Reticulocyte Hemoglobin Vis-À-Vis Immature Reticulocyte Fraction, as the earliest Indicator of Response to Therapy in Iron Deficiency Anemia

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Abstract
Aim: To evaluate reticulocyte hemoglobin (RET-Hb) vis-à-vis immature reticulocyte fraction (IRF) as an earliest indicator of response to iron therapy in iron deficiency anemia (IDA), by assessing change in RET-He and IRF at 48 hours after initiation of intravenous iron therapy.

Material and methods: A hospital based interventional, analytic study was conducted among 144 patients (age group 15-65 years) with newly diagnosed and untreated IDA admitted in medicine ward and not suffering from any inflammatory disorders (excluded by C-reactive protein). Patient having other forms of anemia/hemoglobinopathies/ malignancy, MCV >80 fL and pregnant female were excluded. All patients were subjected to automated CBC, RET-He, iron studies and iron staining of bone marrow aspirates. Then intravenous iron sucrose was given along with oral antioxidants. After 48 hours, CBC, RET-He and IRF were repeated for each patient.

Result: Total 144 patients were included. Of these, 42 patients were excluded due to aparticulate bone marrow aspirate. Remaining 102 patients were classified in to Group A (grade 0 and 1- depleted iron stores) and Group B (grade 2 and 3 - functional iron deficiency). RET-He and IRF increased significantly at 48 hours after initiation of intravenous iron therapy (post therapy) as compared to baseline (pre therapy) in both the two groups as well when all patients were considered together. Post therapy, the mean increase in RET-He was significantly smaller in magnitude in group B than in group A. The increase in IRF was not significantly different between the two groups.

Conclusion: RET-Hb, a real time indicator of iron supply (hemoglobinization) to the developing RBC’s, is the earliest marker of response to iron therapy as compared to IRF (representative of reticulocyte count).

Introduction
Iron deficiency is one of the most common nutrient deficiencies and a leading cause of anemia worldwide.¹ The challenge in iron deficiency anemia (IDA) is not only to diagnose it early but also to monitor and evaluate its response to iron therapy at the earliest. Response to iron therapy is classically assessed by increase in peripheral blood Reticulocyte count which occurs in 3-4 days and rise in hemoglobin within the first week.²

Automated counters form an integral part of modern day hematology. Immature Reticulocyte Fraction (IRF) is one of the newer parameters of automated haematology analyzers and is a sensitive measure of erythropoiesis.³ However, in certain situations like bleeding, IRF will increase despite no improvement in Haemoglobin (Hb). Moreover, IRF does not give any information on the incorporation of iron into developing red blood cells.

A newer reticulocyte parameter Ret-He is a measure of hemoglobin content of the freshly produced red blood cells and offers real-time information on iron supply for erythropoiesis.⁴ RET-He is not an acute phase reactant and has been found to be useful in detecting
response to iron therapy, changing as early as 3rd – 4th days. IRF denotes fraction of developing RBC’s (reticulocytes) which have high content of mRNA with least maturity. The utility of IRF in monitoring anemia has been reported in various studies.

This study was undertaken to evaluate Ret-Hb vis-à-vis IRF as an earliest indicator of response to iron therapy in iron deficiency anemia (IDA), by assessing change in reticulocyte haemoglobin (RET-He) and IRF at 48 hours after initiation of intravenous iron therapy.

### Materials and Methods

This hospital based interventional, analytic study was conducted at a tertiary care center in Rajasthan, during September 2013 to December 2014, after obtaining due permission from Research Review board/ Institutional Ethics committee and informed written consent of the study participants. Hundred forty four patients (age 15 to 65 years) with male: female ratio 1:1.83. Of the 102 patients, 73 (71.6%) had no stainable iron in bone marrow (grade 0), 10 (9.8%) had grade 1 stainable iron, 8 (7.8%) had grade 2 stainable iron and 11 (10.8) had grade 3 stainable iron. Based on the grading of bone marrow iron store, the patients were classified into Group A (grade 0 and 1- depleted iron stores) and Group B (grade 2 and 3 -functional iron deficiency).

In comparison to Group B, patients of Group A had significantly lower RET-He (Group A 17.84 ± 2.39 vs. Group B 25.08 ± 4.42; P< 0.0001) and lower serum ferritin (Group A 8.68 ± 2.80 vs. Group B 15.61 ± 4.68; P < 0.0001) before start of iron therapy (Table 1).

The patients were then started on intravenous iron sucrose (300mg dissolved in 250 ml Normal Saline IV infusion over 4 hours on Day 0 and Day 1) along with oral antioxidants. After 48 hours, CBC, RET-He and IRF were repeated for each patient.

Blood samples from patients were drawn in EDTA vials for CBC including RET-He, IRF and peripheral blood smear; and in plain vials for serum ferritin, serum iron and TIBC. Patients having microcytic hypochromic anemia, serum ferritin below 20 ng/ml and transferrin saturation < 20% were selected. Patient’s clinical history, findings of physical examination and other relevant data, including lab test results, were recorded in structured forms. CBC, RET-He and IRF were tested on Sysmex XT 4000i automated analyzer. Serum ferritin was measured on IMMULITIE 2000 Systems analyzer using a solid-phase, two-site chemiluminescent immunometric assay. Serum iron was measured using colorimetric assay. TIBC was measured using saturation – precipitation method. Transferrin saturation (TSAT) was calculated as TSAT = (serum iron/TIBC) x100 and expressed as percentage.

Under strict aseptic precaution bone marrow aspirates were obtained from posterior iliac crest and sent for Wright – Giemsa staining along with Prussian blue staining for estimation of iron store.

The patients were then started on intravenous iron sucrose (300mg dissolved in 250 ml Normal Saline IV infusion over 4 hours on Day 0 and Day 1) along with oral antioxidants. After 48 hours, CBC, RET-He and IRF were repeated for each patient.

### Statistical Analysis

Microsoft Excel® and SPSS® 20 for Windows® were used for data storage and analysis. The qualitative data were expressed in percentages and quantitative data were expressed as mean ± standard deviation. Student’s t test was used to determine statistical difference between variables. Results were considered significant if P < 0.05.

### Results

A total of 144 patients were included in the study. Of these, 42 (29.2%) patients were excluded from final statistical analysis as their bone marrow aspirates were aparticulate and therefore, their iron stores could not be assessed. From the bone marrow aspirates of remaining 102 patients, Prussian blue stained films were examined and graded.

These 102 study participants had mean age 35.63 ± 15.96 years (range 15 to 65 years) with male: female ratio 1:1.83. Of the 102 patients, 73 (71.6%) had no stainable iron in bone marrow (grade 0), 10 (9.8%) had grade 1 stainable iron, 8 (7.8%) had grade 2 stainable iron and 11 (10.8) had grade 3 stainable iron. Based on the grading of bone marrow iron store, the patients were classified into Group A (grade 0 and 1- depleted iron stores) and Group B (grade 2 and 3 -functional iron deficiency).

<table>
<thead>
<tr>
<th>Total</th>
<th>Group A (N = 83)</th>
<th>Group B(N = 19)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>35.63 ± 15.96</td>
<td>39.16 ± 16.07</td>
<td>0.297</td>
</tr>
<tr>
<td>Hb (g/dL)</td>
<td>4.26 ± 1.16</td>
<td>4.142 ± 1.2371</td>
<td>0.651</td>
</tr>
<tr>
<td>MCV (fL)</td>
<td>72.17 ± 3.55</td>
<td>73.847 ± 3.3180</td>
<td>0.022*</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>19.38 ± 1.23</td>
<td>19.400 ± 1.2715</td>
<td>0.944</td>
</tr>
<tr>
<td>RDW-CV (%)</td>
<td>18.96 ± 1.11</td>
<td>18.289 ± 1.2371</td>
<td>0.001*</td>
</tr>
<tr>
<td>Iron (µg/dl)</td>
<td>19.22 ± 5.59</td>
<td>19.45 ± 5.643</td>
<td>0.396</td>
</tr>
<tr>
<td>Ferritin (ng/ml)</td>
<td>9.97 ± 4.19</td>
<td>15.611 ± 4.6753</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>TIBC (µg/dl)</td>
<td>429.32 ± 36.07</td>
<td>432.21 ± 33.710</td>
<td>0.022*</td>
</tr>
<tr>
<td>TSAT (%)</td>
<td>4.51 ± 1.39</td>
<td>4.211 ± 1.3300</td>
<td>0.291</td>
</tr>
<tr>
<td>IRF</td>
<td>27.21 ± 3.08</td>
<td>27.46 ± 3.29</td>
<td>0.711</td>
</tr>
</tbody>
</table>

@ Significant
RDW-CV 18.965 ± 3.627 vs. 20.169 ± 2.124, P = 0.000 @

Mean difference in IRF:

Parameter | Overall | Group A | Group B | P value
--- | --- | --- | --- | ---
IRF | 3.8133 ± 0.3966 | 3.08 | 3.05 | 3.35 | 0.000 @
IRF | 3.5806 ± 0.0252 | 3.5067 | 3.5067 | 5.0707 | 0.000 @

All values are mean ± SD; @ Significant

in RET-He was significantly smaller in magnitude in group B than in group A. The increase in IRF was not significantly different between the two groups (Table 3).

### Discussion

All the patients included in our study had microcytic hypochromic anemia with low serum ferritin and low transferrin saturation. Those with grade 0 and 1 were considered to have depleted iron stores and, therefore, represented absolute iron deficiency (deplete iron store). Those with grade 2 and 3 in our study had functional iron deficiency.9

Reticulocytes are non-nucleated immature red blood cells (RBCs) in peripheral blood. Reticulocytes are sensitive in detecting erythropoietic activity as they have a more rapid turnover in circulation than mature red cells (1–2 vs. 120 days). Reticulocyte indices provide a real time evaluation of the bone marrow activity, reflecting the balance between iron and erythropoiesis of the preceding 48 hours.6,10 Response to iron therapy could be detected at an earlier stage, when RBC indicators are still as pretreatment level but the iron stores are sufficient to the point of affecting hematopoiesis and inducing production of a certain percentage of reticulocytes with increased Hb content, resulting in a progressive increase of RET-He.11-14

The current study revealed that RET-He and IRF increase significantly after intravenous iron therapy as early as 48 hours after initiation of treatment irrespective of the status of the iron stores (deplete or functional iron deficiency). The mean increase in RET-He (3.8133 ± 0.3966 vs. 1.4947 ± 2.1309, P < 0.001) was of significantly smaller magnitude in group B than in group A. The increase in IRF was not significantly different between the two groups (8.5169 ± 5.0252 vs. 8.4000 ± 5.3905, P > 0.01). Thus those patients who had depleted iron stores showed greater increase in RET-He in response to intravenous iron therapy as compared to those with functional iron deficiency. These results were similar to the ones reported by Brugnara et al.6 who showed that reticulocyte hemoglobin content (CHR) increased within 4 days of intravenous iron therapy. Mittman et al15 had also reported similar increase in CHR within 48 hours of intravenous iron therapy. Another study also reported increase in CHR with intravenous iron therapy.16 RET-He and CHR both are comparable to each other.17 Identifying patients who show low RET-He after IV iron therapy makes it possible to identify the patients who are not responding to IV iron. Thus these patients may be offered additional diagnostic tests and management strategies.

IRF just reflects the erythropoietic activity of bone marrow but does not show the actual incorporation of iron in developing RBC’s (hemoglobinization of mature RBC’s). RET-He is not only sensitive but also a real time indicator of iron supply to the developing RBC’s. In other words, RET-He is a real time parameter of hemoglobinization.

In our study we observed that mean increase in IRF did not show significance between the two groups (iron depleted v/s functional iron deficiency) but there was significant increase in RET-Hb which confirms that mean magnitude change in RET-Hb is a real time indicator of iron supply to the developing RBC’s.

The aim of iron therapy in IDA is to improve iron supply (Hemoglobinization) to the developing RBC’s. As the traditional parameter of treatment response – reticulocyte count and newer parameter – IRF only reflect erythropoietic activity of bone marrow and they do not reflect the incorporation of iron in maturing RBC’s, which is the primary aim of iron therapy. RET-Hb is a real time indicator of iron supply (Hemoglobinization) to the developing RBC’s and is a
useful marker of response to iron therapy. RET-He had the advantage of being measured on routine haemogram at a little increment of cost and the results can be obtained readily.

All these studies support utility of RET-He as an early indicator of response to iron therapy in IDA. Our previous article also showed utility of RET-He as a marker of bone marrow iron store in iron deficiency anemia. On the basis of our observations, we can conclude that RET-He can be used as a marker of bone marrow iron store as well as an earliest indicator of response to therapy in iron deficiency anemia.

Limitations

Our study had certain limitations. We included patients only with severe anemia in our study. Whether our findings can be extrapolated to those with mild to moderate degree of anemia or with iron deficiency state is debatable. We studied effect of intravenous iron in IDA so the results may not applicable on oral iron therapy. This study was done at a tertiary care centre, and recruited inpatients only, resulting in a limited sample size, thus the nature of the investigation and the results do not imply a general case, and further studies with a larger sample size are needed.

Conclusion

Both RET-He and IRF increase as early as 48 hours after initiation of intravenous iron therapy. However, the mean change in IRF was not significant while mean change in RET-Hb had statistical significance. In essence, RET-Hb, a real time indicator of iron supply (hemoglobinization) to the developing RBC’s, is the earliest marker of response to iron therapy.

Conflict of Interests

None of the authors have conflict of interest.

References