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BIOMARKERS OF OXIDATIVE STRESS IN RED BLOOD CELLS

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Background. Exposure to high concentrations of oxygen radicals, the lack of nucleus and mitochrondria, inability to synthesise new protein and degradation of detoxifying enzymes makes red blood cells (RBCs) uniquely vulnerable to oxidative stress. This review summarizes the changes in biochemical parameters that primarily contribute to alterations in red blood cells during oxidative stress.

Methods. PubMed, Science Direct and Springer online databases and updates from the Indian Council of Medical Research (ICMR).

Results and Conclusion. As one of the first cells to be affected by changes in the redox status of the body, alterations in red blood cells are widely used in first step-diagnoses of a number of pathological conditions. The information presented in this review provides an update on biomarkers of redox balance in red blood cells. These biomarkers may be used for assessment of oxidative stress during human health and disease.

INTRODUCTION

Red blood cells (RBCs) are unique, highly specialized and the most abundant cells in the human organism. Although their primary function is transportation of the respiratory gases, O_2 and CO_2, between lungs and tissues, these circulatory cells are equipped with effective anti-oxidative systems that make them mobile free radical scavengers, providing antioxidant protection not only to themselves but also to other tissues and organs in the body[1,2].

All cells living under aerobic conditions are continuously exposed to a large number of oxidants derived from various endogenous as well as exogenous sources, collectively referred to as Reactive Oxygen Species (ROS). Endogenous sources of ROS include the respiratory chain in the mitochondria, immune reactions, enzymes such as xanthine oxidase and nitric oxide synthase and transition metal mediated oxidation[3]. The diverse range of exogenous sources of ROS encompasses ionizing and non -ionizing radiation, pollutants, natural toxic gases such as ozone, drugs and toxins including oxidizing disinfectants[4,5]. However, a poor diet containing inadequate amounts of nutrients may also indirectly result in oxidative stress which impairs the cellular defense mechanisms[3].

ROS are neutralized by endogenous antioxidants such as reduced glutathione (GSH), α-tocopherol, vitamin C, and other antioxidant enzyme systems. However, a condition of oxidative stress develops when the critical balance between oxidants and antioxidants is disrupted due to depletion of antioxidants and/or excess accumulation of ROS[6].

A certain amount of oxidative stress is useful to the body for growth and cell signaling, but excess levels have deleterious effects on cell components including proteins, lipids and nucleic acids and alter the redox status of the cell. Oxidative stress has been reported to play an important role in the development and progression of a number of human diseases such as diabetes, cancer, cardiovascular disorders, influenza, Down's syndrome, hepatitis, rheumatoid arthritis, neurological diseases, ulcers, pneumonia, cataract, glaucoma and human aging[7-9].

RBCs are highly susceptible to oxidative damage due to the high cell concentration of oxygen and hemoglobin, a powerful promoter of the oxidative process[10]. They are one of the first cells to be affected by adverse conditions. As an oxygen shuttle, the RBCs must continue to perform this essential task while being exposed to a wide range of environments for each vascular circuit and to a variety of xenobiotics across its lifetime. In the laboratory, RBCs are a reliable model for the study of oxidative stress[2]. The present review focuses on red blood cell biomarkers which can be used as measures of oxidative stress.

RBC: PRODUCTION, STRUCTURE AND METABOLISM

Red 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[11]. The mature red cell is biconcave disc shaped with a diameter of 8 micron and thickness of 2 micron[12]. Its shape is determined by membrane proteins, especially the spectrin network but also the lipid bilayer content[11]. RBCs are able to maintain their discoid shape and yet

allow cytoskeletal rearrangements that permit them to pass through capillaries and then normalize their shape without cell fragmentation[13].

RBCs are unable to generate ATP using molecular oxygen because of lack of mitochondria. Glycolysis is the only source of ATP generation in mature RBCs. It has been found that under normal conditions about 90% of total imported glucose is used to generate ATP through glycolysis and the remaining 10% of glucose is directed down the hexose monophosphate shunt. Cells use this alternative pathway to generate reducing equivalents (NADPH) which are used by RBCs primarily to reduce glutathione[12].

Human RBCs have an average life span of 120 ± 20 days. During their lifespan, RBCs are exposed to a large number of stressful situations. On average RBCs pass once a minute through the lungs where it is exposed to oxidative stress. More than once an hour it travels through the kidney medulla where it faces osmotic shock. Erythrocytes have to squeeze through capillaries which are smaller than the cells[14]. Thus, the integrity of erythrocytes is constantly challenged.

The RBC membrane is similar to most animal cell membranes and composed of 19.5% (w/w) of water, 39.5% of proteins, 35.1% of lipids and 5.8% of carbohydrates[13]. Both types of proteins, intrinsic as well as extrinsic, and membrane lipids are susceptible to oxidative modification. The mature red cell, by virtue of its specialization, is simultaneously restricted in its ability to respond to oxidative stress since it cannot synthesize new protein or replace irreversibly damaged cellular components[12]. The resultant preservation of membrane structure and function is essential for maintaining membrane fluidity and flexibility as well as ionic balance between the intracellular and extracellular compartments.

A number of *in vitro/ in vivo* studies have shown that several RBCs parameters are negatively affected by increased oxidative stress including inactivation of membrane bound receptors and enzymes[7], ionic parameters[15], increase in oxidation of glutathione (GSH), proteins and lipids[16,17]. Owing to its importance, any abnormality in the red blood cell has major consequences.

ALTERATIONS IN RBC DURING OXIDATIVE STRESS

Hemoglobin oxidation

Hemoglobin is the major protein in RBCs, densely packed in cytoplasm and constitutes about 90% of the dry weight of the red cell[12]. Despite an effective antioxidant system, the ferrous iron of hemoglobin is exposed continuously to high concentrations of oxygen and undergoes slow oxidation to methemoglobin (metHb). MetHb is unable to bind or carry oxygen. Under normal conditions, the level of metHb in RBCs is maintained at less than 1% of total hemoglobin. However in high stress conditions, it increases many fold[2]. The oxidation of hemoglobin also causes the formation of disulfide cross-links between adjacent globin chains, ultimately distorting the primary structure of hemoglobin which eventually leads to visible precipitates known as Heinz bodies. At limited oxidative insult, these aggregates of membrane bound denatured protein can be excised by reticulo-endothelial macrophages but greater degrees of oxidation ultimately result in hemolysis of RBCs, if unchecked[12]. In the hyperglycemic condition, there is oxidative binding of glucose to hemoglobin to form glycated hemoglobin (HbA1c). The HbA1c level is an indicator of the close relation between protein oxidation and glycation in type 2 diabetic patients[8,18]. Kostolanska et al. showed that both glycative and oxidative stress were increased in a poor glycemic control diabetic group compared with controls and this contributed to the development of diabetic complications[19].

Oxidation of membrane proteins

Besides hemoglobin, ROS critically affects other proteins in RBCs since they are easy targets for ROS. The cytoskeleton part of the red cell membrane is composed of several proteins including spectrin, ankyrin, actin, and protein 4.1, forming a quasi-two-dimensional meshwork under the lipid bilayer[10]. Spectrin and actin are the two main structural proteins that form a sub-membrane cytoskeletal meshwork responsible for the viscoelastic properties of the red cell membrane. The cytoskeleton of RBCs is bound to the RBCs membrane through high-affinity protein-protein and protein–lipid interactions[11].

ROS can lead to oxidation of amino acid residue side chains, formation of protein-protein cross-linkages, and oxidation of the protein backbone resulting in protein fragmentation and generation of many protein oxidation products[20]. Oxidative attack on the polypeptide backbone is initiated by an •OH-dependent abstraction of the α-hydrogen atom of an amino acid residue to form a carbon-centered radical. On the other hand, the generation of alkoxyl radicals sets the stage for cleavage of the peptide bonds although peptide bond cleavage can also occur as a result of ROS attack on glutamyl, aspartyl, and prolyl side chains[21].

Cysteine and methionine residues are particularly sensitive to oxidation by almost all forms of ROS. Formation of protein carbonyls occurs by oxidative modification of proteins either by the α-amidation pathway or by oxidation of glutamyl side chains which leads to formation of a peptide in which the N-terminal amino acid is blocked by an α-ketoacyl derivative. However, direct oxidation of lysine, arginine, proline and threonine residues may also generate carbonyl derivatives[2,21]. Among many protein oxidation products such as branched-chain amino acids, advanced oxidation protein products and lipofuscin , protein carbonyls are considered generic markers of damage to proteins by ROS in oxidative stressed situations because of their stability and relatively early formation[22,23].

Although ROS damage all types of cell components damage to proteins is the most harmful. Oxidation of amino acid residues at active sites of an enzyme can lead to their inactivation. Considerable evidence indicates that the maintenance of protein redox status is of fundamental importance for cell function. For this reason, structural

changes in proteins are considered a priori to be among the molecular processes leading to pathological complications[18]. Torres-Ramos et al.[24] reported that RBC membrane damage and decreased band 3 phospho-tyrosine phosphatase activity may be markers of chronic obstructive pulmonary disease progression. Alterations in RBC proteins have been found in aging, diabetes and a number of neurodegenerative conditions[5,25].

Membrane lipids

RBCs have a plasma membrane rich in polyunsaturated fatty acid (PUFA) chains. Thus they are highly susceptible to oxidation. The RBC membrane consists of two domains, cytoskeleton and lipid bilayer. The phospholipids are asymmetrically dispersed in the bilayer and cholesterol is distributed evenly throughout the lipid domain[10]. The presence of cholesterol allows flexibility and provides stability to the membrane. The cell membrane contains proteins and glycoproteins embedded in the lipid bilayer. The RBC membrane is composed of 60% phospholipids, essentially phosphatidylcholine, phosphatidylethanolamine, sphingomyelin and phosphatidylserine. Non-sterified cholesterol represents about 30% of the lipids making the RBC composition, and the remaining 10% are glycolipids[11].

Lipids are considered crucial in the maintenance of the RBCs shape. Even minimal changes in the surface area may lead to morphological and functional abnormalities. Red cell membrane fluidity is also important for proper red cell function. ROS attack causes lipid peroxidation and formation of an array of unwanted products[5].

Malondialdehyde (MDA) is a major lipid peroxidation product. Several hypotheses in relation to the in *vivo* formation of MDA have been proposed. Pryor and Stanley[26] proposed that oxidized lipids are able to produce MDA as a decomposition product and the mechanism is thought to involve formation of prostaglandins, like endoperoxides from PUFA with two or more double bonds. In 1990, Esterbauer and Cheeseman suggested an alternative mechanism for the generation of MDA, based on successive hydroperoxide formation and β cleavage of PUFA which is the main source of MDA generation *in vivo*[27]. However other minor sources of MDA formation also exist such as byproducts of free radical generation by ionizing radiation and biosynthesis of prostaglandins[3]. Measurement of thiobarbituric acid reactive substances (TBARS) is also a useful parameter for the assessment of the extent of lipid-peroxidation. During oxidative stress and in many pathological events, enhanced levels of TBARS have been reported[3,5]. A study performed by Altuntas *et al*., on patients with schizophrenia reports an increased level of lipid peroxidation evidenced by enhanced MDA content[28].Very recently Skoumalová et al. show a significant increase in the end-products of lipid peroxidation, called lipofuscin-like pigments (LFP) in the red blood cells of Alzheimer's disease (AD) patients compared to controls[29]. Since at present there are no reliable diagnostic biomarkers of AD in the blood, to measure these specific products of lipid peroxidation in the red cells of AD patients may be a reliable marker of this pathological condition. The involvement of lipid peroxidation in alterations involving the red cells during oxidative stress is diagrammatically presented in (Fig. 1).

Antioxidative non-enzymatic defense systems

High levels of cytoplasmic antioxidants both enzymatic and non-enzymatic are found in RBCs. Both types of antioxidants work against ROS in order to protect the RBCs from the deleterious effects of oxidative stress. Reduced glutathione (GSH), ascorbic acid (ASC), α-tocopherol and other thiols groups are the major endogenous non-enzymatic antioxidants.

α-Tocopherol serves as potent scavenger of peroxyl radicals to protect PUFA present in RBC membranes against peroxidation[30]. A decreased level of α-tocopherol has been reported in many clinical conditions involving oxidative stress[31]. ASC is the primary cellular antioxidant, it protects the membrane and other hydrophobic compartments from oxidative damage by regenerating the antioxidant form of α-tocopherol[32]. Reduced levels of ASC and decrease in α-tocopherol have been reported in diabetic as well as in dropsy patients[31,33,34].

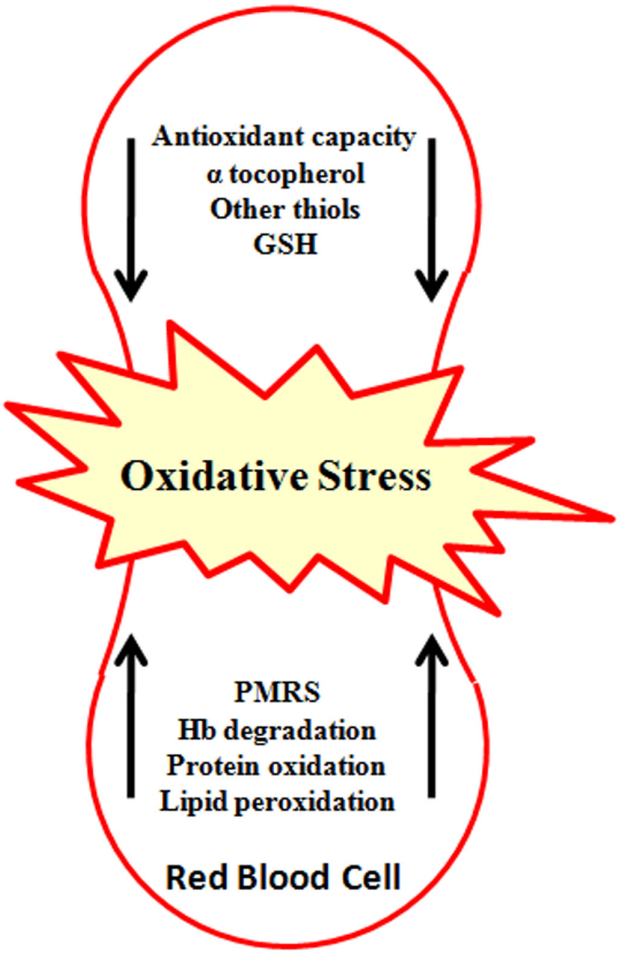

Fig. 1. Alterations in red blood cells during condition of oxidative stress. Several parameters of red cells get negatively affected as evidenced by decreased/increased level of markers of oxidative stress. GSH-Reduced glutathione; PMRS- Plasma Membrane Redox System; Hb-Hemoglobin.

GSH provides first degree protection against oxidants in cells and is considered a molecule with diverse functions. GSH levels in cells reflect the dynamic equilibrium between its synthesis and utilization. The primary role of GSH in erythrocytes is to maintain hemoglobin in its native form in cells at higher concentrations. Peroxidation of the RBCs membrane is known to cause impaired membrane integrity. Reduced GSH also plays a role in the maintenance of membrane thiol groups[17].

Besides a direct role in protection against oxidative stress, GSH is also functions as cofactor for a number of protective enzymes, such as GSH peroxidase and GSH-S-transferases[35]. ROS induced oxidative stress causes GSH depletion as a result of which the overall redox system of the cell is altered. Under oxidative conditions, GSH is reversibly oxidized to glutathione disulfide (GSSG) that can pass through red cell membrane due to oxidative stress induced membrane damage. This mechanism may be responsible for the decreased red cell GSH levels in oxidative stress condition[36].

It is assumed that the capacity of GSH to neutralize oxidants is due to the nucleophilicity of the thiol group and its high reaction rate with oxidants[37]. It has also been observed that cells with low levels of GSH are more sensitive to the effects of irradiation and stress than cells with normal levels of GSH[5]. Depleted levels of GSH have been reported in a number of pathological conditions such as Parkinson's disease, liver disease, cystic fibrosis, sickle cell anemia, AIDS, cancer, heart attack, stroke, diabetes and aging (Fig. 2)(ref.[38]).

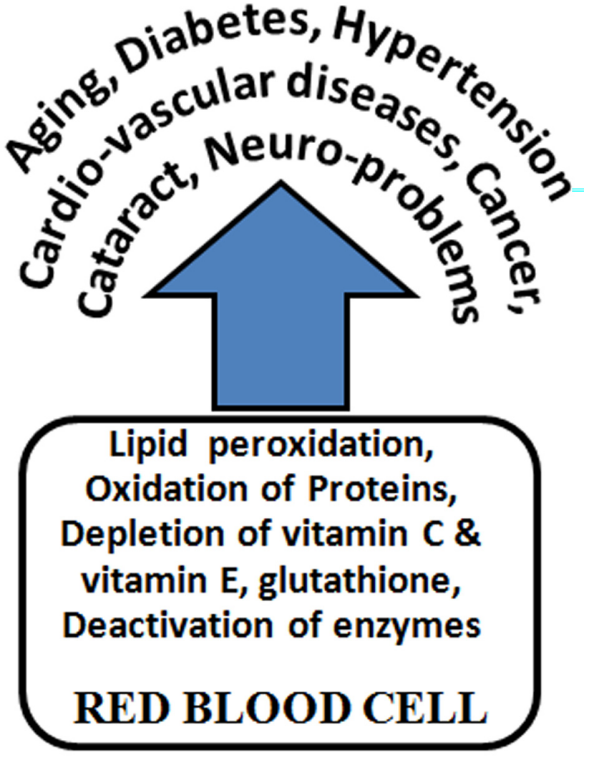

Fig. 2. Oxidation of biomolecules followed by deactivation of enzymes in red blood cells lead to development of various human diseases.

Antioxidative enzymatic defense systems

To cope with the injurious potential of ROS, RBCs possess effective antioxidative enzyme systems that neutralize the reactive oxidants into non/less reactive species. Superoxide dismutase (SOD), glutathione peroxidase (GPx), glutathione reductase (GR) and catalase (CAT) are some of the main endogenous enzymatic defense systems in all aerobic cells. They give protection by directly scavenging superoxide radicals and hydrogen peroxide. Superoxide dismutase is the best known enzymatic antioxidant. This enzyme is located in the cytoplasm of the cell and catalyzes the dismutation of superoxide radical ($\bullet O_2$) in to hydrogen peroxide (H_2O_2). Although H_2O_2 is not a radical, after its formation it rapidly converts to the $\bullet OH$ radical by Fenton reaction. Catalase breaks H_2O_2 to water and molecular oxygen. However glutathione peroxidases reduce H_2O_2 to water by oxidizing two molecules of glutathione into GSSG[5,39,40].

CAT supercede GPx because of their ability to degrade H_2O_2 without consuming cellular reducing equivalents (NADPH) which is an energy efficient way of removing H_2O_2. This mechanism of action results in a net gain of reducing equivalents. The altered activities of these antioxidative enzymatic systems have been documented during oxidative stress condition, making them reliable markers of oxidative stress. Ample experimental reports exist in support of the elevation of oxidative stress during imbalance in redox hemodynamics leading to the development of a number of pathological changes in RBCs[5,28,33]. Altered activities of SOD, GPx and CAT have been reported in aging populations[41,42]. A significant decrease in the activities of enzymatic antioxidant defense system has also been reported in diabetic patients[43].

Plasma membrane redox system

Most eukaryotic cells including RBCs have a plasma membrane redox system (PMRS) that transfers electrons from intracellular substrates to extracellular electron acceptors which may be NADH or/and vitamin C[44]. Several vital functions of PMRS have been proposed including maintenance of homeostasis and recycling of ascorbic acid[32,45,46]. Since ascorbic acid is a primary antioxidant in the body and interestingly humans are unable to biosynthesize it, due to lack of functional enzyme, L-gulonolactone oxidase, the role of PMRS becomes vital[47].

It has been reported that PMRS is a compensatory/protective mechanism that operates to maintain the ascorbate level in plasma and thereby minimize oxidative stress[32]. An altered PMRS status has been found in the RBCs during condition of oxidative stress[5]. The activity of PMRS has been shown to be elevated in RBCs from patients with diabetic nephropathy[48], type 2 diabetes mellitus[49] and during aging in humans[5,44].

CONCLUSION

Oxygen radicals and other reactive species, generated as by-products of aerobic metabolism and through exposure to various environmental toxicants, cause oxidative

stress when the antioxidant defense of the body is overwhelmed. Oxidative stress is now known to be a major factor in the development of most pathological events associated with neurological disorders, CHD, diabetes, cancer, and human aging. RBCs are prone to oxidative stress being the first cells in the body to be exposed to stressful stimuli. The information presented in this review provides an overview on biomarkers of redox balance in RBCs. These biomarkers may be used for assessment of oxidative stress during human health and disease.

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