RESEARCH ARTICLE

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

Manali Roy[1#], Baishali Basak[1,2#], Payel Biswas[1], Rajen Haldar[1*]

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 (Hb). Increased methemoglobin (metHb) formation rate (higher co-oxidation rate) and increased metHb content and membrane-bound Hb 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 still not cleared.

Therefore, a different angle is needed to know a bit about the health status of hemoglobin and erythrocytes in IDA cases. However, the extent to which hemoglobin is safe within the altered erythrocyte, is still unhooked. Surprisingly, the health and functional state of hemoglobin and erythrocytes in IDA patients are scarcely reported in the literature. So, the current study explores the health status of hemoglobin and erythrocytes in the IDA cohort.

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Materials And Methods

Materials Used

Sephadex G-100, glutaraldehyde, acrylamide, and bis-acrylamide were purchased from Sigma-Aldrich (St.

Louis, MO). Nitro-blue tetrazolium chloride, triton X-100, dinitrophenylhydrazine, and guanidine hydrochloride were purchased from SRL. An iron-TIBC kit from Coral Clinical System was used for analysis. The other chemicals were of analytical grades.

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, tranquilizers, 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.[12] The method is based on the oxidation of Hb and its derivatives (except sulfhemoglobin) to met-Hb in the presence of alkaline potassium ferricyanide [$K_3Fe(CN)_6$]. 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.[13] 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.[14]

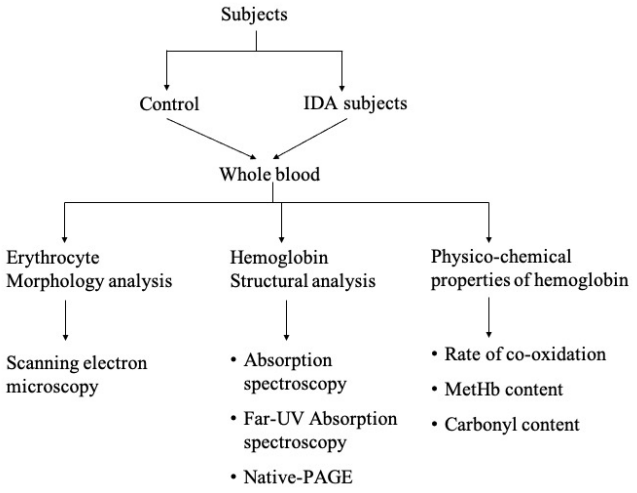

Figure 1: The study design employed in the current study

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 tetraoxide.[15] 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[7].

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 K_2EDTA, 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 K_2EDTA, 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.[16]

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 (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, 1×10^4 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 2022) for 30 mins. The formation of metHb content was measured using extinction co-efficient,[19] $\varepsilon_{630\ nm} = 3.7\ mM^{-1}\ cm^{-1}$.

Estimation of Erythrocyte Methemoglobin (MetHb) Content

Firstly, erythrocytes were separated from leukocytes and thrombocytes using the heparinized whole blood using the αcellulose and microcrystalline cellulose column. The erythrocyte thus obtained was lysed with doubled-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 method[21] 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 2022). The concentration was calculated using the molar extinction coefficient[22] $\varepsilon 415nm = 125 mM^{-1}\ cm^{-1}$.

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 pallets 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 ($\varepsilon_{370nm} = 22{,}000\ M^{-1}cm^{-1}$)[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 metHb, 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 metHb percentage (Figure 5) demonstrated that the total metHb

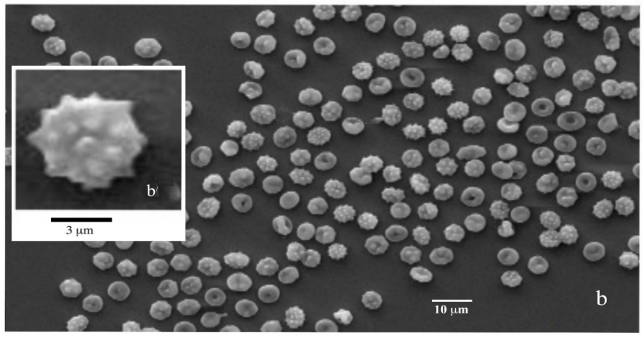

Figure 2: The panel (b) indicates the field of IDA's erythrocytes and the inset indicates the magnified individual (b′) morphology of IDA's erythrocyte

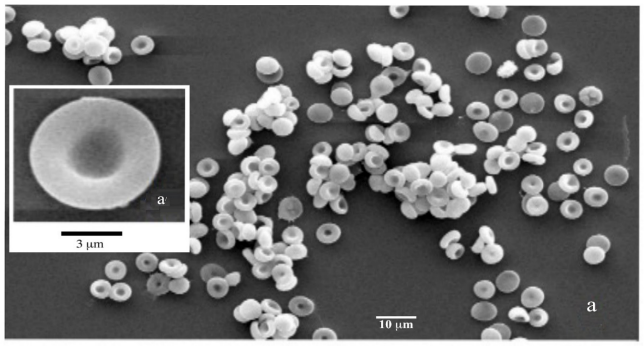

Figure 3: The panel (a) indicates the field of the control's erythrocytes, and the inset indicates the magnified individual (a´) morphology of the control's erythrocyte

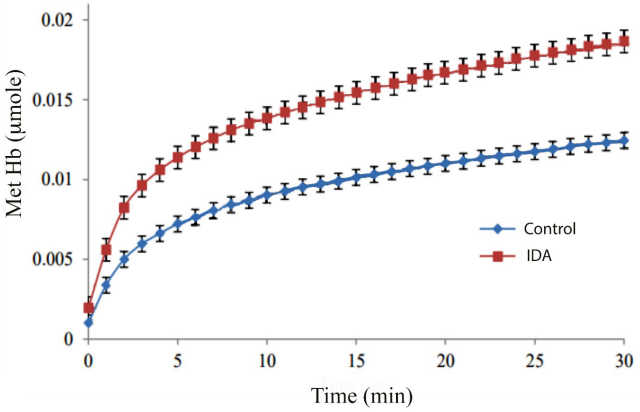

Figure 4: The co-oxidation study of hemoglobin (4 × 10−5 M) in both control and IDA groups in presence of NBT (1 × 10 4 M). The results are represented as mean ± SEM

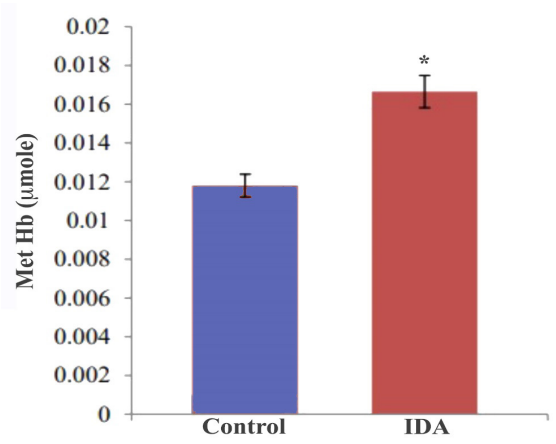

Figure 5: Estimation of methemoglobin percentage in control and IDA subjects. Results are expressed as the mean ± SEM. * indicates p< 0.05.

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

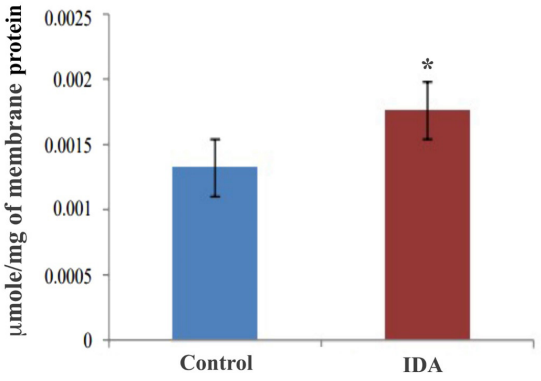

Figure 6: Estimation of membrane-bound hemoglobin content in control and IDA individuals. Results are expressed as the mean ± SEM. * indicates p< 0.05.

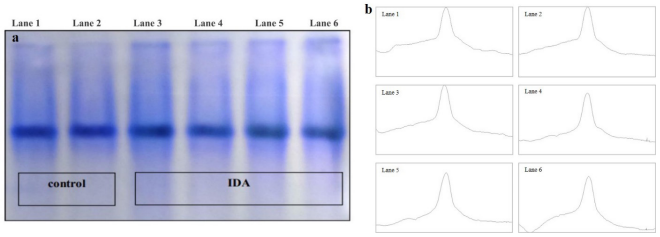

Figure 7: (a) Native polyacrylamide gel electrophoresis (12%) study of control and IDA's hemoglobin (100µg) and (b) band profile of individual lanes of the gel electrophoresis. Lanes 1 and 2 contain the control's hemoglobin, and Lanes 3, 4, 5 & 6 contain IDA's hemoglobin

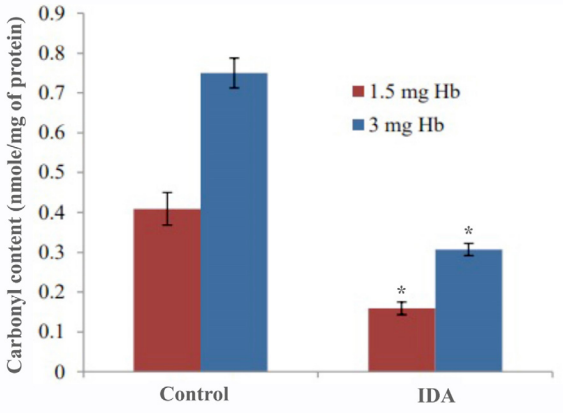

Figure 8: Determination of carbonyl content with two different concentrations of hemoglobin (1.5 and 3 mg) of both control and IDA subjects. Results are expressed as the mean ± SEM. * indicates p< 0.05 when compared to respective controls

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

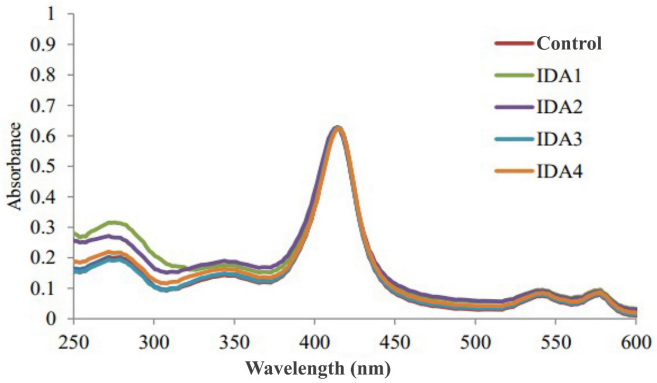

Figure 9: Spectrophotometric analysis of hemoglobin (5 × 10–6 M) of one control subject and 4 different IDA subjects. The absorption spectrum was recorded at UV-Vis range from 250-600 nm wavelength

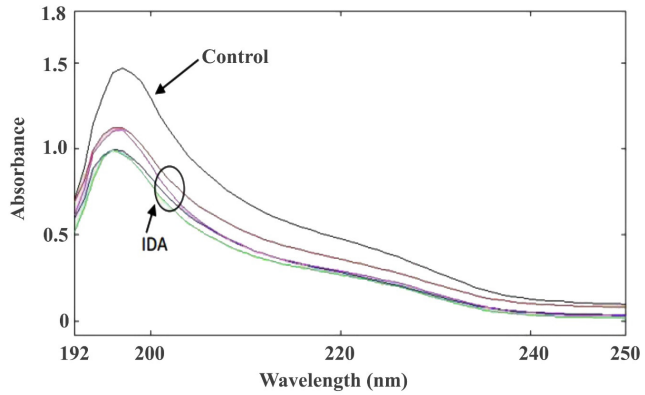

Figure 10: Spectrophotometric analysis of hemoglobin (1 × 10–6 M) of one control subject and 4 different IDA subjects. The absorption spectrum was recorded at Far-UV range from 190-250 nm wavelength

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.[25] This spectrin-actin complex helps to maintain the morphology of erythrocyte.[26] However, the low iron hinders spectrin-actin-protein 4.1 junctional complexes,[27-29] 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.[30-33] 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.[34,35] 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.[36] 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.[37,38] Moreover, band3 clustering also reduces the life span of erythrocytes.[9]

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.[39-41] 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 sepctrum (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 metHb 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

1. Global Health Metrics. Anaemia–Level 1 impairment. Lancet. 2019; 393. Available at: https://www.healthdata.org/results/gbd_summaries/2019/anemia-level-1-impairment. Accessed on: 25th November, 2023.
2. GBD 2021 Anaemia Collaborators. Prevalence, years lived with disability, and trends in anaemia burden by severity and cause, 1990–2021: findings from the Global Burden of Disease Study 2021. *Lancet Haematol*. 2023;10(9):e713-34. DOI: 10.1016/S2352-3026(23)00160-6.
3. Jana A, Chattopadhyay A, Saha UR. Identifying risk factors in explaining women's anaemia in limited resource areas: evidence from West Bengal of India and Bangladesh. *BMC Public Health*. 2022;28;22(1):1433. DOI: 10.1186/s12889-022-13806-5.
4. Ramachandran P. Prevention and management of anaemia in pregnancy: Multi-pronged integrated interventions may pay rich dividends. *Ind J Med Res*. 2021;154(1):12-5. DOI: 10.4103/ijmr.IJMR_994_20.
5. Miller J. Iron Deficiency Anemia: A common and curable disease. *Cold Spring Harb Perspect Med*. 2013;3(7):a011866. DOI:10.1101/cshperspect.a011866.
6. Zhang Y, Zhang W, Wang S, *et al*. Detection of human erythrocytes influenced by iron deficiency anemia and thalassemia using atomic force microscopy. *Micron*. 2012;43:1287-92. DOI: 10.1016/j.micron.2011.10.018.
7. Diez-Silva M, Dao M, Han J, Lim CT, Suresh S. Shape and biomechanical characteristics of human red blood cells in health and disease. *MRS Bull*. 2010;35(5):382-8. DOI:10.1557/mrs2010.571.
8. Park Y, Best CA, Auth T, *et al*. Metabolic remodeling of the human red blood cell membrane. *PNAS*. 2010;107(4):1289-94. DOI: 10.1073/pnas.091078510.
9. Giosheva I, Strijkova V, Komsa-Penkova R, *et al*. Membrane lesions and reduced life span of red blood cells in preeclampsia as evidenced by atomic force microscopy. *Int J Mol Sci*. 2023;24(8):7100. DOI: 10.3390/ijms24087100.
10. Nagababu E, Gulyani S, Earley CJ, Cutler RG, Mattson MP, Rifkind JM. Iron-deficiency anaemia enhances red blood cell oxidative stress. *Free radic res*. 2008;42(9):824-9. DOI: 10.1080/10715760802459879.
11. Zidova Z, Garcia-Santos D, Kapralova K, *et al*. Oxidative stress and increased destruction of red blood cells contribute to the pathophysiology of anemia caused by DMT1 deficiency. *Blood*. 2014;124:4027. DOI: 10.1182/blood.V124.21.4027.4027.
12. Dacie JV and Lewis, SM. Practical Haematology. 4th Edition J. & A, Churchill, 1968; 37.
13. Siedel J, Wahlefeld AW, Ziegenhorn J. Improved, ferrozine-based reagent for the determination of serum iron (transferrin iron) without deproteinization. *Clin Chem*. 1984;30(6):975.
14. Mukherjee KL. Hematology *In: Medical Laboratory technology*. Tata Mcgraw-Hill Publishing Company Limited 2003;1:228-324. ISBN, 1259000753, 9781259000751.
15. Pretorius E, Oberholzer HM, van der Spuy WJ, Swanepoel AC, Soma P. Qualitative scanning electron microscopy analysis of fibrin networks and platelet abnormalities in diabetes. *Blood Coagul Fibrinolysis*. 2011;22(6):463-7. DOI: 10.1097/MBC.0b013e3283468a0d.
16. Rocha-Pereira P, Santos-Silva A, Rebelo I, Figneiredo A, Quintanitha A, Teixeira T. Erythrocyte damage in mild and severe psoriasis. *Br J Dermatol*. 2004;150:232-44. DOI: 10.1111/j.1365-2133.2004.05801.x.
17. Bhattacharyya J, Bhattacharyya M, Chakraborti AS, Chaudhuri U, Poddar RK. Structural organisations of hemoglobin and myoglobin influence their binding behaviour with phenothiazines. *Int J Biol Macromol*. 1998;23(1):118. DOI: 10.1016/s0141-8130(98)00006-3.
18. Wallace WJ, Maxwell JC, Caughey WS. A role for chloride in the autoxidation of hemoglobin under conditions similar to those in erythrocytes. *FEBS Lett* 1974;43:336. DOI: 10.1016/0014-5793(74)81099-9.
19. ElmerJ, Harris DR, Sun G, Palmer AF. Purification of hemoglobin by tangential flow filtration with diafiltration. *Biotechnol Prog*. 2009;25:140210. DOI: 10.1002/btpr.217.
20. Arashiki N, Kimata N, Manno S, Mohandas N, Takakuwa Y. Membrane peroxidation and methemoglobin formation are both necessary for band 3 clustering: Mechanistic insights into human erythrocyte senescence. *Biochemistry*. 2013;52:57609. DOI: 10.1021/bi400405p.
21. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem*. 1951;193:265–27. PMID: 14907713.
22. Gebicka L, Banasiak E. Flavonoids as reductants of ferryl hemoglobin. *Acta Biochim Pol*. 2009;56(3):509-13. PMID: 19774231.
23. Righetti PG, Castagna A, Antonioli P, Boschetti E. Prefractionation techniques in proteome analysis: the mining tools of the third millennium. *Electrophoresis*. 2005;26(2):297-319. DOI: 10.1002/elps.200406189.

24. Levin D E, Fields FO, Kunisawa R, Bishop JM, Thorner J. A candidate protein kinase c gene, pkc1, is required for the *S. cerevisiae* cell cycle. *Cell.* 1990;62(2):213-24. DOI: 10.1016/0092-8674(90)90360-q.
25. Tiffert T, Lew VL. Dynamic morphology and cytoskeletal protein changes during spontaneous inside-out vesiculation of red blood cell membranes. *Pflügers Arch-Euro J Physiol*. 2014;466:2279-88. DOI: 10.1007/s00424-014-1483-5.
26. Chan MM, Wooden JM, Tsang M, *et al*. Hematopoietic protein-1 regulates the actin membrane skeleton and membrane stability in murine erythrocytes. *PLoS One*. 2013;8(2):e54902. DOI: 10.1371/journal.pone.0054902.
27. More TA, Kedar P. Unravelling the genetic and phenotypic heterogeneity of SPTA1 gene variants in Hereditary Elliptocytosis and Hereditary Pyropoikilocytosis patients using next-generation sequencing. *Gene*. 2022;843:146796. DOI: 10.1016/j.gene.2022.146796.
28. Bayhan T, Ünal Ş, Gümrük F. Hereditary Elliptocytosis with Pyropoikilocytosis. Turk *J Haematol*. 2016;33(1):86-7. DOI: 10.4274/tjh.2015.0054.
29. Bandaru SS, Killeen RB, Gupta V. Poikilocytosis. *StatPearls*. 2023. PMID: 32965812.
30. Fortier N, Snyder LM, Garver F, Kiefer C, McKenney J, Mohandas N. The relationship between in vivo generated hemoglobin skeletal protein complex and increased red cell membrane rigidity. *Blood*. 1988;71(5):1427-31. DOI: 10.1182/blood.V71.5.1427.1427.
31. Kiefer CR, Trainor JF, McKenney JB, Valeri CR, Snyder LM. Hemoglobin-spectrin complexes: interference with spectrin tetramer assembly as a mechanism for compartmentalization of band 1 and band 2 complexes. *Blood*; 1995;86(1):366-71. PMID: 7795245.
32. Lux IV SE. Anatomy of the red cell membrane skeleton: unanswered questions. *Blood*. 2016;127(2):187-199. DOI: 10.1182/blood-2014-12-512772.
33. Gevorkian SG, Allahverdyan AE, Gevorgyan DS, Ma WJ, Hu CK. Can morphological changes of erythrocytes be driven by hemoglobin?. *Physica A: Statistical Mechanics and its Applications*. 2018;508:608-12. DOI: 10.1016/j.physa.2018.05.118.
34. Kaneko JJ, Harvey JW, Bruss ML, editors. *Clinical biochemistry of domestic animals*. 6th Edition. Academic Press; 2008;4. ISBN: 978-0123704917.
35. Hoffman R, Benz EJ, Silberstein LE, *et al*, editors. Hematology: Basic Principles and Practice. 8th Edition. Elsevier Health Sciences; 2022;27. ISBN: 9780323733885.
36. Bahabri A, Moradi J, Choong K, Pai N, Bhatt M. Food protein-induced enterocolitis syndrome presenting with life-threatening methemoglobinemia: a case report and review of the literature. *Int J Clin Pediatc.* 2020;9(2):35-40. DOI: 10.14740/ijcp366.
37. Sikdar J, Seal P, Roy A, Haldar R. Cigarette smokers develop altered erythrocyte membrane composition: an investigation unmasking the role of membrane bound integral protein GLUT 1. *Free Rad Res*. 2017;51(4):375-88. DOI: 10.1080/10715762.2017.1321744.
38. Biswas P, Seal P, Sikdar J, Haldar R. Oxidative degradation perturbs physico-chemical properties of hemoglobin in cigarette smokers: a threat to different biomolecules. *Inhal Toxicol*. 2021;33(6-8):275-84. DOI: 10.1080/08958378.2021.1991529.
39. Delobel J, Prudent M, Rubin O, Crettaz D, Tissot JD, Lion N. Subcellular fractionation of stored red blood cells reveals a compartment-based protein carbonylation evolution. *J proteomics*. 2012;5;76:181-93. DOI: 10.1016/j.jprot.2012.05.004.
40. Leal JK, Adjobo-Hermans MJ, Bosman GJ. Red blood cell homeostasis: Mechanisms and effects of microvesicle generation in health and disease. *Front physiol.* 2018;8(9):703. DOI: 10.3389/fphys.2018.00703.
41. Bujok J, Wajman E, Trochanowska-Pauk N, Walski T. Evaluation of selected hematological, biochemical and oxidative stress parameters in stored canine CPDA-1 whole blood. *BMC Vet Res.* 2022;18(1):255. DOI: 10.1186/s12917-022-03353-x.

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.