SPECTROSCOPIC STUDY ON OXIDATIVE REACTIONS OF NORMAL AND PATHOGENIC HEMOGLOBIN MOLECULES

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Abstract. Hemoglobin (Hb) of normal erythrocytes undergoes oxidative reactions with constant rate. In anemia, hemoglobin is exposed to oxidative denaturation. Oxidation of oxy-hemoglobin gives O^{-}_{2} and met-hemoglobin (met-Hb). If the globin structure of hemoglobin molecule is destabilized, met-hemoglobin can be converted to hemichrome. Reaction of hemoglobin with radical or oxidant-generating system and structure denaturation can be followed by spectral analysis. In this study, different oxidation products of hemoglobin were measured in three types of anemia; severe iron deficiency, glucose-6-phosphate dehydrogenase deficiency and β-thalassemia. The results showed that hemoglobin suffers from an increase in the auto-oxidation rate in case of anemia followed by an enhancement in both met-hemoglobin and hemichrome levels and a decrease in the oxy-hemoglobin concentration.

Key words: anemia, hemoglobin, absorbance, oxidation.

INTRODUCTION

Hemoglobin readily undergoes oxidation and reduction and it can act as a source of sink of free radicals. Auto-oxidation of hem groups produces O^{-}_{2} and, indirectly, hydrogen peroxide (H_2O_2) [23]. The reaction of excess H_2O_2 with oxy-hemoglobin (oxy-Hb) forms ferryl species as an intermediate, but the final product is met-hemoglobin [22]. Hemoglobin also interacts with redox-active xenobiotic and metabolites, forming the xenobiotic radical and initiates a series of reactions that generate other radicals and oxidant species and often result in oxidative denaturation of Hb [3]. Oxidation of oxy-Hb gives O^{-}_{2} and met-Hb. If the globin structure is destabilized, met-Hb converts to hemichrome (the main constituent of Heinz bodies), in which either the distal histidine or an external ligand occupies the sixth coordination position of the ferric hem [17]. Hyperhemolysis was observed during evolution of uncomplicated acute painful episodes in some patients with

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anemia. Presence of free plasma Hb, consequent to hyperhemolysis, reduces nitric oxide bioavailability, promotes endothelial dysfunction, and contributes to the development of pulmonary hypertension and vasoocclusion [19] Absorbance studies quickly denote if the colored hem and globin groups are present in normal form or take irregular forms of Hb as a result of oxidative reactions [24]. Hb has a characteristic absorption spectrum. The conformational substrates of Hb molecule appeared from its normal absorption spectrum indicating the stabilization intensity of Hb as a macromolecule. Thus, any change in the characteristic absorption spectrum of Hb reflects the changes in the spin state of iron hem. Absorbance of this spin state band gives a clear report about hem-hem interaction and consequently its affinity to O_2 and its delivery to tissue. Studying Hb oxidation by spectral analysis is advisable to record spectral change over the range of 200–700 nm and attempt to recognize features which are characteristic of different products [2, 9]**.**

This work is interested in investigating the oxidative products of hemoglobin by spectral analysis in three types of anemia: iron deficiency, glucose-6-phosphate dehydrogenase and β-thalassemia.

MATERIALS AND METHODS

The specimens investigated in this work were classified into four groups:

(1) Healthy (control) group: thirty normal healthy persons (15 males and 15 females, 5–35 years old) were investigated. All of the control subjects had normal hematological data with normal Hb typing and had no family history of blood diseases.

(2) Severe iron deficiency anemia patients group*:* thirty patients (15 males and 15 females, 10–35 years old) with severe iron deficiency anemia were reported. None of these patients had received chelate therapy prior to this study.

(3) Glucose-6-phosphate dehydrogenase (G6PD) deficiency patients group: twenty-five patients (20 males and 5 females, 10–35 years old) with G6PD enzyme deficiency anemia were analyzed.

(4) β-thalassemia patients group: thirty patients (16 males and 14 females, 5–25 years old) with β-thalassemia major were recruited from the division of hematology. None of these patients had received chelate therapy and non-splenectomized prior to this study.

The blood samples were collected from the patients of the hematology divisions, Suez Canal and Al-Azhar Universities hospitals where the medical states of the patients were reported. Venous blood samples (each of 5 ml) were collected into tubes containing ethylene diamine tetra-acetic acid (EDTA) (100 μL/mL blood sample). For each sample, complete blood picture was measured for evaluating Hb concentration by automatic cell counter. Hemoglobin was then extracted [11] and an appropriate dilution with phosphate buffer of concentration 0.1 M and pH 7.4,

was used to adjust the concentration of Hb to be 4×10^{-5} M to define the different oxidation products of Hb, and to 2.6×10^{-4} M to study globin structure and dynamic motion of Hb [9, 24].

There are absorption bands characteristic for absorption spectra of Hb molecule: (1) choleglobin is indicated by an increase in absorbance at 700 nm, (2) met-Hb gives a shoulder at 630 nm, (3) ferryl Hb is distinguished from met-Hb by its lack of a shoulder at 630 nm, (4) oxy-Hb at 577 and 542 nm bands, (5) hemichrome gives a shallower trough at 560 nm, (6) hem-hem interaction band (soret band) at 420 nm, (7) globin-hem interaction band at 340 nm and (8) constant globin at 275 nm which is characteristic to dynamic motion of Hb [2, 19].

To calculate the micromolar concentrations of oxy-hemoglobin (oxy-Hb), met-hemoglobin (met-Hb) and hemichrome the following equations were considered [20]:

$$Oxy-Hb = 119A_{577} - 39A_{630} - 89A_{560} \tag{1}$$

$$Met-Hb = 28A_{577} + 307A_{630} - 55A_{560} \tag{2}$$

$$Hemichrome = -133A_{577} - 114A_{630} + 233A_{560} \tag{3}$$

where A_{577} , A_{630} and A_{560} are the absorbance at 577, 630 and 560 nm respectively.

RESULTS

Table 1 shows the mean values of hemoglobin (of concentration 4×10^{-5}M) absorbance at different absorption band peaks at 630, 577, 542, 420 and 340 nm. The table also shows the ratio between the absorbance at 577 and 420 nm. It can be noticed that there was a significant increase ($P < 0.001$) in the absorbance values at 630 nm, which corresponds to the met-hemoglobin in case of the studied types of anemia if compared to the control value. Table 1 also shows significant decreases in the absorbance values at 577 and 542 nm which correspond to the oxy-hemoglobin in case of anemia if compared to control. Concerning the Soret band (420 nm), which corresponds to the hem-hem interaction, there was a significant decrease in the absorbance values in G6DP deficiency and β-thalassemia while there was no significant change in iron deficiency if also compared to control. At 420 nm, the globin-hem interaction band, there was also a gradual significant decrease in each of iron deficiency, G6PD deficiency and β-thalassemia if compared to control. A decrease in the ratio (A_{577}/A_{542}) which indicates the conversion of oxy-hemoglobin to met-hemoglobin can be also observed in the three types of anemia. These changes in the absorbance values at the mentioned peaks can be also illustrated in Fig. 1.

Table 1

Mean values ± S.D. of Hb (4×10^{-5} M) absorbance at different bands of three types of anemia

Bands / Groups	630 nm	577 nm	542 nm	577/542 nm	420 nm	340 nm
Control group	0.0103± 0.0005	0.547± 0.028	0.534± 0.028	11.025± 0.006	3.61± 0.42	1.29± 0.22
Iron deficiency anemia	0.0299± 0.0012	0.456± 0.018	0.465± 0.016	0.098± 0.009	3.71± 0.39	0.93± 0.25
G6PD deficiency anemia	0.0164± 0.0009	0.498± 0.016	0.491± 0.016	11.014± 0.005	2.97± 0.23	0.88± 0.12
β- thalassemia major	0.0359± 0.0019	0.349± 0.018	0.368± 0.017	0.948± 0.009	2.67± 0.17	0.74± 0.14

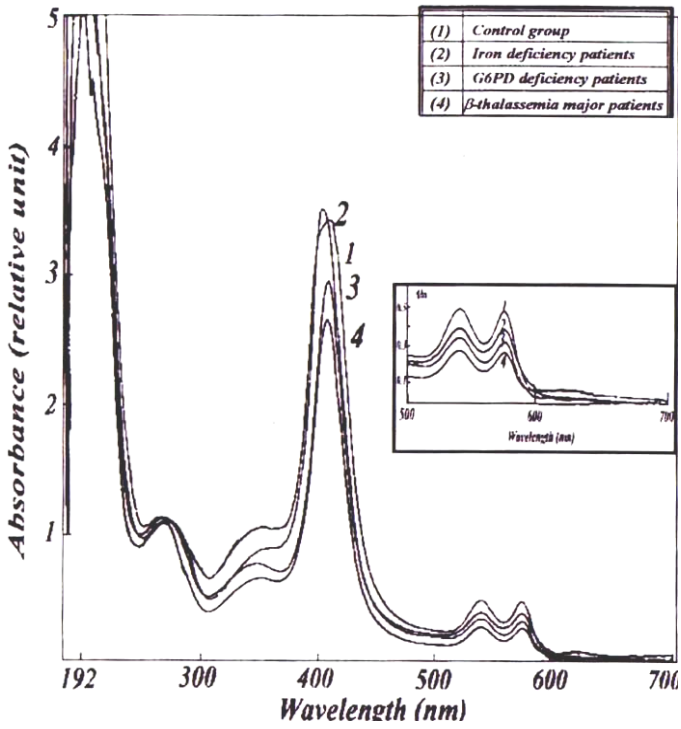

Fig. 1. The absorption spectra of Hb molecules, of concentration 40 μM in phosphate buffer at pH 7.4, at constant globin (275 nm) for control and anemic groups.

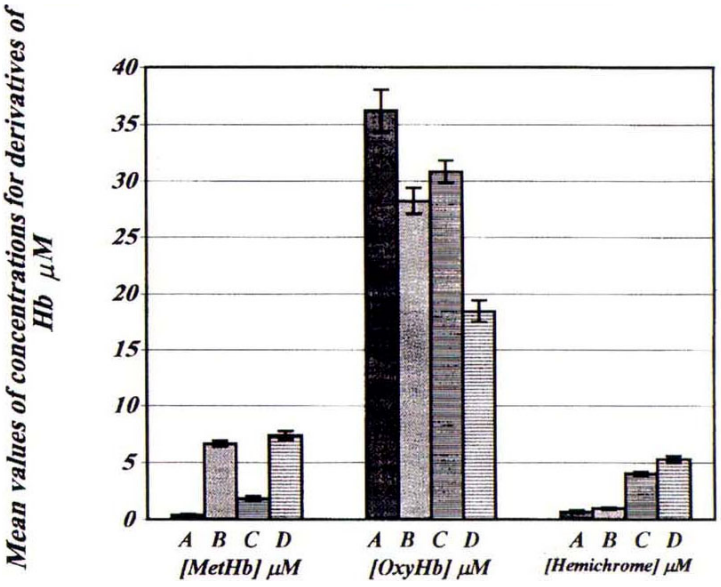

Fig. 2. The mean values of Hb derivatives concentration for the control and anemic groups.

Table 2

Mean values ± S.D. of the concentration (μM) of some Hb derivatives in control and anemia cases

Groups \ Hb derivatives	Met-Hb	Oxy-Hb	Hemichrome
Control group	0.38± 0.05	36018± 1.82	0.70± 0.08
Iron deficiency anemia	6.63± 0.26	28.23± 1.13	0.99± 0.08
G6PD deficiency anemia	1.80± 0.17	30.83± 1.01	4.02± 0.13
β-thalassemia major	7.35± 0.41	18.39± 0.98	5.24± 0.29

Table 2 shows the mean values of some Hb derivatives concentrations in the studied types of anemia as compared to control. A significant increase in the concentrations of both met-hemoglobin and hemichrome with a decrease in the concentration of oxy-hemoglobin can be observed in the investigated cases of anemia if compared to the control case. The change in the concentrations of hemoglobin derivatives can be also shown in Fig. 2. The mean values of the hemoglobin (2.6×10^{-4} M) absorbance at 275 nm, which corresponds to the constant globin, and the half soret band width in cases of anemia compared to control are shown in Table 3. Significant increases in the absorbance values at 275 nm in both iron deficiency and β-thalassemia were detected. No significant change could be

detected in case of G6PD deficiency. The table also demonstrates a significant increase and a decrease in the half Soret band width in β-thalassemia and iron deficiency cases, respectively. There was also no significant change observed in case of G6PD deficiency if compared to control. These changes can be also seen in Fig. 3.

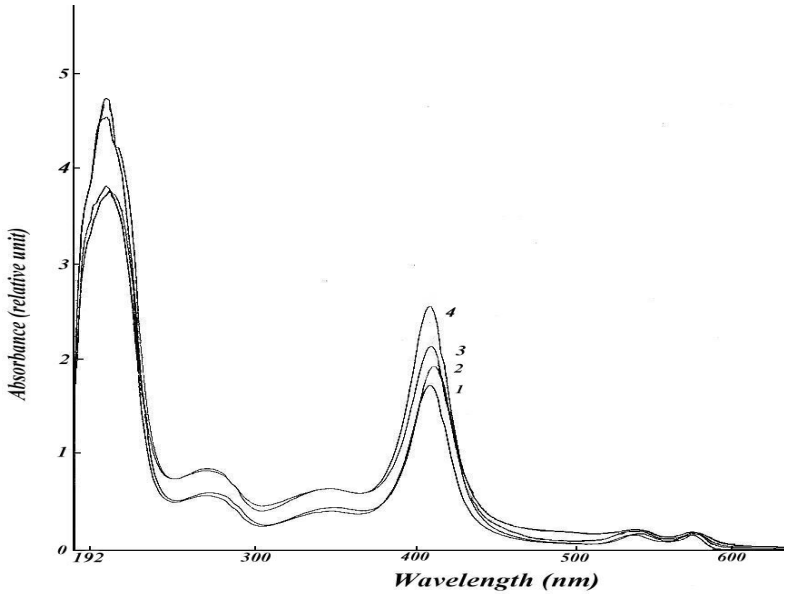

Fig. 3. The absorption spectra of Hb molecules, of concentration 26 μM in phosphate buffer at pH 7.4, at constant hem (577 nm) for control and anemic groups.

DISCUSSION

The oxidative damage of erythrocytes in case of thalassemia was reported to be related to the generation of free radicals by an excess of denaturated β-globin chains, intracellular iron overloaded and a low concentration of normal hemoglobin [21]. In G6PD deficiency anemia, diminished G6PD activity impairs the ability to form the reducing compounds of nicotine amide adenine dinucleotide phosphate (NADPH) and glutathione (GSH) which are essential in the detoxification of free radicals and peroxides formed within the cell [7]**.** In iron deficiency anemia, elevation in HbA_2 values was striking [13]**.** HbA_2 exhibited increased susceptibility to auto-oxidation to met-Hb [18]**.** Beta-thalassemia trait was suggested to be indicated by a mean corpuscular volume of less than 80 fL and/or a mean corpuscular hemoglobin level of less than 27 pg and a hemoglobin A_2 level of more than 3.2% [6]. Antioxidant enzyme levels also decrease in

children with iron deficiency anemia. Consequently, the presence of this oxidative stress results in oxidation of oxy-Hb to met-Hb [4]. Met-Hb formation and recycling are accompanied by release of superoxide. As a result, oxidation to met-Hb enhances, more importantly, there is a rapid conversion of met-Hb to hemichrome [10]**.** Generally, the degree of conversion of Hb to met-Hb depends on the degree of unfolding and leads to the existence of a hybrid of low spin and high spin states as it appears from the shift toward shorter wavelength of the soret band. An increase in the content of parallel and antiparallel beta-sheets and changes in the tyrosine ring absorption band were also observed in the structure of hemoglobin from β-thalassemia patients [12]**.** Changes in the spin state of Hb during disorders of a red blood cell including thalassemia and sickle cell anemia were detected [25]

In anemia, acidity increases directly in proportion to the concentration of 2,3 DPG augments causing a decrease in O_2-affinity of Hb [14]**.** The ratio of zinc protoprophyrine/hem (ZPP/H) is elevated in common hereditary Hb disorders that mimic the microcytic anemia of iron deficiency, low concentration of Hb and high levels of met-Hb as inactive Hb cause impaired to rate of O_2–transport and decrease of O_2-affinity. So, the concentration of oxy-Hb decreases [5]. Hemoglobin polymerization was shown to be inhibited by the formation of asymmetric Hb in anemia [15]**.**

Absorbance ratio (A_{577}/A_{542}) and absorbance of Soret band at 420 nm markedly decreased concomitantly with the appearance of a new band at 630 nm in anemia groups. This decrement means the conversion of oxy-Hb to met-Hb. The degree of conversion depends on hem-hem interaction at 577 nm and the elevation in absorbance of the band detected at 630 nm. Free oxygen radicals and hydrogen peroxide (H_2O_2) induced damage to the protein globule of Hb associated with cleavage of the prophyrin ring and hem loss [20]**.** Decrement in absorbance band at 340 nm refers to the stretching or weakness of the non-covalent bond between histidine of globin and hem iron.

Proteins are dynamic systems and their motions are essential to their function [8]. Enhancement in the absorbance at 275 nm, as an indication for this abnormal motion, reflects its deviation from a normal structure and function, depending on the degree of globin unfolding and random motion of the Hb molecule under the different degrees of oxidative stress. Globin chain imbalance was observed to be an important determinant of the clinical severity of anemia, especially thalassemia [1]. The band at 275 nm, as it was reported [8], is assigned to $\pi \rightarrow \pi^*$ transitions in the aromatic amino acids**.**

Elevation in the half soret band width and shifting towards shorter wavelength indicate the stretching of iron and nitrogen bonds in prophyrin ring and the imbalance between protein and hem in the Hb molecule. These are due to coupling with a new band at 630 nm [9]**.** The broad absorption centered at 192 nm is a characteristic of the amid linkage in polypeptide and increases the absorbance

in this region by the free amino acid. The absorption spectra in the region around 190 nm are due to certain side chains, especially the aromatic ones [16]**.**

CONCLUSION

In anemia, hemoglobin undergoes an enhancement of autoxidation rate followed by increase in the concentration of met-hemoglobin and hemichrome. This indicates a case of hemoglobin oxidation instead of its oxygenation leading to a state of hypoxia. These changes clear up the hem iron exists in a hybrid of low and high spin states.

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