Biochemical and biophysical characterization of hemoglobin of erythrocytes from patients suffering from iron deficiency anemia: A pilot study

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ABSTRACT

Background: Iron deficiency anemia (IDA) is a substantial global health concern, affecting nearly 1 billion individuals worldwide, and women are the more vulnerable due to their various reproductive phases. This study examines physicochemical aspects of erythrocytes in IDA women, especially in reproductive-age women in the North 24 Parganas district of West Bengal. Methods: Fifteen IDA women and eight healthy women aged 25 to 45 years were selected, and only ten volunteers fulfilled the IDA criteria. Observations: Scanning electron microscopic study revealed the formation of echinocytes, indicating asymmetry in the lipid bilayer of the erythrocyte membrane. Although native-PAGE did not show any remarkable change in hemoglobin structure, absorption spectra indicated structural abnormality in the globin chain of hemoglobin. Increased methemoglobin (metHb) formation rate (higher co-oxidation rate) and increased metHb content in the membrane-bound hemoglobin are clear indications of the alteration of physicochemical property of hemoglobin, which may affect erythrocyte life span in IDA. Conclusion: The hemoglobin of IDA individuals is not only quantitatively low, but the quality is also compromised by its structure, and the morphology of erythrocytes is also altered.

Keywords: Iron deficiency anemia, erythrocyte, hemoglobin, morphology, physicochemical property.

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INTRODUCTION

Globally impacted by 1 billion individuals, lack of iron, iron deficiency anemia (IDA) is still a serious worldwide medical issue.¹,² In West Bengal, it is concerning that approximately 63.2% of young women still experience the adverse effects of IDA.³ Lack of iron impacts the menstrual cycle, pregnancy risks, mental ability, lower work capacity, and efficiency.⁴ Various biological cycles, for example, oxygen transport, energy regulation, aging, and DNA synthesis, rely upon iron.⁵ Low iron levels and microcytic hypochromic erythrocytes are signs of a lack of iron. Even as efforts to combat iron deficiency have progressed in some regions, addressing the conditions ensuring sufficient iron intake, especially among vulnerable populations, remains essential. This underscores the need for ongoing efforts and innovative solutions.

Structural alterations in erythrocytes in IDA and its restoration to some extent with proper supplementation for a certain period have been reported.⁶ To maintain this biconcave structure of erythrocyte, ATP plays a vital role by connecting the spectrin network and lipid bilayer, and its depletion triggers the morphological transformation of erythrocyte.⁷,⁹ This altered erythrocyte goes through an oxidative insult by generating reactive oxygen species (ROS) with increased lipid peroxidation and methemoglobin (metHb) production, leading to a shorter life span of erythrocyte.¹⁰ The increased oxidative insult and accelerated destruction of erythrocytes contribute to the pathophysiology of IDA.¹¹ On the other hand, in IDA, the formation of hemoglobin is impaired, and the quantity is also low, but the health status of hemoglobin is also compromised by its structure, and the morphology of erythrocytes is also altered.

Materials and Methods

Materials Used

Sephadex G-100, glutaraldehyde, acrylamide, and bis-acrylamide were purchased from Sigma-Aldrich (St.
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Selection of Subjects and Collection of Blood Samples
The study was conducted in a small cohort of North 24 Parganas district of West Bengal, as depicted in Figure 1. At first, by measuring hemoglobin concentration, about 15 anemic women and eight age-matched healthy women (between 25-45 years) were recruited. The hemoglobin concentration for anemic women was about 7-9 gm/dl (moderate anemia), and control women were above 11 gm/dl. After performing the baseline parameters (such as hemoglobin concentration, serum iron, and total iron binding capacity (TIBC)), the subjects were divided into control and IDA. The subjects who did not fulfill the inclusion criteria were excluded from the study. Besides this, the exclusion criteria for subject selection were devoid of any other illness, use of any medication, tranquillizers, and anesthetics, as well as any smoking or drinking habits or any blood-related abnormalities (except IDA). With the permission of the Institutional Human Ethics Committee (IHEC/RH/OL7/2021; dated 12.11.2021), Department of Physiology, University of Calcutta, and as per the experimental design, the blood sample was collected after their duly signed written and oral consents. About 2-3 ml of venous blood was collected by phlebotomy technique in both heparinized and non-heparinized vial.

Hemoglobin Estimation
The volunteers were selectively recruited by estimating the Hb concentration using the cyanmethemoglobin method. The method is based on the oxidation of Hb and its derivatives (except sulfhemoglobin) to met-Hb in the presence of alkaline potassium ferricyanide \([K_2Fe(CN)_6]\). About 20 µl of whole blood was mixed with 5 ml of Drabkin’s reagent. After 20 minutes of incubation, the color intensity was measured (compared with the standard solution) spectrophotometrically at 540 nm against the sample blank.

Estimation of Serum Iron and TIBC
To confirm IDA, serum iron and total iron binding capacity (TIBC) were measured. Transferrin releases the iron in an acidic medium, reducing the ferric ions to a ferrous state. The Fe(II) ions react with ferrozine to form a violet-colored complex. The intensity of the complex formed is directly proportional to the amount of iron present in the sample. The formed product was measured spectrophotometrically (Make: Systronics, Model: Dual Beam Spectrophotometer 2022) at 570 nm.

Preparation of Packed Cell Volume (PCV)
About 2 mL of heparinized blood was taken in a centrifuge tube and washed three times with 0.01 M phosphate buffer saline (PBS; pH 7.4). After gentle mixing, it was centrifuged at around 1800-2000 rpm for 3 minutes at room temperature (24-26°C). After centrifugation, the buffy coat was disposed of, and the packed cell was obtained.

Morphological Analysis of Erythrocyte
To preserve the structure, erythrocytes were fixed, followed by an hour incubation process with 2.5% glutaraldehyde in PBS. After washing for 15 minutes with PBS, it was postfixed for 30 minutes with 1% osmium tetroxide. The specimen was washed with PBS for 15 minutes before dehydration in 30%, 50%, 70%, 90%, and 100% ethanol. The sample was dried, mounted, and platinum-coated before being examined with a scanning electron microscope (Make: Zeiss, Germany, Model: EVO18 special edition) and micrographs obtained at 15 kV accelerating voltage.

Preparation of Erythrocyte Membrane
For complete cell lysis, the packed cells were resuspended in hypotonic buffer solution (1 × 10⁻² M Tris HCl, 1.44 × 10⁻³ M K₂EDTA, Cocktail protease inhibitor), pH 7.4 for 30 minutes. After lysis, the hemolysate was centrifuged (Make: Eppendorf, Germany, Model: 5430R) for 30 minutes at 20,000g at 4°C. The erythrocyte membrane pellet was obtained and carefully washed using isosmotic buffer (1 × 10⁻² M Tris HCl, 1.44 × 10⁻³ M K₂EDTA, 1.7 × 10⁻² M NaCl), pH 7, many times for the complete removal of Hb. Then, it was resuspended in buffer (1 × 10⁻² M Tris HCl), pH 7.4, kept in several aliquots, and preserved at -20 °C for further experiments.

Purification of Hemoglobin
The hemolysate was prepared by resuspending the packed cells in 10 mM phosphate buffer (pH 7.4) and kept for 30 minutes for complete hemolysis at 4°C. The hemolysate was then centrifuged at 14,000 rpm for 30 minutes at 4°C, and the red-colored supernatant (which contains Hb) was obtained. Then, the Hb was purified from the supernatant by size-exclusion chromatography using a Sephadex G-100 column.
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column (12 × 1.5 cm). The middle red fraction was collected/eluted (in the aliquots) for the highest purification and used for further experiments.17

**Study of Hemoglobin Co-oxidation**
Under healthy circumstances, Hb is slowly oxidized to its met form and superoxide formation (auto-oxidation). However, in the presence of the oxidizing agent, e.g., nitro-blue tetrazolium (NBT), Hb is oxidized faster (co-oxidation).18 To carry out this study, 1 × 10^6 M NBT was added to 40 × 10^6 M purified Hb in 0.01 M PB, pH 7.4, and the absorbance at 630 nm was monitored in UV-Vis Dual Beam Spectrophotometer (Make: Systronics, Model: Dual Beam Spectrophotometer 2202) for 30 mins. The formation of methHb content was measured using extinction co-efficient 19 ε_{630nm} = 3.7 mM^{-1} cm^{-1}.

**Estimation of Erythrocyte Methemoglobin (MetHb) Content**
Firstly, erythrocytes were separated from leukocytes and thrombocytes using the heparinized whole blood using the acellular and microcrystalline cellulose column. The erythrocyte thus obtained was lysed with double-distilled water and centrifuged at 1000 g for 10 mins. The supernatant was then treated with 2% potassium ferricyanide to estimate the methemoglobin following the method of Arashiki et al.20

**Quantification of Erythrocyte Membrane Protein**
The isolated erythrocyte membrane protein was quantified using the standard Lowry method21 using bovine serum albumin as standard protein. The quantified protein was then subjected to further experimental procedures.

**Estimation of Membrane-bound Hemoglobin**
According to the method of Rocha-Pereira et al.22 the amount of membrane-bound hemoglobin (MBHb) was measured. The erythrocyte membrane was incubated with 1% Triton X-100 for the dissociation of Hb, and the absorbance of Hb was measured at 415 nm spectrophotometrically (Make: Systronics, Model: Dual Beam Spectrophotometer 2202). The concentration was calculated using the molar extinction coefficient 22 ε_{415nm}=125mM^{-1} cm^{-1}.

**Native Polyacrylamide Gel Electrophoresis of Hemoglobin**
For the detection of the native form of Hb, about 12% polyacrylamide discontinuous gel matrix was prepared, and 100 µg of Hb from both healthy and IDA subjects were loaded and run at 90-100 volts. The gel was stained with Coomassie Brilliant blue.23

**Estimation of Carbonyl Content of Hemoglobin**
Carbonyl content of Hb was measured using 4-dinitrophenyl hydrazine (DNPH) according to the method of Levine et al.24 Fifty microlitres of 10 × 10^{-3} M DNPH was added and thoroughly mixed with 1.5 mg and 3 mg of Hb in 500 µl reaction mixture and incubated at 37°C for 1 hour. After adding 500 µl 20% TCA, the reaction mixtures were centrifuged at 11,000 g for 5 minutes. The pellets were washed three times with a 1:1 mixture of ethanol and ethyl acetate. After that, the pellets were dissolved in 1-mL of 6 M guanidine hydrochloride and incubated for 15 minutes at 37°C. After centrifugation, the supernatants were collected and measured spectrophotometrically at 370 nm (ε_{370nm} = 22,000 M^{-1} cm^{-1}) 24.

**Analysis of the Absorption Spectrum of Hemoglobin**
The absorption spectra at UV-Vis range of 5×10^{-6} M Hb (in 0.01 M phosphate buffer, pH 7.4) of both control and IDA groups were recorded from 250-600 nm wavelength using a dual beam spectrophotometer (Make: Systronics, Model: Dual Beam Spectrophotometer 2202) and the absorption spectra at far-UV range of 1×10^{-6} M Hb was also recorded from 190-250 nm wavelength using another spectrophotometer (Make: Shimadzu, Kyoto, Japan, Model: UV-1800).

**Statistical Analysis**
All the data were expressed as mean ± standard error of the mean (SEM), and an unpaired Student’s t-test was used to assess the differences between two mean values in Microsoft Excel (Office 2019). P values of less than 0.05 were considered significant.

**Results**
Among the selected anemic women, ten were diagnosed with IDA after estimation of Hb concentration, serum iron content, and TIBC. The scanning electron microscopic results showed that a good number of echinocytes (structurally altered erythrocytes) are formed in IDA cases (Figure 2) as compared with the control cases (erythrocytes remain at their discoid shape, Figure 3). The alteration in structure might be due to modification in erythrocyte membrane properties. The co-oxidation rate, i.e., the formation of methHb, was significantly higher in IDA cases compared with the control group (Figure 4). As methemoglobin formation accompanies superoxide formation, a higher co-oxidation rate in IDA is a clear indication of augmentation of oxidative stress within erythrocytes. The graphical representation of the methHb percentage (Figure 5) demonstrated that the total methHb

![Figure 2](image-url)
content in the presence of K3Fe(CN)6 is about 33% higher in IDA individuals than in control subjects. The membrane-bound hemoglobin content of erythrocytes (Figure 6) is significantly higher in IDA individuals (p<0.05). The presence of excess metHb may cause an increase in membrane-bound Hb content. The native polyacrylamide gel electrophoresis of hemoglobin of control and IDA subjects does not show any significant difference (Figures 7a and 7b) as the protein remained in its native state in both cases. The carbonyl content of hemoglobin is significantly deceased (p<0.05) in IDA cases in both concentrations of Hb as compared with the control groups (Figure 8), which denotes a reduction of oxidative degradation of the Hb. UV-Vis absorption spectra
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of purified Hb of both groups are shown in Figure 9. The spectrum showed the shifting of the Soret peak (415 nm), the variation in the changes of globin chain structure (250-350 nm), and a prominent change in the 538 and 577 nm peaks, i.e., oxyhemoglobin and carboxyhemoglobin. For further confirmation, we performed the Far-UV spectrum and found an abrupt alteration in the globin structure in IDA cases, as depicted in Figure 10.

**Discussion**

The spectrin (bands 1 and 2) and actin (band 7) are erythrocytes’ major cytoskeleton proteins, accounting for 75% of the membrane cytoskeleton. This spectrin-actin complex helps to maintain the morphology of erythrocyte. However, the low iron hinders spectrin-actin-protein 4.1 junctional complexes, affecting erythrocyte morphology. Moreover, hemoglobin, which is encapsulated within the erythrocyte, may also be affected by altered erythrocyte morphology. This may be due to the formation of a hemoglobin-spectrin complex, which can change the membrane’s elasticity. Therefore, it is pertinent to reveal the physicochemical status of hemoglobin, which is already low in amount due to the deficiency of iron in IDA.

The erythrocyte morphology by scanning electron microscopy demonstrates a significant increase in echinocyte formation in IDA patients (Figures 2 and 3). Echinocytes are mainly formed when the symmetry of both sides of the lipid bilayer is hampered, i.e., when the surface area of the outer lipid layer is increased compared to the inner lipid layer. These echinocytes do not remain in circulation for long, and cellular lysis occurs.

On the other hand, in an attempt to assess the different biochemical and biophysical properties of hemoglobin, the rate of co-oxidation was found to be elevated in IDA patients (Figure 4). This indicates a higher rate of metHb formation along with the formation of superoxides. However, metHb is converted to its native deoxy form by metHb reductase. Moreover, total metHb content was also higher in IDA (Figure 5), indicating that the metHb formation exceeded the capacity of metHb reductase to bring down excess metHb. As the metHb content increased, the possibility of alteration in the content of membrane-bound Hb was suggested, and experimentally, membrane-bound Hb content was also found to be higher in IDA cases compared with the healthy instances (Figure 6). A higher concentration of metHb interacts with Band 3 (metHb-Band3 cluster), the integral membrane protein of erythrocyte membrane, and is reflected as membrane-bound Hb. This increased content of membrane-bound Hb reduces the elasticity of erythrocyte membrane. Moreover, band3 clustering also reduces the life span of erythrocytes.

Notably, the Native-PAGE of Hb showed no distinct alterations of its native form of both groups (Figure 7). As the rate of co-oxidation was higher, the production of superoxide was also higher, which might have increased the intracellular oxidative load and affected the intracellular biomolecules, including hemoglobin itself. Interestingly, the carbonyl content of Hb from IDA patients was less than that of the control (Figure 8), which was not expected, and no proper justification was available from the literature. However, it is worth mentioning that the proteasomes, to some extent, help to repair protein from carbonylation by further accumulation of carbonylated products. Through the vesiculation procedure, erythrocytes may then expel the damaged protein aggregates. However, the possibility was not assessed in the current study.

The Soret peak (415 nm) shifting (Figure 9) indicated that the heme–globin interaction was hampered. The spectra at 250-350 nm indicated abnormalities in globin structure, which was inconsistent among all the IDA patients. Similarly, the 538 nm and 577 nm peaks were also changed in IDA. The 538 nm and 577 nm peaks are due to oxyhemoglobin and carboxyhemoglobin. Accordingly, it is hypothesized that the change in the globin chain may affect the interaction with heme, resulting in the shifting of the Soret peak and finally affecting the oxygen binding capacity of Hb. Alteration in
the globin chain was also confirmed by the Far-UV spectrum (Figure 10); however, the degree of alteration was not assessed.

The current study shows that the erythrocytes of IDA women showed echinocyte formation with increased methHb content, higher co-oxidation rate, increased membrane-bound Hb content, and decreased carbonyl content. The echinocytes may be formed due to modifying the erythrocyte membrane’s cytoskeleton proteins and the lipid bilayer’s asymmetry. The physicochemical properties of Hb and erythrocytes are altered in IDA cases and reduced the life span of erythrocytes. Nevertheless, the amount of oxidative load within the erythrocyte is still unclear from this study and warrants further studies to evaluate these aspects. Overall, an abrupt alteration in the globin structure, along with distressing heme-globin interaction, is significantly noted. This study is based on studies with blood samples from a tiny cohort of North 24 Parganas district of West Bengal; therefore, the inference from this study may have a limitation of small sample size. Even with this limitation, observations of this study have the potential to be a new entrant to the diagnostic and therapeutic world arena related to IDA.

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**REFERENCES**


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PEER-REVIEWED CERTIFICATION

During the review of this manuscript, a double-blind peer-review policy has been followed. The author(s) of this manuscript received review comments from a minimum of two peer-reviewers. Author(s) submitted revised manuscript as per the comments of the assigned reviewers. On the basis of revision(s) done by the author(s) and compliance to the Reviewers’ comments on the manuscript, Editor(s) has approved the revised manuscript for final publication.