7. Hypoxia/reoxygenation phenomenon. Under conditions of hypoxia, hypoxanthine and XO are generated. During reoxygenation, these two products can be converted into superoxide. Abbreviation: O$_2$ -2, superoxide ( source : Erica et al 2012 )
**Role of Antioxidants in SCD:**
Antioxidants are substances that prevent neutralize, or kill free radical. More specifically antioxidants are vitamins, minerals, coenzymes and herbs that help our body fight and prevent damage from toxins and free radicals.

How do antioxidants work?

Broadly, the possible mechanisms by which antioxidants work are:

- Prevention of formation of ROS.
- Enzymatic interceptions of generation of free radicals.
- Facilitating the repair of damage caused by free radicals.
- Providing (e.g. as a cofactor or by acting to maintain a suitable redox status) a favourable environment for the effective functioning of other antioxidants.

As the oxygen species that are formed in SCD, the protective mechanisms such as antioxidants are decreased. Those that provide enzymatic defense, including SOD, GPX, and catalase, those that scavenge free radicals, such as glutathione, vitamin C, and vitamin E, are most affected.

Types of antioxidant defense:

- **Endogenous** - Speroxide dismutase (SOD), Catalase, GPX, Bipirubun, Uric acid etc.
- **Exogenous** - Vitamin C, Vitamin A, Vitamin E
THE FIRST LINE OF ANTIOXIDANT DEFENSE IN CELLS IS PROVIDED BY ENZYMES-

1. Superoxide Dismutase (SOD)

SOD appears in three forms:

- Cu-Zn-SOD in the cytoplasm with two subunits.
- Mn-SOD in the mitochondrion
- A third extracellular SOD has recently been described which contains copper (Cu SOD)

Mechanism of Action

Most prevalent of all forms ins Cu-Zn SOD. In this ‘Cu’ is the catalytic metal while ‘Zn’ helps to maintain the enzyme structure.

The metals bound to SOD Catalyses the reaction of two superoxide (O$_2^-$) molecules with H$^+$ ions to form H$_2$O$_2$ and O$_2$. this reaction occurs slowly at pH 7.4

\[ 2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \]

But SOD accelerate is by 10,000 times (Guttridge et al 1996)

2. Catalase (CAT).

Mechanism of Action

Catalase removes H$_2$O$_2$ by breaking it down directly into O$_2$

\[ 2 H_2O_2 \rightarrow 2 H_2O + O_2 \]
3. **Glutathione Peroxidase (GPX)**

Mechanism of Action

GPx reduces $\text{H}_2\text{O}_2$ to $\text{H}_2\text{O}$ by oxidizing glutathione

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

Rereduction of the oxidized form of glutathione (GSSG) is then catalysed by glutathione reductase (GR)

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

Selenium dependent glutathione peroxidase also inactivates lipid peroxides within cells. It converts lipid peroxides (LOOH) into water and relatively harmless fatty acid alcohols (LOH), at the expense of reduced glutathione.

$$2\text{GSH} + \text{LOOH} \rightarrow \text{GSSG} + \text{LOH} + \text{H}_2\text{O}$$

**Glutathione Reductase (GR)**

Glutathione reductase belongs to the flavor protein oxidoreductase a family of enzymes that possess a disulphide bond that is alternately oxidized and reduced as part of the catalytic mechanism.

Mechanism of action:

Glutathione reductase uses NADPH to reduce oxidized glutathione in cells.

Glutathione Peroxidase reduces $\text{H}_2\text{O}_2$ to $\text{H}_2\text{O}$ by oxidizing glutathione (GSH). Rereduction of the oxidized form of glutathione (GSSH) is then catalysed by glutathione reductase.

$$\text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow 2\text{GSH} + \text{NADP}^+$$ [Halliwell 1989]
Figure 8: Mechanisms of oxidant production in sickle RBCs. Sickle RBCs, through the auto-oxidation of hemoglobin (Hb)S, produce O$_2^-$, which is metabolized to H$_2$O$_2$ by superoxide dismutase (SOD). H$_2$O$_2$ is then metabolized to O$_2$ and H$_2$O by catalase and GPx. Deficiencies in SOD, catalase, and GPx in sickle RBCs lead to increased O$_2^-$ and H$_2$O$_2$ production. GSSG, oxidized glutathione. (Elizabeth et al 2001)
1. **REDUCED GLUTATHIONE (GSH)**

Reduced glutathione (GSH) a tripeptide, a gamma- glutamyl cysteinyl Glycine. Reduced glutathione is characterized by its relative thiole group and its gamma- glutamyl bond, which makes its resistance to peptidase attack.

**MECHANISM OF ACTION:**

GSH is a major antioxidants produced by the cell, protecting it from free radicals. It plays a critical role in detoxification reaction. It is a specific substrate for GPx, which catalyses reaction of $\text{H}_2\text{O}_2$ to $\text{H}_2\text{O}$ at the expense of reduced glutathione.

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

GSSG is either converted into reduced from by GR or transported out of the cells.

$$\text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow 2\text{GSH} + \text{NADP}^+ \quad \text{[Knapen et al 1998]}$$

**Figure 9.** GSH mechanism in SCD (source: google.com)
EXOGENOUS ANTIOXIDANTS IN SCD

1. Vitamin C

It is the most potent water soluble antioxidant and its concentration in serum is about 50-60µm (4-20mg/l). Humans are unable to synthesize l-ascorbic acid from d-glucose due to the absence of enzyme L-gulacolactone oxidase. Hence, humans must therefore obtain it from dietary sources from citrus fruits, potatoes, tomatoes and green leafy vegetables.

Mechanism of Action

The chemoprotective action of vitamin C is attributed to two of its functions. It is a water soluble chain breaking antioxidant (Kootathep et.al.1991). As an antioxidant it scavenges free radicals and reactive oxygen species. It also prevents formation of carcinogens from precursor compounds.

One important property is its ability to act as a reducing agent with hydrogen potential of +0.08V, making it capable of reducing compounds like molecular oxygen, nitrate, cytochrome a and c. Ascorbate reacts rapidly with O2•⁻ and even more rapidly •OH to give dehydroascorbic acid (DHA). DHA, itself can act as a source of vitamin C.

\[
\text{Ascorbic acid} + 2\text{O}_2\cdot + 2\text{H} \rightarrow \text{H}_2\text{O}_2 + \text{DHA}
\]
Vitamin E

It acts as a lipid-soluble antioxidant in cell membranes, where many of its functions can be provided by synthetic antioxidants, and is important in maintaining the fluidity of cell membranes. It also has a (relatively poorly defined) role in cell signaling. Vitamin E is the generic descriptor for two families of compounds, the tocopherols and the tocotrienols.

Mechanism of action

The main function of vitamin E is as a chain-breaking, free-radical-trapping antioxidant in cell membranes and plasma lipoproteins by reacting with the lipid peroxide radicals formed by peroxidation of polyunsaturated fatty acids.

Figure 11.: Mechanism of action. Tocopherol. (Harper 38th Edition)