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Quantitative analysis for estimating injury effects of metal-catalyzed oxidation on human erythrocytes

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ABSTRACT

Objective: To investigate whether human erythrocyte proteins were susceptible to oxidative effects of pharmacological doses of iron and whether resulting damages affect their structure.
Methods: Conformational changes in hemoglobin were indicated by spectrophotometric analysis from 300 to 650 nm. Carbonyl assay was performed for estimating the protein oxidation in erythrocytes. Oxidative injury in erythrocyte membrane was investigated by evaluation of the structural changes in cytoskeleton proteins by sodium dodecyl sulfate polyacrylamide gel electrophoresis in presence of 2-mercaptoethanol and staining with Coomassie briliant blue G-250.
Results: A significant increase in absorbance at 630 nm represented the formation of methemoglobin. Increase in absorbance at 340 nm was indicated by interaction between globin and heme group, which predicted for low oxygen affinity. A decrease in absorbance at 420 nm showed the conversion of oxygen hemoglobin to methemoglobin and significant decrease in oxygen hemoglobin concentration. There was marked elevation in hemichrome compared with control group. Of interest, a positive correlation was observed between iron concentration and hemoglobin absorbance at 340 nm. Elevated levels of carbonyl groups confirmed the oxidative damage to erythrocyte proteins. Analysis of membrane proteins using sodium dodecyl sulfate polyacrylamide gel electrophoresis, showed molecular aggregates in the range of 150 to 180 kDa and slight decrease in the intensity of α-spectrin band.
Conclusions: It is possible to predict the situation of everyone who exposed to oxidant agent via a simple blood analysis. In this way, contents of oxidative products in blood samples would be assessed by this method.

1. Introduction

Iron deficiency anemia is one of the most deleterious diseases in the world and affects women more often than men (9.9% versus 7.8%). Approximately one billion people suffer from this disease. Moderate degree of iron deficiency anemia affected approximately 610 million people or 8.8% of the world's population[1]. Treatment of iron deficiency anemia involves oral supplementation with ≤195 mg per day of elemental iron as ferrous sulfate, -fumarate or -gluconate[2].

There have been always controversies over the supplementation of iron given to patients. During iron administration, reactive oxygen species (ROS) may be produced through Fenton and Haber-Weiss reactions. ROS is involved in aging and pathologic conditions such as cardiovascular diseases[3-6], chronic vascular diseases[7], kidney diseases[8], diabetes[9,10], muscle atrophy[11], neurodegeneration[12], schizophrenia[13], fetal distress[14], aging eye[15], chronic lung diseases[16] and Friedreich ataxia[17]. ROS is neutralized by exogenous and endogenous antioxidants such as glutathione, nicotinamide adenine dinucleotide phosphate, antioxidative enzymes and vitamins. Oxidative stress occurs when the balance between pro-oxidant molecules and antioxidant systems is disrupted.

Several studies have shown that iron participates in site-specific metal-catalyzed reactions[18]. The most consistent

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finding in patients with iron overload is hepatotoxicity which followed by cardiac disease, endocrine abnormalities, arthropathy, osteoporosis and skin pigmentation[19]. The explanation of iron-mediated oxidation in human erythrocytes is necessary for the understanding of the role of ROS in pathologic conditions of iron overload.

Erythrocytes are highly susceptible to oxidative stress due to the high concentration of oxygen and hemoglobin (Hb), a promoter of the oxidative process[20]. Oxidative reactions may oxidize Hb and have deleterious consequences on the structure and function of Hb. Oxidation of Hb causes the formation of disulfide cross-links between globin chains and leads to Heinz bodies.

On the other hand, ROS can lead to oxidation of amino acid residues and fragmentation of cytoskeleton protein, which disrupts viscoelastic properties of the red cell membrane.

Therefore, administration of iron in anaemic patients may result in complicated conditions and precise monitoring of structural changes in erythrocyte proteins, could confirm oxidative stress and its consequences, especially hematologic abnormalities.

Many of the methods used to assess oxidative stress are based on ease of use and simplicity rather than specificity in clinical diagnosis. Furthermore, many of the techniques employed to estimate oxidative stress do not yield precise and reliable results. This makes comparison between the data obtained in different researches very difficult. For these reasons, there is a critical need for validated techniques that yield accurate and quantitative data. Application of such techniques may provide valuable data on the importance of oxidative stress on structure and function of biomolecules such as enzymes, proteins and lipids.

The objective of this study was to establish a sensitive and quantitative algorithm of methods for estimating oxidative stress in erythrocytes. In the present study, authors determined whether human erythrocyte proteins were susceptible to oxidative effects of clinically prescribed amounts of iron and whether resulting damages affect their structure. Assuming that following iron administration, blood level of iron is suddenly increasing; erythrocytes were exposed to different doses of iron to investigate structural changes related to oxidative stress in erythrocyte proteins. To investigate erythrocyte oxidative damage, methemoglobin (metHb), hemichrome, carbonyl groups, spectral analysis of Hb and electrophoretic profile of membrane proteins were studied.

2. Materials and methods

2.1. Preparation of whole blood and isolation of blood cell fractions

Anticoagulated (citrate or heparin treated) whole blood was collected from healthy volunteers who had given informed consent with the approval of the Human Ethics Committee in Arak University of Medical Sciences and in accordance with the Declaration of Helsinki. Plasma was prepared by centrifugation (4000 r/min) for 10 min at 4 °C. Erythrocytes were washed three times with cold isotonic potassium phosphate buffer (100 mmol/L, pH 6.8, 4 °C) and buffy coat was removed carefully.

2.2. Induction of oxidative stress in erythrocytes

Erythrocytes were incubated in isotonic phosphate buffer pH 7.4, containing 10 mmol/L $MgCl_2$, 90 mmol/L KCl, 25 mmol/L ascorbate and 0.1-18.18 μmol/L $FeCl_3$ for 4-24 h at 37 °C with shaking under aerobic condition. In this MCO system ferric ions convert to ferrous ions which result in ROS production. The ascorbate and $FeCl_3$ solutions were freshly prepared. In control groups the above solution was made without ascorbate and $FeCl_3$. After incubation, the oxidation reaction was stopped by separating the erythrocytes with centrifugation at 4 °C and washing with cold isotonic phosphate buffer pH 7.4 for 3 times. All samples put on ice and Hb was estimated by Drabkin method. Hb concentration was adjusted to 4×10^{-5} mol/L with phosphate buffer and absorbance of each sample was measured at 340, 420, 542, 560, 577 and 630 nm. One hundred micro liter of each sample was divided into aliquots and stored at −80 °C for further use.

2.3. Determination of Hb derivatives concentrations

Micromolar concentrations of oxygen Hb (oxy-Hb), metHb and hemichrome were calculated according to the following equations[21]:

$$\text{Oxy-Hb}=-89A_{560}+119\ A_{577}-39A_{630}$$

$$\text{MetHb}=-55A_{560}+28A_{577}+307A_{630}$$

$$\text{Hemichrome}=233A_{560}-133A_{577}-114A_{630}$$

Where A is the absorbance of Hb at indicated wavelengths.

2.4. Investigating the spectral changes

The sum of oxy-Hb and metHb concentrations should be remain constant during the course of incubation. If not, the reaction is more complex and optical densities at more wavelengths including 340, 420 and 542 nm must be considered[22].

2.5. Carbonyl assay in erythrocyte proteins

Protein samples were suspended in acidic solution of 2,4-dinitrophenylhydrazine (10 mmol/L in HCl, 2 mmol/L) and incubated at room temperature. Derivatized proteins were precipitated with trichloroacetic acid (20% w/v) and dissolved in guanidinium hydrochloride (6 mol/L, pH 2.3). Carbonyl