DIAGNOSTIC SIGNIFICANCE OF MEASURING RETICULOCYTE MATURITY INDICES IN IRON DEFICIENCY ANAEMIA

Tanweer Ahmed, Asad Mahmood, Nasir Uddin*, Helen Mary Robert, Rafia Mahmood, Usman Tahir Swati

Armed Forces Institute of Pathology/National University of Medical Sciences (NUMS) Rawalpindi Pakistan, *Combined Military Hospital Lahore/
National University of Medical Sciences (NUMS) Pakistan

ABSTRACT

Objective: To evaluate the diagnostic significance of reticulocyte maturity indices in iron deficiency anaemia.

Study Design: Comparative cross-sectional study.

Place and Duration of Study: Department of Hematology, Armed Forces Institute of Pathology, Rawalpindi, from Sep 2019 to Jun 2020.

Methodology: A total of 340 children, ages between 1 and 5 years of either gender, were divided into two groups based on haemoglobin and ferritin level. Group A comprised of 203 children with haemoglobin >11 g/dL andferritin level >7 ng/mL. Group B comprised of 137 children with haemoglobin <11g/dL and ferritin <7 ng/mL. Red blood cell and reticulocyte parameters were compared.

Results: Iron deficiency anaemia was found to be 37.6% in the study population. Comparison revealed that the children in groups B had significantly higher mean red cell distribution width (17.1 ± 1.3% vs. 13.3 ± 1.3%) and significantly lower hemoglobin (9.5 ± 1.0 g/dL vs. 12.7 ± 1.0 g/dL), mean corpuscular hemoglobin (21.8 ± 2.5 pg vs. 28.6 ± 2.3 pg), mean corpuscular volume (69.4 ± 3.5 fL vs. 80.4 ± 3.3 fL), mean corpuscular hemoglobin concentration (31.5 ± 1.8 g/dL vs. 32.7 ± 1.6 g/dL) and serum ferritin level (4.5 ± 2.6 mg/mL vs. 55.9 ± 32.1 ng/mL). No significant differences in the mean total red blood cell count, hematocrit and reticulocytes between two groups were noted (p>0.05) and significant differences were noted in terms of low, medium & high fluorescence ratios in both groups (p<0.05).

Conclusion: Our findings suggest that medium fluorescence ratio and high fluorescence ratio were higher among children with iron deficiency anaemia.

Keywords: High fluorescence ratio, Iron deficiency anaemia, Medium fluorescence ratio, Reticulocyte maturity indices.

INTRODUCTION

Anaemia is described as hemoglobin (Hb) level below normal but the criteria vary according to age and gender. Among children under the age of 5 years, "A secondary analysis of National Nutrition Survey Data 2011-2012" published in 2016, revealed prevalence of iron deficiency anaemia as 33.2%.[1] As per "World Health Organization (WHO)" in children of 6 months to 4 years, anaemia is labeled as hemoglobin less than or equal to 11 g/dL.[2] Peripheral blood reticulocyte count is considered helpful in diagnosis, classification and evaluation of individuals with anaemia while it also holds the center stage for evaluation and bone marrow response to anaemia treatment.[3]

Iron deficiency anaemia (IDA) is among the top 5 leading causes of years spent with disability.[4] Not only IDA is a major concern in pediatric age groups, pre-menopausal and pregnant ladies, but it is also considered a clinical issue affecting individuals who present with different medical and surgical morbidities.[5] It needs prompt and vigilant evaluation for the diagnosis and management of IDA to improve well-being of the affected patients. For identifying high-risk population, early detection of anaemia and evaluation of response to therapy, new laboratory parameters are being studied.[6] These new parameters are only available in modern automated hematology analyzers that are costly and not available at many places in developing countries like Pakistan. These new parameters are also not part of routine lab reports at the moment due to lack of standardization and studies on large number of population samples.

Reticulocytes are known to be non-nucleated immature red blood cells (RBCs) in the peripheral blood and contain reticulo-filamentous material composed of residual ribonucleic acid (RNA).[7] For reticulocyte counts, manual method is mostly adopted in diagnostic haematology laboratories. RNA has the propensity of

Correspondence: Dr Tanweer Ahmed, Resident Haematology, Combined Military Hospital Lahore Pakistan

reacting with certain supravital stains like New Methylene Blue or Brilliant Cresyl Blue. This results in formation of blue to purple granular or filamentous precipitate that can be viewed under microscope. Such reticulocytes are counted under high power field and reported as percentage of red blood cells.[8] This manual method is inherently inferior to newer automated reticulocyte analysis as it is time consuming and requires manual labor, skillful technician and microscopist. This method also does not provide any information about newer reticulocyte parameters.

Recently, Automated Reticulocyte Analysis (ARA) using flow cytometry is commonly used as a substitute to manual counts. ARA is considered a more rapid and accurate method which is easy to perform and provides number of reticulocytes count as well as numerous other indices that can help in diagnosing various pathologies and also in monitoring of bone marrow recovery.[9] Many modern automated hematology analyzers are equipped with ARA. As reticulocyte indices need standardization and labeling of reference values, their regular and proper use in clinical settings is yet to be seen. With ARA, reticulocytes can be classified into three subpopulations as per fluorescence intensity, reflecting maturity i.e. more the RNA content and fluorescence intensity, less mature the reticulocytes. These subpopulations or indices are Low Fluorescence Ratio (LFR), Medium Fluorescence Ratio (MFR) and High Fluorescence Ratio (HFR). With this classification, intracellular RNA levels are directly linked to fluorescence intensity which show degree of maturation of reticulocytes.[10]

Newer parameters related to reticulocytes have not been researched in our setup. International studies have demonstrated promising research in this regard and have suggested the routine use of these parameters for evaluation of anemias including IDA. Keeping this in view, this study was planned to evaluate diagnostic significance of measuring LFR, MFR and HFR in children aged 1-5 years with IDA.

METHODOLOGY

This comparative cross-sectional study was conducted at Department of Hematology, Armed Forces Institute of Pathology, Rawalpindi, from September 2019 ot June 2020. Approval from Institutional Review Board (FC-HEM18-1/READ-IRB/20/571 Dated 23 Aug 2019) was obtained before starting this study. Using non-probability consecutive sampling technique, a sample size of 340 participants was calculated

with 33% prevalence of IDA in our population of interest and confidence interval of 95%.

Inclusion Criteria: Children of either gender with age 1-5 years were included in the study.

Exclusion Criteria: Children taking any iron or vitamin supplements (as confirmed by parents/guardians of the children) were excluded.

All the study participants were divided into 2 groups. Group A (n=203) comprised of children with Hb>11 g/dL and serum ferritin level >7 ng/mL. Group B (n=137) comprised of children with Hb <11 g/dL and serum ferritin level <7 ng/mL. The purpose of study and procedures were explained and informed consent was sought from parents/guardians of all the study participants.

Venous whole blood samples from all children were drawn in commercially available Ethylenediaminetetraacetic acid (EDTA) containing whole blood collection tube (for complete blood counts and reticulocyte parameters) and Gel tube (for serum ferritin level). Samples were properly labelled and dealt within 3 hours of collection. For every blood sample from the participants, the hematology analysis was performed on a "Sysmex XN-3000 automated hematology analyzer" while serum ferritin level was measured using a Chemiluminescence-based automated IMMULITE 1000 immunoassay analyzer. Reticulocyte count was corrected by using following formula, keeping normal hematocrit at 45%: corrected reticulocyte count (%) = (Patient's Hematocrit/40) x (Reticulocyte count %).

Children having anaemia (group B) were compared with those who did not have anaemia (group A). All the data was processed through IBM Statistical Package for Social Sciences (SPSS), version 26. Mean and standard deviations of were calculated for quantitative variables including parameters related to red blood cells and reticulocytes. Independent sample t-test used to compare groups considering *p*-value of ≤0.05 as statistically significant.

RESULTS

Out of 340 participants of the study, 184 (54.1%) were males and 156 (45.9%) were females. The mean age at the time of study was 1.59 years. Frequency of IDA in the population under study was noted to be 128 (37.6%). Comparison of red blood cell parameters between the two groups was shown in Table-I. When compared to group A, the children in groups B had significantly higher mean red cell distribution width (RDW%) and significantly lower haemoglobin (g/dl),

mean corpuscular haemoglobin (MCH), mean corpuscular volume (MCV), mean corpuscular haemoglo-bin concentration (MCHC) and serum ferritin levels. No statistically significant difference in mean total red blood cell count (TRBC) (p=0.08) and Haematocrit (HCT) (p=0.09) between the two groups was noted.

Table-I: Red blood cell parameters and serum ferritin in both groups .

Parameters	Group A (n=203)	Group B (n=137)	p-value
Total Red Blood Cell Count (TRBC, x 109/L)	4.7 ± 2.6	4.3 ± 0.4	0.080
Hemoglobin (Hb, g/dL)	12.7 ±1.0	9.5 ± 1.0	<0.001
Hematocrit (Hct, %)	36.4 ± 4.0	35.6 ± 3.9	0.090
Mean corpuscular hemoglobin (MCH, pg)	28.6 ± 2.3	21.8 ± 2.5	<0.001
Mean Corpuscular Volume (MCV, fL)	80.4 ± 3.3	69.4 ± 3.5	<0.001
Mean Corpuscular Hemoglobin concentration (MCHC, g/dL)	32.7 ± 1.6	31.5 ± 1.8	<0.001
Red Cell Distribution width (RDW, %)	13.3 ± 1.3	17.1 ± 1.3	<0.001
Serum Ferritin (ng/mL)	55.9 ± 32.1	4.5 ± 2.6	<0.001

Table-II shows comparison of reticulocyte parameters between the two groups. There was no statistical difference in mean reticulocytes (%) between group A and group B (p=0.100). In terms of reticulocyte indices, significant difference was observed in LFR, MFR and HRF (p<0.05) between two groups.

Table-II: Reticulocyte parameters in both groups.

Parameters	Group A (n=203)	Group B (n=137)	p-value
Reticulocytes (%)	1.1 ± 0.3	1.1 ± 0.3	0.100
Low Fluorescence Ratio (LFR, % total reticulocytes)	91.9 ± 3.7	85.9 ± 4.0	<0.001
Medium Fluorescence Ratio (MFR, % total reticulocytes)	7.0 ± 3.6	11.2 ± 3.9	<0.001
High Fluorescence Ratio (HFR, % total reticulocytes)	1.0 ± 0.7	2.8 ± 0.8	<0.001

DISCUSSION

While traversing reticulocyte channel in Sysmex automated hematology analyzers, cell membranes of red blood cells (RBCs), white blood cells (WBCs) and platelets are perforated by a specific lysing reagent. A fluorescent marker then penetrates these cells and tags the nucleic acids in cytoplasm and nuclei. Intensity of fluorescent signal is directly proportional to the amount of nucleic acids in these cells. This is followed by separation of reticulocytes from mature RBCs. Reti-culocyte maturity indices (LFR, MFR &and HFR) are also measured. This is based on the flow cytometric prin-ciples of forward scatter and fluorescent intensity.[11] These indices are known to highlight reticulocyte im-maturity and bone marrow activity.

This study has been carried out with an aim to evaluate reticulocyte maturity indices among children with and without IDA. We measured these reticulocyte maturity indices with a modern and automated state of the art Sysmex XN-3000 Hematology Analyzer and found statistically significant difference in terms of reticulocyte maturity indices between patients with IDA and those without IDA. It seems that monitoring of effective erythropoiesis in response to anaemia can be assessed employing quantitative measurements of reticulocyte subpopulations. Our findings highlighted that evaluating reticulocyte maturity indices using quantification of fractions of reticulocytes as LFR, MFR or HFR can be performed easily where facilities of resources and newer automated hematology analyzers exist. Reticulocyte fluorescence intensity has been found to be directly linked with the quantity of intrace-llular ribonucleic acid.[12] Immature reticulocytes fraction (IRF) is the sum of medium fluorescence ration (MFR) and high florescence ratio (HFR). IRF indicates young reticulocytes released into peripheral circulation prematurely and these parameters can be effectively used for the evaluation of erythropoietic efficiency during anaemias including IDA.[13,14]

A possible explanation for the findings of our study is that a rise in the immature reticulocytes in the blood of a patient with IDA significantly highlights body's response to iron deficiency anaemia provided that the medullary tissues and cortical elements nee-ded for erythropoiesis cycle are conserved. Anaemic hypoxia is also thought to stimulate release of erythro-poietin from the kidneys which results in increased cell proliferation and differentiation.[15] This explanation also highlights that the presence of functioning eryth-ropoiesis cycle along with erythropoietin's response to anaemic hypoxia can be ensured by measuring reticu-locyte maturity indices and IRF during initial evalua-tion and subsequent monitoring of the response to therapy.

Maturation time of reticulocytes might decrease in bone marrow in case of severe anaemia,[16] as higher quantity of immature reticulocytes are discharged in the peripheral blood and stay there for an estimated more than 48 hours until these get transformed into mature RBCs, hence, number of immature reticulocy-

tes in the peripheral blood is increased.[17] Indices linked to immaturity of reticulocytes are increased in IDA demonstrating a decline in raw materials required for the production of hemoglobin and transformation of reticulocytes into mature RBCs. Findings of our study are in agreement with other international studies which reported increase in MFR and HFR in patients with anaemias including IDA. Sunkara *et al*, demonstrated that diagnosis of IDA can be made helpful if various reticulocyte parameters are adopted without using any further biochemical investigations. The study found that, owing to ineffective erythropoiesis, immature reticulocyte fraction and immature reticulocyte subpopulations were significantly higher in IDA.[18] Wollmann *et al*, demonstrated that reticulocyte indices related to maturity may be utilized as early markers of IDA and anemia based on their findings that in comparison to control group, individuals suffering from IDA presented with an increased proportion of mean fluorescence ration (10.3 ± 4.7% vs. 6.0 ± 3.4%; *p*-value= 0.003) and high fluorescence ratio (2.3 ± 0.87% vs. 0.9 ± 0.9%; *p*-value=0.03).[19] Choi *et al*, compared reticulocyte maturity indices & iron-related parameters between healthy and iron deficient females. The study demonstrated that middle & high fluorescence ratios started increasing when serum iron and serum ferritin levels were depleted. The increment in reticulocyte maturity indices was greatest when iron deficiency was overt.[14] Zhao *et al*, compared various red blood cell & reticulocyte parameters during evaluation of anemias and found that the low fluorescent reticulocyte sub-population significantly decreased, while, medium & high fluorescent reticulate subpopulations increased in all cases of anemia compared to control population. The study suggested utilizing mean corpuscular volume, red cell distribution width and reticulocyte maturity-related parameters for the evaluation of various anemias.[20] Velasco-Rodríguez *et al*, analyzed various reticulocyte parameters in delta-beta thalassemia trait, beta thalassemia trait and IDA and correlated them with the corresponding pathophysiologic features. The study demonstrated that patients with IDA had much more immature reticulocytes and less absolute reticulocyte count than beta thalassemia trait. The study suggested that not only red cell indices but also reticulocyte maturity indices may be utilized to differentiate the three clinical entities.[21]

Since average lifecycle of an RBC in peripheral circulation is about 120 days, the usual renewal of RBC mass equates with 1% of RBCs in circulation, therefore, identification of abnormality in the routine hematimet-

ric indices of patients with IDA may require weeks-to-months.[12,18] On the other hand, reticulocyte maturity indices show changes much earlier with the benefits of automation and more accuracy. For these reasons, reticulocyte maturity indices can prove helpful as significant and early markers of anemias including IDA.

LIMITATION Of STUDY

Limitations in the usage of reticulocyte maturity indices in the laboratory practices at some facilities, because of either definitions or issues due to standardization and correlation between different methods employed, could pose a significant challenge. The findings of this study warrant further research involving different age groups with larger sample size of both healthy individuals and population at high risk of developing IDA at different centers of Pakistan so that benefits of automation and newer reticulocyte parameters may be reaped by inducting these tests in routine catalogues of haematology laboratories.

CONCLUSION

Our findings suggest that MFR and HFR are high among children with IDA, highlighting increased erythropoietic activity. These indices can be adopted as rapid, less time consuming, less labor intensive and lesser invasive procedure for early diagnosis of iron deficiency anaemia.

Conflict of Interest: None.

Authors' Contribution

TA: Direct contribution to conception, desing, analysis & interpretation, AM: Intellectual contribution to analysis, literature review & manuscript preparation, NU: Intellectual contribution to analysis, literature review & manuscript preparation, HMR: Intellectual contribution to analysis, literature review & manuscript preparation, RM: Intellectual contribution, manuscript preparation, UTS: Manuscript preparation & data analysis.

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