REVIEW OF LITERATURE

Red blood cells (RBCs)

Red blood cells are also known as RBCs, red cells, red blood corpuscles, haematids, erythroid cells or erythrocytes (from Greek erythros for "red" and kytos for "hollow vessel", with -cyte translated as "cell" in modern usage). Red blood cells (RBCs), also called erythrocytes, are the most common type of blood cell and the vertebrate organism's principal means of delivering oxygen (O_2) to the body tissues—via blood flow through the circulatory system. RBCs take up oxygen in the lungs or gills and release it into tissues while squeezing through the body's capillaries.

Red blood cells are the product of a differentiation process that starts in the bone marrow where hematopoietic stem cells differentiate into nucleated RBCs. After extrusion of nuclei and degradation of endoplasmic reticulum, mitochondria and other organelles, reticulocytes emerge in the circulation. The rate of generation of RBCs is closely coordinated with their removal by the reticulo-endothelial system\(^{25}\).

The cytoplasm of erythrocytes is rich in hemoglobin, an iron-containing biomolecule that can bind oxygen and is responsible for the red color of the cells. The cell membrane is composed of proteins and lipids, and this structure provides properties essential for physiological cell function such as deformability and stability while traversing the circulatory system and specifically the capillary network.
The Erythrocyte Membrane structure

The red blood cell membrane consists of three basic components: a lipid bilayer, transmembrane (integral) proteins and a cytoskeletal network.

Lipid Bilayer

The lipid bilayer is a semipermeable, incompressible, two dimensional liquid crystal which is asymmetric in composition and separates the cytoplasm, within the cell, from the extracellular medium. Phosphatidylcholine (PC), sphingomyelin and the sterol cholesterol are the dominant extra leaflet components while phosphatidylserine (PS) and phosphatidylethanolamine (PE) are the dominant inner leaflet components \(^{26}\). Interestingly enough there are proteins in the bilayer called flippases which maintain the correct lipid asymmetry.

Transmembrane Proteins

Transmembrane proteins are solutes in a two dimensional fluid, the bilayer, and thus have varying degrees of lateral mobility in the plane of the membrane dynamics. They have a transbilayer domain and either or both an extrafacial domain, which contributes to the
glycocalyx and a cytoplasmic domain. The major transmembrane proteins are glycoproteins, band 3 and glygophorin. Band 3 (90-100 kDa) is a mult spanning ion transport channel and exists in a dimer / tetramer equilibrium. It is structurally important because band 3 tetramers, rather than dimers, tether the bilayer to the skeleton via an interaction between its cytoplasmic domain and ankyrin (215 KDa) which is associated with spectrin. Glycophorins have a single spanning alpha helix and are a general class of proteins which contribute the major portion of glycosylation (sugar) at the extracellular domain. Glycophorin C (GPC) is another bilayer / skeleton tethering point via its interaction with protein 4.1 within the junctional complex. Glycophorin A (GPA) is partially associated with band 3. There are a host of other transmembrane proteins. Aquaporin, as the name suggests, is a water channel protein existing as a homotetramer. Rh is a protein complex thought to associate with band 3. One class of proteins, which are not strictly transbilayer, are Glycosylphosphatidylinositol (GPI)-linked proteins. GPI is a lipid analog which links an extrafacial protein, for example, CD59.

The Cytoskeleton

The cytoskeleton is an irregular hexagonal lattice of polymeric spectrin molecules which are tied together by actin (subunit mass of 43 kDa), 4.1 and other numerous proteins at nodes called junctional complexes. Two isoforms of spectrin, alpha (260 kDa) and beta (225 kDa), form a loosely wound helix. Two alpha-beta helixes are linked end to end to form a single tetramer, which has binding sites for several other proteins, including other spectrin molecules. Ankyrin is itself connected to a transmembrane protein called 'band 3' or anion exchanger protein (90 to 100 kDa). The purpose of band 4.2 (palladin, 72 kDa) may be to stabilize the link between ankyrin and the anion exchanger. Spectrin is also linked to a
transmembrane protein called glycophorin C (25 kDa) by the protein known as 'band 4.1.
The skeleton makes a two dimensional network which is very flexible, compressible with obvious structural importance.\textsuperscript{28}

![Figure 2: Erythrocyte Membrane structure](image)

**Figure 2: Erythrocyte Membrane structure**

**Structure of hemoglobin**

Haemoglobin is found in red blood cells. The haemoglobin molecule is a tetramer consisting of 4 polypeptide chains, known as globins, which are usually: 2 alpha chains that are each 141 amino acids long and 2 beta chains that are each 146 amino acids long, attached to each chain is an iron-containing molecule known as haem.

**Red blood cells metabolism**

The function of the red cell is to mediate the exchange of respiratory gases, oxygen and carbon dioxide, between the lungs and the tissues. The oxygen-transport protein
hemoglobin, accounting for about 90% of the dry weight of the mature cell is of fundamental importance in this process. At rest, we consume about 250 ml of oxygen and produce 200 ml of carbon dioxide per minute. This increases tenfold during exercise. The iron in the heme moiety of hemoglobin must be maintained in the reduced (ferrous) state in order to bind oxygen reversibly, despite exposure to a variety of endogenous and exogenous oxidizing agents. The red cell maintains several metabolic pathways to prevent the action of these oxidizing agents and to reduce the hemoglobin iron if it becomes oxidized. These mechanisms may fail under certain circumstances including as found in red cells of sickle cell disease and thalassemia. Oxidized hemoglobin, methemoglobin is unable to bind oxygen. Known mechanisms for preventing or reversing oxidative denaturation of hemoglobin in the erythrocyte include involvement of enzymes such as methemoglobin reductases, superoxide dismutase, glutathione peroxidase, and catalase \(^\text{29, 30}\). Most of the physiologically important methemoglobin reduction occurs enzymatically but may be reduced nonenzymatically by certain compounds found in erythrocytes, such as ascorbic acid and glutathione. Glutathione is the principal reducing agent in erythrocytes and the essential cofactor in the glutathione peroxidase reaction. Reduced glutathione (GSH) is a tripeptide (glutamyl-cysteinyl-glycine). Two enzymatic reactions are required for the de novo synthesis of glutathione: glutamic acid + cysteine to glutamyl-cysteine followed by reaction with glycine to GSH. In the course of reactions protecting hemoglobin from oxidation, GSH is oxidized, forming oxidized glutathione (GSSG), which consists of two GSH molecules joined by a disulfide linkage, and mixed disulfides with hemoglobin. GSSG rapidly leaves the erythrocyte. Thus, if a continuous supply of GSH is to be maintained, there is need for a system to reduce the oxidized form of glutathione. Such a system is
provided by glutathione reductase, which catalyzes the reduction of GSSG by NADPH, a product of the pentose phosphate pathway. Glutathione reductase also catalyzes the reduction of hemoglobin-glutathione disulfides, yielding GSH and hemoglobin.

Since there are no mitochondria in erythrocytes, these cells must depend on two much less efficient pathways for production of high-energy compounds, the anaerobic glycolytic (Embden-Meyerhof) pathway, which is also known as the hexose monophosphate shunt or the phosphogluconate pathway. Under normal circumstances, about 90% of glucose entering the red cell is metabolized by the anaerobic pathway and 10% by the aerobic pathway. Three important products are formed by the anaerobic glycolytic pathway: NADH, a cofactor in the methemoglobin reductase reaction. ATP, the major high-energy phosphate nucleotide that powers the cation pump; and 2,3-DPG, a regulator of hemoglobin function. For each molecule of glucose that enters the pathway, two molecules of NADH are generated. The yields of ATP and 2,3-DPG vary depending on the activity of the Rapoport-Luebering shunt. The most important product of the pentose phosphate pathway in erythrocytes is reduced nicotinamide-adenine dinucleotide phosphate (NADPH). The red cell lacks the reactions to utilize NADPH for energy; instead, NADPH, by serving as a cofactor in the reduction of oxidized glutathione (GSSG), is a major reducing agent in the cell and the ultimate source of protection against oxidative attack. The maintenance of a reducing state in the red cell is also essential to keep oxidant attack to other red cell (membrane) proteins and lipids in check. In addition ATP is needed for a myriad of active processes in the red cell from maintaining ion transport across the membrane and energy dependent enzyme reactions needed to maintain composition and organization of cytosol and red cell membrane.
Oxidative stress

Oxidative stress reflects an imbalance between the systemic manifestation of reactive oxygen species and a biological system's ability to readily detoxify the reactive intermediates or to repair the resulting damage. Disturbances in the normal redox state of cells can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids, and DNA. Oxidative stress from oxidative metabolism damages cells and that is caused by reactive oxygen species (ROS) generated, e.g. $O_2^-$ (superoxide radical), OH$^-$ (hydroxyl radical) and $H_2O_2$ (hydrogen peroxide).

Reactive oxygen species

The reduction of molecular oxygen ($O_2$) produces superoxide ($\cdot O_2^-$) and is the precursor of most other reactive oxygen species:

- $O_2 + e^- \rightarrow \cdot O_2^-$

Dismutation of superoxide produces hydrogen peroxide ($H_2O_2$)

- $2H^+ + \cdot O_2^- + \cdot O_2^- \rightarrow H_2O_2 + O_2$

Hydrogen peroxide in turn may be partially reduced to hydroxyl radical ($\cdot OH$) or fully reduced to water.

- $H_2O_2 \rightarrow HO^- + \cdot OH$
- $H_2O_2 + H_2O_2 \rightarrow H_2O + H_2O + O$

1. $\cdot O^-2$, superoxide anion - One-electron reduction state of $O_2$, formed in many autoxidation reactions and by the electron transport chain. Rather unreactive but can release Fe2+ from iron-sulfur proteins and ferritin. Undergoes dismutation
to form H$_2$O$_2$ spontaneously or by enzymatic catalysis and is a precursor for metal-catalyzed •OH formation.

2. H$_2$O$_2$, **hydrogen peroxide** - Two-electron reduction state, formed by dismutation of •O$^{-2}$ or by direct reduction of O$_2$. Lipid-soluble and thus able to diffuse across membranes.

3. •OH, **hydroxyl radical** - Three-electron reduction state, formed by Fenton reaction and decomposition of peroxynitrite. Extremely reactive, will attack most cellular components

4. ROOH, **organic hydroperoxide** - Formed by radical reactions with cellular components such as lipids and nucleobases.

5. RO•, **alkoxy and ROO•, peroxy radicals** - Oxygen centred organic radicals. Lipid forms participate in lipid peroxidation reactions. Produced in the presence of oxygen by radical addition to double bonds or hydrogen abstraction.

6. HOCl, **hypochlorous acid** - Formed from H$_2$O$_2$ by myeloperoxidase. Lipid-soluble and highly reactive. Will readily oxidize protein constituents, including thiol groups, amino groups and methionine.

7. ONOO-, **peroxynitrite** - Formed in a rapid reaction between •O$^{-2}$ and NO$’$. Lipid-soluble and similar in reactivity to hypochlorous acid. Protonation forms peroxynitrous acid, which can undergo homolytic cleavage to form hydroxyl radical and nitrogen dioxide.
Phenylhydrazine (PHZ)

Phenylhydrazine hydrochloride (PHZ) was the first hydrazine derivative characterized by Hermann Emil Fischer in 1875. This compound is used worldwide mainly as a chemical intermediate in the pharmaceutical, agrochemical, and chemical industries. PHZ, C6H8N2 (see structural diagram below) has a molecular weight 108; it exists as yellow to pale brown crystals or as a yellowish oily liquid, with a freezing point of 19.6°C and a boiling point of 243.4°C. PHZ metabolism seems to occur via ring hydroxylation and conjugation, excretion is primarily via the urine.

![Chemical structure of PHZ](image)

**Figure 3: Chemical structure of PHZ**

**PHZ in medicine**

PHZ derivatives were used firstly as antipyretics but the toxic action on red blood cells made their use dangerous. For many years phenylhydrazine was used for experimental induction of anaemia in animals until Morawitz and Pratt suggested it as a drug for polycythemia vera, a clonal disorder which is known by a net increase in the total number of erythrocytes in the body. Earlier in the last century, PHZ and phenylhydrazine hydrochloride were administered orally (usually around 100-200 mg/day)
for the treatment of blood disorders. In some cases, treatment was effective; in others, however, the outcome was fatal. PHZ decreases haemoglobin level, red blood cell concentration, and packed cell volume, and impairs erythocyte deformability. It induces reticulocytosis, increases osmotic resistance, free plasma haemoglobin, mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), and erythropoietin levels, and extramedular haematopoiesis in the spleen and liver.

**PHZ and Haemolytic Anaemia**

Phenylhydrazine (PHZ) induces hemolytic anemia to study erythropoietin regenerative response through clinical, pathological, and morphological studies. PHZ absorbed by inhalation, oral and dermal routes causes oxidative stress within erythrocytes resulting in oxidation of oxyhemoglobin to methemoglobin which is subsequently converted into irreversible hemichromes leading to the precipitation of hemoglobin in the form of Heinz bodies. PHZ causes damage in cytoskeletal protein, lipid peroxidation, ATP depletion, cation imbalances, and reduced membrane deformability. All these symptoms are exhibited in hemolytic anemia.

Phenylhydrazine (PHZ) intoxication leads to hemolysis resulting in severe hemolytic anemia and reticulocytosis, and to hepatic and splenic iron overload causing a number of pathophysiological changes, e.g. fatty liver and hepatocyte necrosis. PHZ-induced free iron release, followed by free radical generation, is a likely mechanism of its toxicity. It is known, for example, that PHZ induces oxidative damage to hemoglobin, to membrane phospholipids and proteins in human erythrocytes and it generates free radicals and reactive
oxygen species. PHZ is one of the most potent carcinogens belonging to the hydrazine family of molecules.

Heinz (1890) likewise found that mixing either nucleated (from cold blooded animals) or nonnucleated erythrocytes with PHZ, turned them green-brown. He also discovered that inclusion bodies (now termed Heinz bodies) were formed in erythrocytes exposed to PHZ and that a number of compounds closely related to PHZ, e.g., Nacetylphenylhydrazine, could induce similar effects.

Yeshoda (1942) induced anaemia in rats following a single phenylhydrazine intraperitoneal administration at a dose of 20 mg/kg b.w. (aqueous solution): erythrocyte concentration lowered to about 50% and haemoglobin level to about 60% of normal values in the course of 4 days.

Phenylhydrazine is used for the induction of haemolytic anaemia and the study of its mechanism in many species: rabbit, rat, mouse, calf, chicken, duck, rainbow trout, *Xenopus*, goldfish, and *in vitro* also in both rat and human erythrocytes.

**Mechanism at cellular level**

PHZ increases reactive oxygen species (ROS) and lipid peroxidation, and decreases glutathione (GSH). ROS production was associated with extensive binding of oxidized and denatured haemoglobin to the membrane cytoskeleton. Thus, PHZ-induced haemolytic injury seems to be derived from oxidative alterations to red blood cell proteins rather than to membrane lipids. PHZ induces Heinz body formation and oxidative degradation of spectrin without any crosslinking of membrane proteins; both these findings impair erythrocyte
deformability. Formation of phenyl radicals and the replacement of haem with phenyl-substituted protoporphyrins, causes destabilisation of haemoglobins to induce Heinz bodies and haemolytic anaemia with PHZ.

PHZ treatment increases the transport rates in Na-K pump, Na-H exchange, Na-Li exchange, and K-Cl cotransport in vivo, while a decrease in Na-K pump, Na-H exchange, Na-Li exchange and increase K-Cl cotransport were found in rabbit red cells.

*Terminalia arjuna*

TA is a 20-30 m deciduous tree of the Combretaceae family. It is found in abundance throughout the mountainous area of North India. It is also found in the forests of Sri Lanka, Burma and Mauritius. Remarkably the tree is pest and disease free. It has huge, often buttressed trunk and horizontally spreading branches. The bark is smooth, pinkish-grey from outside and flakes off in large, curved and rather flat pieces. The size of each piece may vary up to 15 cm or more in length, 10 cm in width and 3-10 mm in thickness. The bark has been used in Ayurvedic medicine for over three centuries, primarily as a cardiac tonic 46.

![Figure 4: Different useful parts of *Terminalia arjuna* tree](image)
Constituents of TA bark

TA contains triterpenoids including arjunin, arjunic acid, arjunolic acid, arjungenin, and terminic acid. The bark also contains glycosides, including arjunetin, arjunoside I, arjunoside II, TAphthanoloside and terminoside A; sitosterol; flavonoids including arjunolone, arjunone, bicalein, luteolin, gallic acid, ethyl gallate, quercetin, kempferol, pelargonidin, oligomeric and proanthocyanidins; tannis and minerals.46

Figure 5: TA Bark and Powder

Therapeutic activities of TA: Oxidative stress and TA

The bark of the tree is useful as an anti-ischemic and cardio-protective agent in hypertension and ischemic heart disease (IHD). Its stem bark contains glycosides, large quantities of flavonoids, tannin and minerals.16 Flavonoids have been detected to exert antioxidant, anti-inflammatory and lipid lowering effects while glycosides are cardiotonic,
thus making TA unique amongst currently used medicinal plants. Considering its anti-ischemic activity and other beneficial effects on coronary artery disease (CAD), it becomes essential to determine the mechanism of action of aqueous or alcoholic extract of the bark of TA by which it provides cardio-protection. Clinical evaluation of TA indicates that it can be of benefit in CAD, heart failure, and possibly hypercholesterolemia. It has been found to possess anti-bacterial and anti-mutagenic effects.

Terminalia’s active constituents include tannins, terpenoids, saponins (like arjunic acid, arjunolic acid, arjunogenin, arjunglycosides), flavonoids (like arjunone, arjunolone, luteoline), gallic acid, ellagic acid, oligomeric proanthocyanidines, phytosterols, calcium, magnesium, zinc and copper. The cardio-protective effects of Terminalia are thought to be brought about by the antioxidant potential of several flavonoids and oligomeric proanthocyanidins (viz., condensed tannins), while the positive ionotropic effect may be caused by saponin glycosides. Arjunolic acid possesses free radical scavenging activity and enhance the cellular anti-oxidant capability against arsenic induce cytotoxicity. Arjungenin and glycoside exhibited a moderate free radical scavenging activity while all compounds showed no effect on the superoxide release from polymorphonuclear (PMN) cells. Further, arjunjenin also exhibited greater inhibitory action on the hypochlorous acid production from human neutrophils. A novel napthanol glycoside, arjunanapthanoloside was isolated from the stem bark of TA and its structure was established. This compound showed more potent antioxidant activity than formers and inhibited lipo-polysacharide (LPS) mediated NO production in rat peritoneal macrophages.

Oral treatment of the active constituents of TA at a dose of 50mg/kg body weight for seven days prior to CCl₄ administration significantly restored the antioxidant enzyme
activity and enhanced cardiac intracellular antioxidant activity. Methanolic extract of TA exerted gastro-protective effect on diclofenac sodium-induced gastric ulcer. Bark stem powder of TA when administered to patients of post-myocardial infarction angina and anti-ischemic cardiomyopathy showed significant reduction in angina frequency, improvement in left ventricular ejection fraction and reduction in left ventricular mass. Flavonoid and tannin fractions of the methanolic extract of the bark of this plant is also shown to possess the anti-bacterial activity against the growth of Propionibacterium acnes and Staphylococcus epidermidis for protection from acne vulgaris in human.

Arjunolic acid shows cardio-protection against the damage caused by myocardial necrosis. Hypolipidemic effect of this plant was proved by comparing atherogenic index between hypercholesterolemia rats with or without treatment of this same extract of this plant bark. Ethanolic extract of this bark exhibited protection against isoproteranol (ISO) induced chronic heart failure by enhancing the sensitivity of baroreceptors. The ethanolic extract of the same plants prevented hypercholesteroliemia in rats. Ethanolic extract of TA exhibited antioxidant activity through correction of oxidative stress and validates the traditional use of this plant in diabetic animals. The same ethanolic extract of TA also exerted a protective effect against N-nitrosodiethylamine-induced liver cancer. Experimental studies have revealed that the ethanolic extract of this plant bark can exert positive ionotropic and hypotensive effect, increased coronary artery flow and protects myocardium against ischaemic damage. Ethanolic extract of the bark of this plant protected rabbit heart from ischaemic-reperfusion injury in semi in vitro system by modulating the activities of antioxidant enzymes and heat shock proteins. The identical pattern of protection exerted by the same extract was found in perfused kidney of albino Wister rats during
treatment with Fenton mixture (containing Fe$^{2+}$, H$_2$O$_2$) at 500/kg dose in semi *in vitro* system.

Aqueous extract of this same plant bark protected the heart against severe chronic congestive refractory failure in human. Aqueous extract of the TA bark protects liver and kidney tissues against CCL$_4$–induced oxidative stress probably by increasing antioxidant defense activities. Pre- treatment with aqueous extract of the bark of TA protected male rats from uraemia, that was induced by hydration $^{58}$. Aqueous extract of bark of this plant ameliorated oxidative stress in goat heart mitochondria against Cu-ascorbate induced oxidative stress in *in vitro* system $^{24}$. 