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Original Article

The functional connotations of iron deficiency-effect on neutrophil oxidative burst activity in preschool children

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Abstract: Iron deficiency anaemia (IDA) makes an individual prone to bacterial infections. The antimicrobial defence mechanism of neutrophils is orchestrated by Nicotinamide Adenine Dinucleotide Phosphate Hydrogen (NADPH) oxidative burst which is iron-dependent. The few previous studies documenting a decrease in neutrophil oxidative burst in iron-deficient children have been based mainly on the Nitro blue tetrazolium test (NBT). Very few studies have been conducted using the more robust flow cytometry-based dihydro rhodamine (DHR) assay in this regard worldwide and none in India. Aim: To estimate the effect of iron deficiency on neutrophil oxidative burst activity in children under 5 years of age by flow cytometry-based dihydro rhodamine (DHR) assay and compare it with the control group. Methods: Thirty-six children between 6 months to 5 years of age diagnosed with moderate (Hb 7-10 gm/dl) to severe (Hb <7 gm/dl) iron deficiency anaemia were selected as cases with equal number of sex/age matched controls. The peripheral blood was analyzed for hematological and biochemical parameters such as complete iron profile, serum vitamin B12, and folate levels. The oxidative burst activity of neutrophils in peripheral blood was assessed using a flow-cytometry-based Dihydrorhodamine (DHR) assay. Results: The percentage of neutrophils showing stimulation, Mean Fluorescence Index in stimulated neutrophils, and Neutrophil oxidative index (NOI) were significantly reduced in iron deficiency anaemia patients as compared to controls. In cases, haemoglobin showed significant positive correlation with NOI and percentage of neutrophils showing stimulation. Conclusion: To conclude, a significant decrease in neutrophil oxidative burst parameters depicts an insufficient innate immune response to pathogens and makes Iron deficiency anaemia patients more susceptible to infections, further aggravated by the severity of anaemia.

Keywords: Preschool children, iron deficiency anaemia, neutrophil oxidative burst, flowcytometry, Dihydrorhodamine assay

Introduction

Anaemia is a major public health issue responsible for the loss of approximately 50 million years of healthy life due to disability by anaemia in the year 2019. Globally, 39.8%% of children aged 6-59 months (<5 years age), suffered from anaemia with iron deficiency being the most common and widespread cause of anaemia in children [1, 2]. As per National Family Health Survey (NFHS-5), on Indian children from 6-59 months age, overall, 67.1% of children had some degree of anaemia (<11 g/dl) which was higher than that quoted in NFHS-4 [3].

Iron deficiency anaemia is a problem of serious public health significance that affects mental and physical development, homeostasis, and work performance. Iron is a necessary element for the normal development of the immune system having a role in multiple pathways of both Innate and Adaptive immune response. It is documented that the incidence of infections increases in the presence of Iron deficiency anaemia, however the exact underlying mechanism is unknown [4]. Iron deficiency is associated with the impairment of cell-mediated immunity caused by reduced CD4+ lymphocyte levels and low CD4:CD8 ratio. However, the effect of iron deficiency on humoral and innate

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immunity is less well described than its role in adaptive immunity [5-7].

Neutrophils being one of the important cells of innate immune response act via Nicotinamide Adenine Dinucleotide Phosphate (NADPH) oxidase 2 (NOX2) mechanism and produce reactive oxygen species to protect against microbes. NOX2 is a heterodimeric enzyme complex in the plasma membrane of neutrophils, which has haem moiety containing ferrous Iron in its cytoplasmic subunit [8].

Several studies found that the phagocytic activity of neutrophils is dependent on serum Iron levels and this activity gets reduced in IDA [9-13]. The reason for a reduction in neutrophil oxidative burst activity in IDA can be due to a decrease in the activity of NADPH oxidase which is a heme-dependent enzyme. Depleted Iron stores and reduced plasma Iron in IDA may affect Iron availability for enzyme activity and thus lead to decreased oxidative burst activity. To support this fact, various studies were conducted to demonstrate the association between iron and oxidative burst activity of neutrophils by comparing iron-deficient individuals with healthy controls [9-13].

A recent ex-vivo study demonstrated that plasma iron modulates the phagocytic capacity as the neutrophils produced during hypoferremia exhibited reduced capacity to phagocytose fluorescent E. coli and staphylococcus aureus [9]. Other studies demonstrated significantly lower NADPH oxidase activities in Iron-deficient patients in comparison to healthy controls [4, 5, 10-13]. Few researchers also found increased oxidative bursts of neutrophils after administration of Iron supplements [4, 5].

Various methods can be used to measure NADPH oxidative burst phenomena, such as 3', 3'-diaminobenzidine (DAB) oxidation, p-nitro blue tetrazolium (NBT) reduction, chemiluminescence of superoxide ions released by neutrophils, Cytochrome c reduction colorimetric assay and the newer flow cytometry-based DHR (Dihydrorhodamine) assay [14-16].

The Chemiluminescence and colorimetric tests are complicated and laborious while NBT is highly subjective and thus prone to inter-observer variability. On the contrary, flow cytometry-based DHR assay (Dihydro rhodamine assay) is more objective, rapid, highly sensitive and specific to assess the oxidative burst activity of neutrophils as it measures the activity of thousands of neutrophils in a short period [17, 18]. Few previous studies documenting a decrease in neutrophil oxidative burst in iron-deficient children have been based mainly on the NBT test.

Very few studies have been conducted using the much more robust DHR assay in this regard all over the world and none in preschool children in India [19, 20]. Also, no study has been conducted to measure the neutrophil burst activity using DHR assay in preschool children in whom IDA is very rampant. Thus, the present study aims to assess neutrophil oxidative burst activity by DHR assay in children with iron deficiency anaemia less than 5 years of age and compare it with healthy age-matched controls.

Material and methods

It was a prospective, cross-sectional, and comparative study conducted in the Department of Paediatrics and Pathology of a 1500-bed tertiary care hospital in Delhi. Ethical approval for the study was obtained from the Institutional Ethics Committee for human research.

Study subjects

All Children between 6 months to 5 years in age presenting to paediatric out patient department and confirmed to have Iron deficiency anaemia were included for the study. Iron deficiency anaemia was diagnosed on the basis of Hb less than 10 gm/dL, microcytic hypochromic blood picture and a serum Ferritin <12 µg/l and/or Transferrin saturation (TSAT) <16%. Subjects with mild anaemia (Hb 10-10.99 gm/dl), coexisting B12 or Folate deficiency, history of blood transfusion, history of iron intake in the past 3 months, severe malnutrition as per WHO criteria, known cases of HIV/chronic granulomatous disease/primary immunodeficiency disease, children on steroids >4 weeks, immunosuppressant (oral methotrexate and chemotherapy) and children with rheumatological illness/chronic renal failure/chronic liver disease were excluded from the study.

Thus, a total of 36 children with Iron deficiency anaemia were included as cases and were fur-

ther classified as moderately (Hb 7-9.9 gm/dl) to severely (Hb <7 gm/dl) anemic. An equal number of healthy age and sex matched children presenting to immunization clinic during that period were recruited as controls. A written informed assent was taken from the parents/guardians of the study subjects participating as cases and controls.

Clinical and demographic details

A baseline assessment including clinical history (immunization history and birth history) and examination was done at the time of enrolment. Demographic details including age, sex, address, contact number, date of enrolment and anthropometry (weight, height and body mass index) were recorded in the case record form.

Laboratory assessment

Laboratory assessment was done using peripheral venous blood obtained under all aseptic and universal precautions. 7 ml of peripheral venous blood was drawn under aseptic conditions and aliquoted in one EDTA and one plain vial. 2 ml blood in EDTA vial was used for complete hemogram by automated haematology analyzer (Mindray BC 6800), peripheral smear examination, ESR (fully automated ALIFAX Roller 20 LC ESR analyser) and flow cytometry based DHR assay performed by Beckman coulter cytomics FC500 flow cytometry analyzer. The reagents DHR and PMA were dissolved in DMSO, 5 µl and 10 µl aliquots were prepared and stored at -20°C. The rest 5 mL blood in iron free plain tube was centrifuged to separate the serum and was stored at -20°C, later used for Iron profile (by spectrophotometry), serum vitamin B12, folate and serum ferritin. The serum ferritin, vitamin B12 and folate were estimated using respective commercially available ELISA kits procured from Cal biotech Pvt Ltd.

Iron profile

The parameters of iron profile were measured according to ICSH 1978 guidelines [10]. Serum Iron (SI) levels less than 60 µg/dL, TSAT levels <16% along with a raised TIBC were taken as Iron deficient. The normal values of SI, TSAT and TIBC considered normal in our laboratory are 60-170 µg/dL, 16-50% and 250-400 µg/dL respectively.

Dihydrorhodamine (DHR) assay

Sample preparation: For each case, 2 tubes were prepared, stimulated and unstimulated, and appropriately labelled. Simultaneously, 2 tubes were prepared for control for each case (**Figure 1**).

Acquisition and analysis: Flowcytometric analysis was carried out on 5 colour 3 laser cytomics FC 500 flow cytometer from Beckman coulter. After excluding the debris, neutrophils were gated on the basis of forward scatter and side scatter characteristics. Care was taken to include only neutrophils by drawing appropriate gates around them. DHR flow histograms on this gated population were analyzed in both cases and controls for the parameters such as Percentage neutrophils that underwent stimulation, Mean fluorescence Intensity (MFI) of stimulated neutrophils, Mean fluorescence Intensity (MFI) of unstimulated neutrophils and Neutrophil oxidative index (NOI = MFI stimulated tube/MFI unstimulated tube).

Statistical analysis

The data was entered into a computer-based spreadsheet and analyzed using SPSS20.0. The neutrophil oxidative burst was estimated by Mean ± Standard Deviation. Student (unpaired) T-test was used for the comparison of hematological parameters, percent stimulated neutrophils, and mean NOI between cases and controls. The correlation of percentage of neutrophil showing stimulation and hemoglobin was done using the Pearson correlation coefficient. The *p*-value less than 0.05 was considered significant.

Results

The age of the patients ranged from 6 months to 5 years with a mean ± SD of 3.9±1.0 in the case group and 2.4±1.2 in the control group. Out of the 36 cases, 26 cases were moderately anemic and 10 cases were severely anemic.

Hematological parameters

Hemoglobin ranged from 3.3 to 9.9 g/dl with a mean ± SD of 7.7±1.6 g/dl in cases and 11.2 to

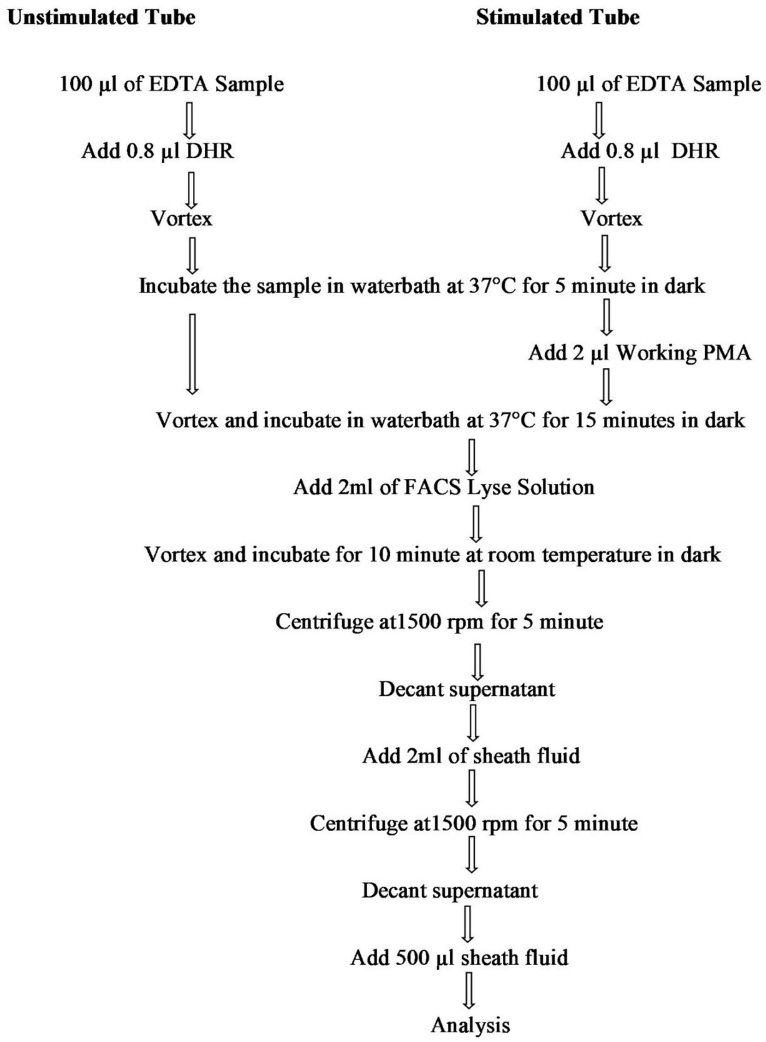

Figure 1. DHR Assay procedure.

13.4 g/dl with mean ± SD of 12.2±0.5 g/dl in controls. The hemoglobin, hematocrit, RBC count, MCV, MCH, MCHC were significantly higher in controls as compared to cases (P= 0.000). Platelets were significantly higher in cases as compared to controls (P=0.000) signifying reactive thrombocytosis. ESR was significantly higher in cases as compared to controls (P=0.000). The Total Leucocyte Count (TLC) of cases ranged from 4300-24600 with a Mean ± SD of 12522±5800. In controls, TLC ranged from 4400-13000 with a Mean ± SD of 7102±1695. The difference was statistically significant (P=0.000) (**Table 1**).

Iron profile

The Serum Iron and TIBC were significantly lower in cases as compared to controls and the TIBC was significantly higher in the former. Also, comparing cases with moderate and severe anaemia, it was found that Serum Iron was significantly lower in cases with severe anaemia as compared to those with moderate anaemia, though no significant difference was found between TIBC and TSAT between the two groups (**Table 2**).

Neutrophil oxidative burst

1. The percentage of neutrophils showing stimulation ranged from 0.67-99 with a Mean ± SD of 83±27 in cases however in controls it ranged from 85-99 with a mean ± SD of 96±2. It was significantly reduced in cases as compared to controls (P=0.002).

2. The MFI in unstimulated neutrophils was 1.0±0.62 in cases and 0.83±0.37 in controls and no significant difference was seen (P=0.166).

3. The MFI in stimulated neutrophils ranged from 6.6-351 (Mean ± SD of 157±71) in cases and 203-432 (Mean ± SD of 1.29±1.4) in controls. Thus the neutrophil MFI post-stimulation was significantly reduced in cases as compared to controls (P=0.000). On further comparing the Neutrophil oxidative index (NOI), it was found significantly reduced in cases (Mean ± SD of 181±108) as compared to controls (Mean ± SD of 422±167) (P=0.000) (**Table 3**; **Figure 2**).

Further, these parameters were compared in children with moderate and severe anaemia. The percentage of neutrophils showing stimulation, MFI in stimulated neutrophils, and NOI were significantly reduced in cases with severe anaemia as compared to cases with moderate anaemia (P=0.03, P=0.016 and P=0.005) respectively (**Table 4**; **Figure 3**).

Then the Hemoglobin and Iron levels were correlated with Neutrophil stimulation parameters.

Table 1. Hematologic parameters in cases and controls

Parameters	Cases (n=36)		Controls (n=36)		*p*-value
	Mean ± SD	Range	Mean ± SD	Range	
Hb (g/dl)	7.7±1.6	3.3-9.9	12.2±0.57	11.2-13.4	0.000*
Hematocrit (%)	26±4.4	14.6-37	36.7±3.31	26.6-46	0.000*
RBC count ($\times 10^{12}$/L)	4.1±0.78	3-6.4	4.4±0.26	4-5.2	0.052*
MCV (fl)	56.5±7.7	43-76	83.1±5.4	72-94	0.000*
MCH (pg)	17±3.6	11-23	27±2	23-31	0.000*
MCHC (g/dl)	28±3.9	20-36.4	32±1.4	27.7-36.6	0.000*
TLC ($\times 10^9$)	12522±5800	4300-24600	7102±1695	4400-13000	0.000*
Platelet count ($\times 10^9$)	4.7±1.8	1.9-8.7	2.5±1.6	1.3-4.4	0.000*
ESR (mm in 1st hour)	22±14.9	7-60	4±2.8	2-15	0.000*

*Denotes statistically significant *p*-value.

Table 2. Serum iron parameters in cases (n=36)

Parameters	Total Cases (n=36)	Moderate Anaemia (n=26)	Severe anaemia (n=10)	*p*-value
	Mean ± SD	Mean ± SD	Mean ± SD	
Serum Iron (µg/dl)	41±23	48±16	25±7	0.001*
TIBC (µg/dl)	439±22.7	437±22.7	442±23	0.567
Transferrin saturation (%)	8.4±2.4	8.5±2.6	8.1±2.06	0.672

*Denotes statistically significant *p*-value.

Table 3. DHR assay in the cases (n=36) and controls (n=36)

DHR assay parameters	Cases		Controls		*p*-value
	Mean ± SD	Range	Mean ± SD	Range	
% Stimulated neutrophils	83±27	0.67-99	96±2	85-99	0.002*
MFI (Unstimulated)	1.0±0.62	0.10-3.12	0.83±0.37	0.37-1.89	0.166
MFI (Stimulated)	157±71	6.6-351	305±58	203-432	0.000*
NOI	181±108	16.1-486	422±167	197-858	0.000*

*Denotes statistically significant *p*-value.

The Hemoglobin level showed a significant positive correlation with NOI and the percentage of neutrophils showing stimulation (r=0.514, P=0.001 and r=0.433, P=0.008) respectively (**Table 5**).

Discussion

The neutrophil oxidative burst or respiratory burst is a series of reactions in which superoxide ions are generated by the activity of NADPH oxidase (NOX2) and then metabolized to hydrogen peroxide, hypochlorous acid, and other reactive oxygen species to counteract the microbes. It is also a participant in the apoptosis of neutrophils and neutrophil extracellular traps (NETs) [21, 22]. NETs play various roles in inflammation, homeostasis and were found to be involved in autoimmunity as well as immuno-thrombotic events in COVID-19 [23-25].

The NOX2 activity is finely tuned and tightly regulated for appropriate immune functions. The lack of functional NADPH oxidase enhances the risk of recurrent infection conversely, excess reactive oxygen species production leads to tissue damage by oxidative stress.

This study was conducted on 36 preschool children with Iron deficiency anaemia and their neutrophil oxidative burst activity was assessed using a flow-cytometry-based DHR assay and was compared with healthy controls. The percentage of neutrophils showing stimulation, MFI before and after stimulation and Neutrophil oxidative index (NOI) was noted for

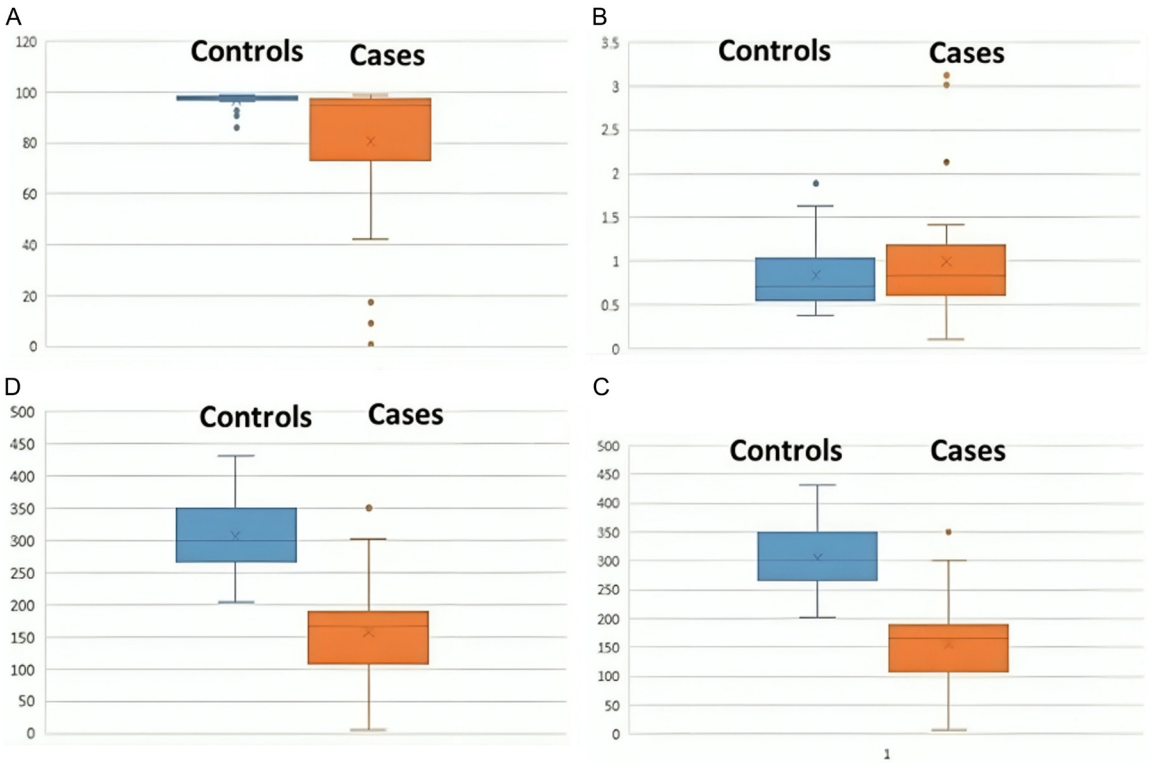

Figure 2. Box plots demonstrating comparison of Mean proportion of neutrophils showing stimulation (A), Mean fluorescence intensity of unstimulated neutrophils (B), Mean fluorescence Intensity of stimulated neutrophils (C), and Mean NOI (D) in cases and controls.

Table 4. DHR assay in cases with moderate and severe anaemia

DHR assay parameters	Moderate Anaemia		Severe Anaemia		*p*-value
	Mean ± SD	Range	Mean ± SD	Range	
% Stimulated neutrophils	87±20	17-99	63±42	0.67-98.23	0.03*
MFI (Unstimulated)	0.9±0.28	0.45-1.42	1.2±1.09	0.1-3.1	0.145
MFI (Stimulated)	173±60	67-351	107±89	0.5-245	0.016*
NOI	212±10	57-486	103±62	16-216	0.005*

*Denotes statistically significant *p*-value.

all subjects. The percentage of neutrophils showing stimulation, mean MFI in stimulated neutrophils and NOI was significantly reduced in children with Iron deficiency anaemia as compared to controls. A decrease in these parameters depicts impaired oxidative burst activity in neutrophils in children with IDA. The reason for a reduction in neutrophil oxidative burst activity in IDA can be due to a decrease in the activity of NADPH oxidase which is a heme-dependent enzyme containing iron in its structure. Depleted Iron stores and reduced plasma Iron in IDA may affect Iron availability for enzyme activity and thus lead to decreased oxidative burst activity. Not many studies have been conducted to assess neutrophil function in children using DHR assay. One similar study using a DHR assay demonstrated a significant decrease in oxidative burst activity in neutrophils in children with IDA (P<0.005) in comparison to healthy children [12]. On an extensive search of the literature, we could not find any more studies on children with IDA measuring neutrophil oxidative burst activity using DHR assay. However, there were few studies done on pregnant females and elderly IDA subjects

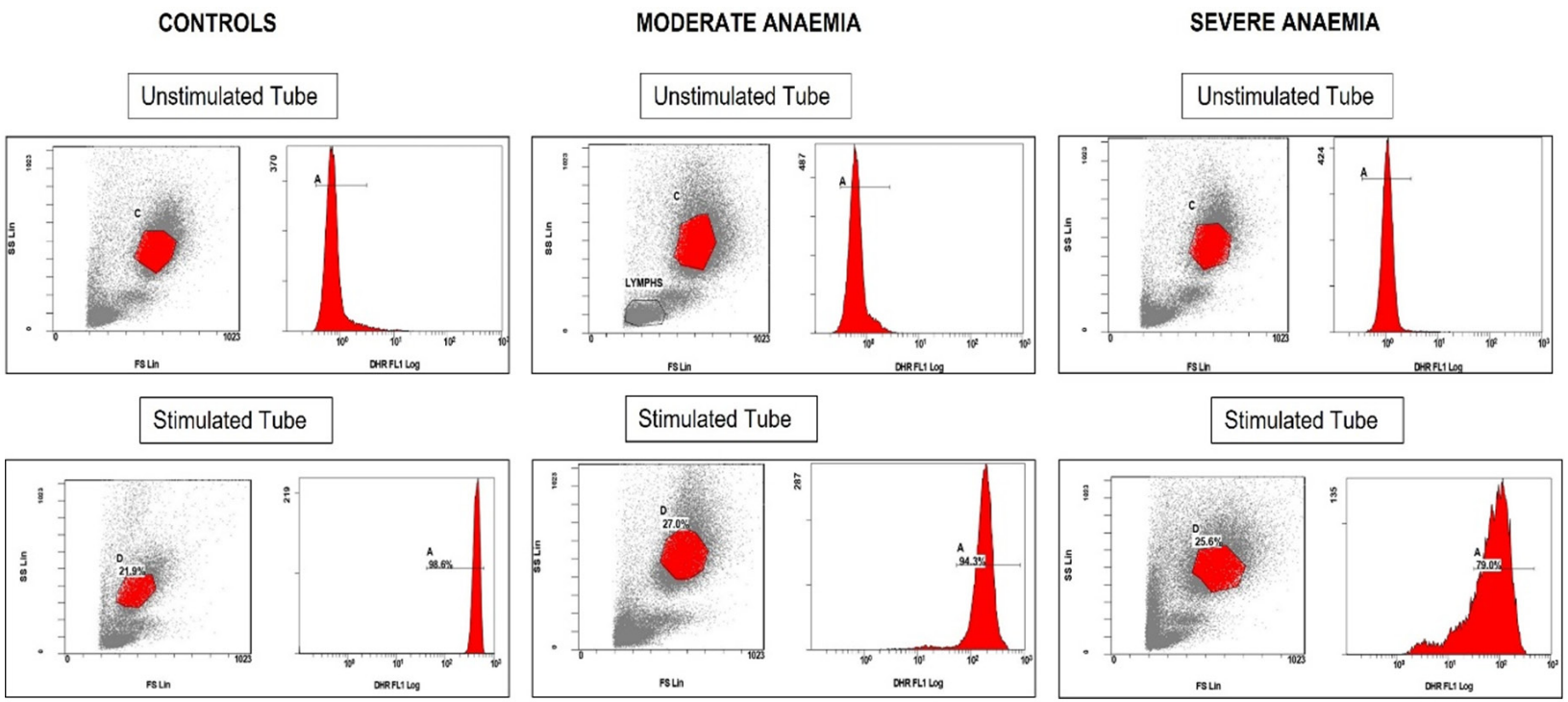

Figure 3. DHR assay histograms depicting a comparison of Mean Fluorescence Intensity in healthy controls, cases with moderate anaemia and severe anaemia before and after stimulation.

Table 5. Correlation of hemoglobin with NOI and % stimulated neutrophils in cases

	Neutrophil Oxidative Index (NOI)		% Neutrophils showing stimulation	
	r value	*p*-value	r value	*p*-value
Hemoglobin	0.514	0.001*	0.433	0.008*

*Denotes statistically significant *p*-value. The r value depicts Pearson correlation coefficient.

for the assessment of neutrophil oxidative burst activity by DHR assay. The study on elderly IDA subjects showed significantly lower (50%) oxidative burst in IDA in comparison to the control group [26] however study on pregnant females with IDA showed no significant difference [13]. The fact that pregnancy is associated with altered homeostasis and thus the results cannot be compared to children.

Few researchers have used the NBT test to assess neutrophil oxidative burst activity in children with IDA and concluded a significant fall in oxidative burst of neutrophils in children with IDA compared to healthy controls [5, 11]. Furthermore, a study by Kurtoglu et al showed significantly lower NADPH oxidase activity in IDA patients than in healthy controls by using a fluorometric procedure [4].

In contrast to our study, there are some studies in which no significant difference was observed in between children with iron deficiency anaemia and healthy controls by NBT [27, 28]. Since, these studies were mostly done using the NBT test to assess neutrophil oxidative burst activity, which is very subjective and thus prone to inter-observer variability, the results of our study and these studies cannot be compared. In contrast, the DHR assay is more objective, rapid, and highly sensitive and specific to assess the oxidative burst activity of neutrophils. Therefore, our data on oxidative burst activity in patients and healthy controls in the paediatric population suggests that neutrophil oxidative burst is reduced in IDA.

In the present study neutrophil oxidative burst activity was further lower in children with severe anaemia as compared to those with moderate anaemia ($P<0.05$). There are very few studies conducted on the association of iron deficiency with neutrophil function and how it varies as the Iron deficiency increases. A study by Chandra et al demonstrated a significant decrease in the oxidative reduction of nitro blue tetrazolium in severe anaemia as compared to moderate anaemia. Also, the bactericidal capacity of neutrophils was significantly lower in children with IDA as compared to healthy controls. The abnormalities in bacterial killing and the NBT were corrected within 4 to 7 days of parenteral iron which implies that iron deficiency is directly proportional to neutrophil function [5]. Thus, similar to our study, this study also demonstrated that neutrophil functions decreased steadily as plasma Iron levels decreased. However, this study was also done using the more subjective NBT assay. Also, the study had fewer subjects as compared to our study. After extensive research, no more studies were found to assess neutrophil oxidative burst in children with moderate and severe IDA.

We found a significant positive correlation of haemoglobin with neutrophil oxidative index and percentages of neutrophil showing stimulation which implies that as haemoglobin levels fall, neutrophil functions further decline. This implies that severely anaemic children are at a higher risk of infections and infection-related morbidity. This is a novel finding of our study as no study could be found correlating these parameters using this highly sensitive and accurate DHR assay. Future large-scale studies are planned to correlate these parameters.

The limitation of our study is that follow-up of cases was not done to find out the effect of iron supplementation on neutrophil oxidative burst. Moreover, this is a hospital-based study with a limited number of patients. Larger community-based studies with more study subjects and follow-up after the correction of Iron deficiency are needed in the future.

Conclusion

The present study found significantly reduced oxidative burst activity of neutrophils in Iron deficient preschool children as compared to healthy age-matched controls. The decrease in these parameters depicts impaired neutrophil

function resulting in poor innate response for pathogens thus further emphasizing IDA children are more susceptible to infections. Moreover, the neutrophil activity directly correlated with the severity of anaemia and fall in iron levels. Community-level measures to correct Iron deficiency will have a direct impact on infection-related morbidity in these children.

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Disclosure of conflict of interest

None.

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References

[1] World Health Organization. Anaemia 2023. https://www.who.int/news-room/fact-sheets/detail/anaemia.

[2] Stevens GA, Paciorek CJ, Flores-Urrutia MC, Borghi E, Namaste S, Wirth JP, Suchdev PS, Ezzati M, Rohner F, Flaxman SR and Rogers LM. National, regional, and global estimates of anaemia by severity in women and children for 2000-19: a pooled analysis of population-representative data. Lancet Glob Health 2022; 10: e627-639.

[3] International Institute for Population Sciences (IIPS) and ICF. National Family Health Survey (NFHS-5) India 2019-21. Demographic and Health Surveys 2022; 1: 1-714.

[4] Kurtoglu E, Ugur A, Baltaci AK, Mogolkoc R and Undar L. Activity of neutrophil NADPH oxidase in iron-deficient anemia. Biol Trace Elem Res 2003; 96: 109-115.

[5] Chandra RK. Reduced bactericidal capacity of polymorphs in iron deficiency. Arch Dis Child 1973; 48: 864-6.

[6] Bhaskaram C and Reddy V. Cell-mediated immunity in iron- and vitamin-deficient children. Br Med J 1975; 3: 522.

[7] Das I, Saha K, Mukhopadhyay D, Roy S, Raychaudhuri G, Chatterjee M and Mitra PK. Impact of iron defIciency anaemia on cell-mediated and humoral immunity in children: a case control study. J Nat Sci Biol Med 2014; 5: 158-163.

[8] Paclet MH, Laurans S and Dupré-Crochet S. Regulation of neutrophil NADPH oxidase, NOX2: a crucial effector in neutrophil phenotype and function. Front Cell Dev Biol 2022; 10: 945749.

[9] Frost JN, Wideman SK, Preston AE, Teh MR, Ai Z, Wang L, Cross A, White N, Yazicioglu Y, Bonadonna M, Clarke AJ, Armitage AE, Galy B, Udalova IA and Drakesmith H. Plasma iron controls neutrophil production and function. Sci Adv 2022; 8: eabq5384.

[10] Berrak SG, Angaji M, Turkkan E, Canpolat C, Timur C and Demiralp-Eksioglu EZ. The effects of iron deficiency on neutrophil/monocyte oxidative burst response in children. Cell Proliferation 2007; 40: 741-754.

[11] Hassan TH, Badr MA, Karam NA, Zkaria M, El Saadany HF, Abdel Rahman DM, Shahbah DA, Al Morshedy SM, Fathy M, Esh AMH and Selim AM. Impact of iron deficiency anemia on the function of the immune system in children. Medicine (Baltimore) 2016; 95: e5395.

[12] Ekiz C, Agaoglu L, Karakas Z, Gurel N and Yalcin I. The effect of iron deficiency anemia on the function of the immune system. Hematol J 2005; 5: 579-583.

[13] Youssef SR, Hendawy SF, Boshnak NH and Sedhom MS. Effect of intra-cellular trafficking on flow cytometric measurement of neutrophil's oxidative status in iron deficient pregnant females. J Clin Lab Anal 2018; 32: e22210.

[14] Chen Y and Junger WG. Measurement of oxidative burst in neutrophils. Methods Mol Biol 2012; 844: 115-124.

[15] Zielonka J, Hardy M, Michalski R, Sikora A, Zielonka M, Cheng G, Ouari O, Podsiadly R and Kalyanaraman B. Recent developments in the probes and assays for measurement of the activity of NADPH oxidases. Cell Biochem Biophys 2017; 75: 335-349.

[16] Murphy MP, Bayir H, Belousov V, Chang CJ, Davies KJA, Davies MJ, Dick TP, Finkel T, Forman HJ, Janssen-Heininger Y, Gems D, Kagan VE, Kalyanaraman B, Larsson NG, Milne GL, Nyström T, Poulsen HE, Radi R, Van Remmen H, Schumacker PT, Thornalley PJ, Toyokuni S, Winterbourn CC, Yin H and Halliwell B. Guidelines for measuring reactive oxygen species and oxidative damage in cells and in vivo. Nat Metab 2022; 4: 651-662.

[17] Donko A, Kuhns DB, Cousin MA, Smith MJ, Sacco KA, Klee EW, Joshi AY, Gavrilova RH, Holland SM, Leto TL and Abraham RS. Interpretation of Dihydrorhodamine-1,2,3 flow cytometry in chronic granulomatous disease: an atypical exemplar. J Clin Immunol 2022; 42: 986-999.

[18] Richardson MP, Ayliffe MJ, Helbert M and Davies EG. A simple flow cytometry assay using dihydrorhodamine for the measurement of the neutrophil respiratory burst in whole blood: comparison with the quantitative nitrobluetetrazolium test. J Immunol Methods 1998; 219: 187-193.

[19] Zavvar M, Zargaran S, Baghdadi H, Poopak P, Poopak AH, Nabatchian F, Fatahi Y, Khosravipour G and Poopak B. Dihydrorhodamine-123 flow cytometry method: time for substantial revision in technical procedure. Lab Med 2024; [Epub ahead of print].

[20] Recommendations for measurement of serum iron in human blood. Br J Haematol 1978; 38: 291-294.

[21] Dupré-Crochet S, Erard M and Nüβe O. ROS production in phagocytes: why, when, and where? J Leukoc Biol 2013; 94: 657-670.

[22] Kenny EF, Herzig A, Krüger R, Muth A, Mondal S, Thompson PR, Brinkmann V, Bernuth HV and Zychlinsky A. Diverse stimuli engage different neutrophil extracellular trap pathways. Elife 2017; 6: e24437.

[23] Veras FP, Pontelli MC, Silva CM, Toller-Kawahisa JE, de Lima M, Nascimento DC, Schneider AH, Caetité D, Tavares LA, Paiva IM, Rosales R, Colón D, Martins R, Castro IA, Almeida GM, Lopes MIF, Benatti MN, Bonjorno LP, Giannini MC, Luppino-Assad R, Almeida SL, Vilar F, Santana R, Bollela VR, Auxiliadora-Martins M, Borges M, Miranda CH, Pazin-Filho A, da Silva LLP, Cunha LD, Zamboni DS, Dal-Pizzol F, Leiria LO, Siyuan L, Batah S, Fabro A, Mauad T, Dolhnikoff M, Duarte-Neto A, Saldiva P, Cunha TM, Alves-Filho JC, Arruda E, Louzada-Junior P, Oliveira RD and Cunha FQ. SARS-CoV-2-triggered neutrophil extracellular traps mediate COVID-19 pathology. J Exp Med 2020; 217: e20201129.

[24] Ackermann M, Anders HJ, Bilyy R, Bowlin GL, Daniel C, De Lorenzo R, Egeblad M, Henneck T, Hidalgo A, Hoffmann M, Hohberger B, Kanthi Y, Kaplan MJ, Knight JS, Knopf J, Kolaczkowska E, Kubes P, Leppkes M, Mahajan A, Manfredi AA, Maueröder C, Maugeri N, Mitroulis I, Muñoz LE, Narasaraju T, Naschberger E, Neeli I, Ng LG, Radic MZ, Ritis K, Rovere-Querini P, Schapher M, Schauer C, Simon HU, Singh J, Skendros P, Stark K, Stürzl M, van der Vlag J, Vandenabeele P, Vitkov L, von Köckritz-Blickwede M, Yanginlar C, Yousefi S, Zarbock A, Schett G and Herrmann M. Patients with COVID-19: in the dark-NETs of neutrophils. Cell Death Differ 2021; 28: 3125-3139.

[25] Olsson LM, Johansson ÅC, Gullstrand B, Jönsen A, Saevarsdottir S, Rönnblom L, Leonard D, Wetterö J, Sjöwall C, Svenungsson E, Gunnarsson I, Bengtsson AA and Holmdahl R. A single nucleotide polymorphism in the NCF1 gene leading to reduced oxidative burst is associated with systemic lupus erythematosus. Ann Rheum Dis 2017; 76: 1607-1613.

[26] Paino IM, Miranda JC, Marzocchi-Machado CM, Cesarino EJ, De Castro FA and De Souza AM. Phagocytosis, oxidative burst, and produced reactive species are affected by iron deficiency anemia and anemia of chronic diseases in elderly. Biol Trace Elem Res 2009; 129: 116-125.

[27] Macdougall LG, Anderson R, McNab GM and Katz J. The immune response in iron-deficient children: impaired cellular defense mechanisms with altered humoral components. J Pediatr 1975; 86: 833-843.

[28] Yetgin S, Altay C, Ciliv G and Laleli Y. Myeloperoxidase activity and bactericidal function of PMN in iron deficiency. Acta Haematol 1979; 61: 10-4.