Reticulocyte Analysis in Iron Deficiency Anemia and Hemolytic Anemia

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Abstract

Reticulocyte analysis was studied in 28 anemic patients, 15 with iron deficiency anemia (IDA), and 13 with hemolytic anemia including 9 glucose 6 phosphate dehydrogenase deficiency (G6PD def.), and 4 with G6PD def. combined with HbE trait or α thalassemia trait (α thal trait). The reticulocyte analysis among these patients showed the increased number of reticulocyte percentage with low degree of maturation in both IDA and G6PD def. patients. The significantly decreased reticulocyte hemoglobin content (CHr) was found in IDA (CHr= 21.74 ± 4.61 pg in IDA *vs* 28.41 ± 1.34 pg in normal; p-value = < 0.0001), whereas, increased CHr was found in G6PD def. patients. In addition, the G6PD def. patients also showed a significant increase in mean corpuscular reticulocyte volume (MCVr) when compared to normal (MCVr = 132.0 ± 8.39 fl. in G6PD def. *vs* 110.39 ± 5.09 in normal; p - value = < 0.0001). However, a significant decrease in MCVr was found in IDA patients (MCVr = 95.89 ± 8.57 fl.; p-value = < 0.0001 *vs* normal). From this study, we can suggest that the reticulocyte hemoglobin content (CHr) and mean corpuscular reticulocyte volume (MCVr) are the important defects in patients with iron deficiency anemia.

Key word : Reticulocyte Analysis, Iron Deficiency Anemia, G6PD Deficiency

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Reticulocyte analysis in peripheral blood samples is the simplest and non-aggressive method available to evaluate erythropoietic activity in the bone marrow. The manual microscope counting of supravital stained cells is imprecise and time consuming[1-3]. The recent automated flow cytometry using RNA-binding fluorochromes has greatly improved accuracy, precision at the expense of high[4-6]. Another advantage of flow cytometry technique is the possibility to measure the degree of maturation within the reticulocyte population based on the proportion between the fluorescent intensity and the amount of RNA present in the cell[7-9]. The Technicon H*3 automated flow cytometer can simultaneously measure the volume and hemoglobin concentration in red blood cells and reticulocytes, and has valuable diagnostic applications for reticulocyte counting on anemic patients with microcytic and macrocytic red cells[10]. Reticulocyte monitoring was also used in patients receiving recombinant human erythropoietin treatment[11], bone marrow transplantation[12], and cancer chemotherapy[13]. We report here the H*3 reticulocyte evaluation in patients with iron deficiency anemia (IDA), and hemolytic anemia (HA), which included glucose 6 phosphate dehydrogenase (G6PD) deficiency, G6PD def. combined with HbE trait and alpha thalassemia trait. The results were compared with normal subjects to assess the anemic status and erythropoietic activity.

PATIENTS AND METHOD

Patients and Subjects

Twenty eight anemic patients consisted of 15 with IDA, 9 with G6PD deficiency, 4 with G6PD deficiency combined with HbE trait or α thalassemia trait, and 25 normal subjects were studied. Diagnosis of various types of anemia was based on hemoglobin typing, DNA analysis, G6PD assay, serum iron (SI) and total iron binding capacity (TIBC). In addition, the WHO criteria for discrimination of iron deficiency anemia was also followed[14].

Hematological analysis

The hematological parameters were analyzed by using Technicon H*3 RTX automated hematology analyzer within 4 hours after venous blood collection using EDTA as an anticoagulant. The Mile H*3 [Mile, Diagnostics Division, Tarrytown, NY] blood analyzer, which combines the capabilities of a routine CBC and fivepart differential blood analyzer with reticulocyte analyzer was utilized. The H*3 used flow cytometric analysis of cells with laser light scattering to quantify cell volume, hemoglobin concentration, and light absorbance of cells stained with Oxazine 750 to detect reticulocytes, and distinguish them from mature red cell[15,16].

Reticulocyte analysis

Reticulocyte analysis was also evaluated by using Technicon H*3 automated hematology analyzer. Three microliters of whole blood was mixed and incubated with 3 ml of reticulocyte reagent for 15 minutes. The reticulocyte cytoplasmic RNA was selectively stained with fluorescent dye, Oxazine 750. The prepared samples were aspirated through the H*3 red cell flowcell and were detected by three detectors, a low angle (2^{o} to 3^{o}), a high angle (5^{o} to 15^{o}), and absorption detectors. By these detectors, the stained reticulocytes were counted separately from unstained erythrocytes, platelets, and leukocytes. The reticulocytes can be classified by fluorescent intensity, which is proportional to their RNA content, into low, medium and high fluorescent absorption reticulocytes (L, M and H reticulocytes respectively), that indicated their maturation. The H*3 reticulocyte analysis can measure reticulocyte mean corpuscular volume (MCVr) in femtoliters, and corpuscular hemoglobin concentration mean (CHCMr) in gram per deciliter separately from MCV and CHCM of mature erythrocytes. Mean hemoglobin content of reticulocyte (CHr) and of erythrocyte (CH) were calculated from the volume and hemoglobin concentration of a single cell[17-19].

Statistical Analysis

Results were presented as mean ± standard deviation. Statistical differences between groups were tested using Wilcoxon rank sum test, ANOVA, and p-value of less than 0.05 were considered to be significant.

RESULTS

Hematological analysis

Erythrocyte parameters in IDA, G6PD def. patients, and normal subjects are shown in Tables 1 and 2. Significant decrease in red blood cell count (RBC), hemoglobin (Hb), hematocrit (Hct), and cellular hemoglobin concentration mean (CHCM) were demonstrated in both types of ane-

Table 1. Mean ± SD of hematological analysis from H*3 hematological analyzer in IDA and G6PD deficiency patients compared with normal subjects.

Types	RBC (10^6/μl)	Hb (g/dl)	Hct (%)	MCV (fl)	CHCM
Normal	4.94±0.42	14.31±1.57	43.40±4.70	87.74±4.86	32.90±1.38
IDA	4.25±0.63	9.10±2.13	30.92±6.0	72.52±9.47	30.40±2.51
p-value	(0.002)	(<0.0001)	(<0.0001)	(<0.0001)	(<0.0001)
G6PD def.	3.45±0.82	10.51±1.46	33.23±5.44	97.93±10.34	29.88±1.65
p-value	(<0.0001)	(0.0032)	(<0.0001)	(0.0026)	(0.0008)

Table 2. Comparison between the percentage of hypochromic RBC (Hypo), Microcytosis (Micro), Macrocytosis (Macro) and red blood cell distribution width (RDW) in IDA, G6PD deficiency patients and normal subjects.

Types	n	Hypo (%)	Micro (%)	Macro (%)	RDW
Normal	25	3.33±1.69	0.88±0.78	0.71±0.19	13.02±0.75
IDA	15	30.46±6.58	21.83±4.87	0.17±0.09	17.61±2.49
p-value		(<0.0001)	(<0.0001)	(NS)	(<0.0001)
G6PD	13	31.17±7.61	2.47±0.91	12.23±4.29	18.67±2.61
p-value		(0.0018)	(NS)	(<0.0001)	(<0.0001)

(NS) = Not significantly different

Table 3. The Mean ± SD of H*3 reticulocyte parameters and reticulocyte indicies measured in IDA and G6PD deficiency patients compared with normal subjects.

Parameters			Normal (n=25)	IDA (n=15)	G6PD def. (n=13)
Reticulocyte (%)	;	Mean ± SD	1.16 ± 0.52	1.39±0.42	2.18±0.2
		p-value		(NS)	(NS)
LReticulocyte (%)	;	Mean ± SD	91.24 ± 3.05	83.33±6.25	78.48±5.66
		p-value		(0.0012)	(0.0006)
MReticulocyte (%)	;	Mean ± SD	6.78 ± 2.55	11.11±3.30	17.28±3.28
		p-value		(0.0061)	(<0.0001)
HReticulocyte (%)	;	Mean ± SD	1.59 ± 1.34	5.57±3.77	4.24±3.11
		p-value		(0.0005)	(NS)
MRet+HRet (%)	;	Mean ± SD	8.36 ± 3.19	16.67±6.27	21.52±5.66
		p-value		(0.0006)	(0.0003)
MCVr (fl)	;	Mean ± SD	110.39 ± 5.09	95.89±8.57	132.0±8.39
		p-value		(<0.0001)	(<0.0001)
CHCMr (g/dl)	;	Mean ± SD	26.61 ± 1.41	23.37±3.53	24.48±2.28
		p-value		(<0.0001)	(NS)
CHr (pg)	;	Mean ± SD	28.41 ± 1.34	21.74±4.61	31.12±3.03
		p-value		(<0.0001)	(NS)

(NS) = Not significantly different

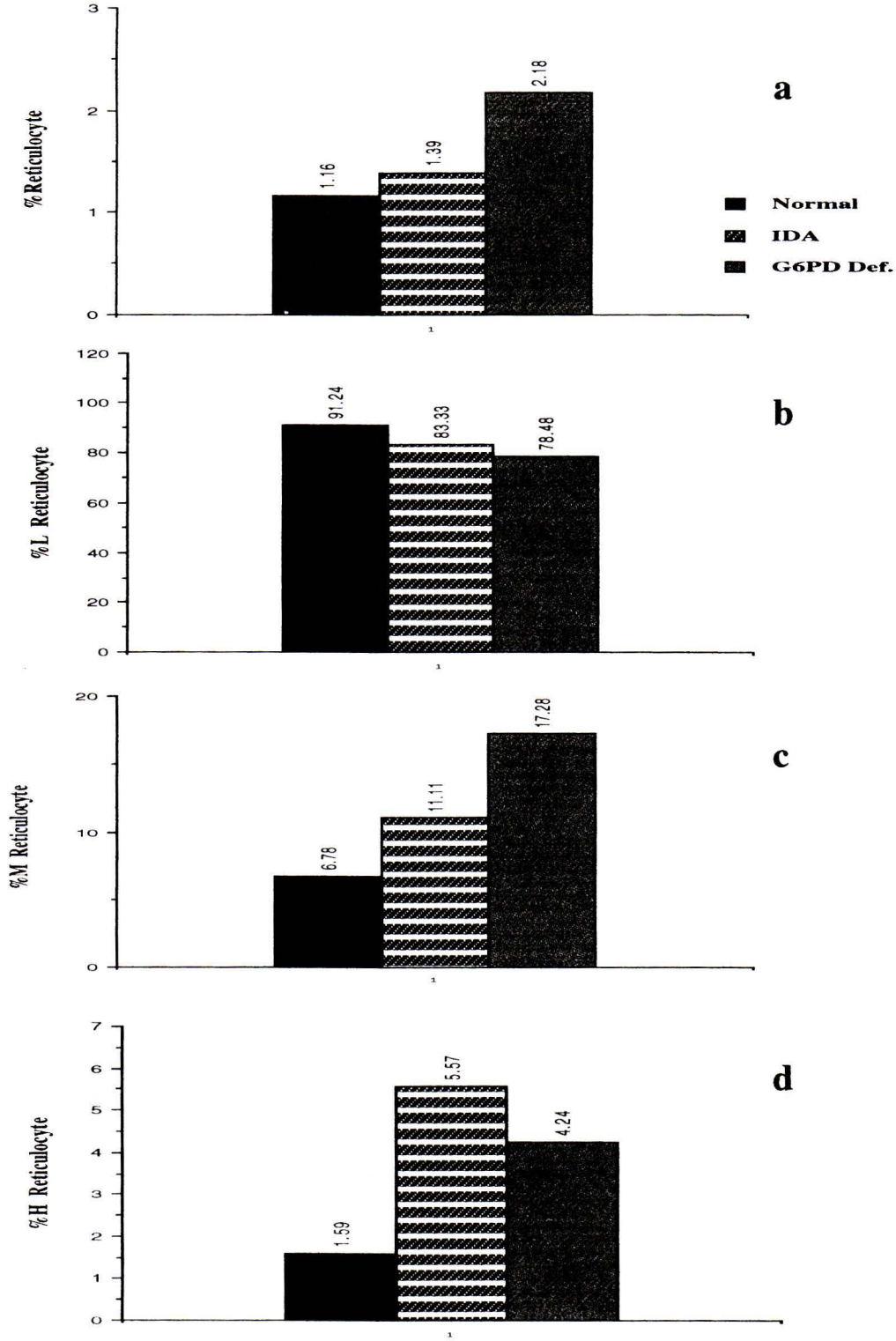

Fig. 1. Comparison between the percentage of reticulocyte (a); LReticulocyte (b); MReticulocyte (c) and HReticulocyte (d) in normal, IDA and G6PD def. patients.

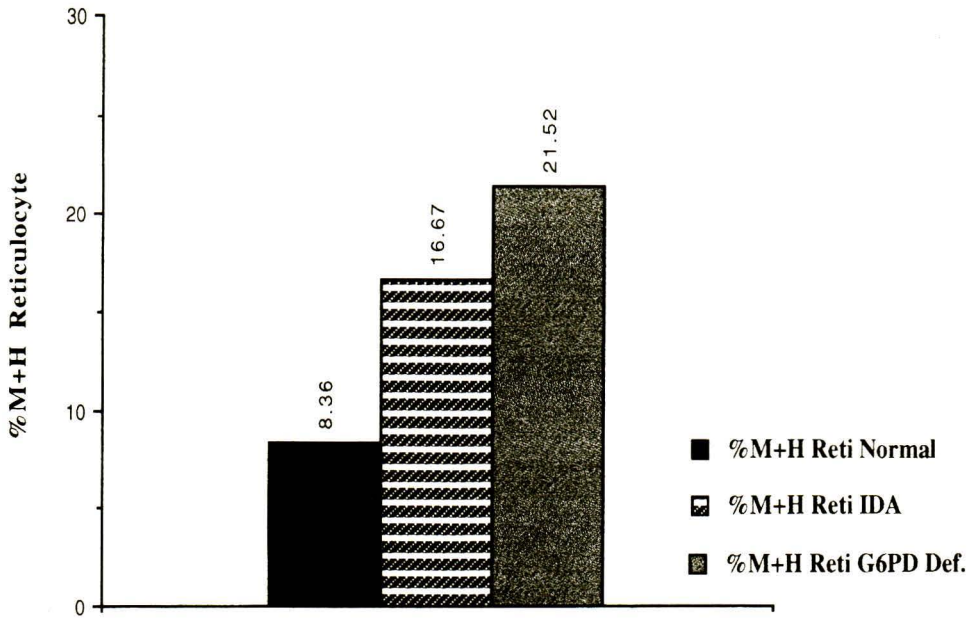

Fig. 2. Comparison between the percentage of M reticulocyte plus HReticulocyte (MRet + HRet) in normal, IDA and G6PD def. patients.

mia when compared to normal (p-value = < 0.0001-<0.002), whereas, significant increase in % hypochromic (Hypo), % microcytic red cells (Micro), and red blood cell distribution width (RDW) were demonstrated in IDA only (p-value = <0.0001). However, the significant increases in the % Hypo, % Macro, and RDW except % Micro were also demonstrated in G6PD def. groups (p-value = 0.0018, <0.0001 and <0.0001 for % Hypo, % Macro and RDW respectively; Table 2). In addition, a significant increase in mean cell volume (MCV) was also found in G6PD def. groups (p-value = 0.0026) when compared to normal. However, MCV was significantly decreased in IDA (p-value = <0.0001; Table 1).

Reticulocyte parameters analysis

Reticulocyte parameters in IDA, and G6PD def. patients, and normal subjects are shown in Table 3. Increased percentage of reticulocyte numbers (Ret %) were detected in both IDA and G6PD def. group when compared to normal. The three populations of reticulocyte (L, M and H Reticulocytes), that represented their maturity showed a significant difference from that of normal in both IDA and G6PD def. patients, except HRet in G6PD def. group (Table 3; Fig. 1). The percentages of L Reticulocyte in IDA and G6PD def. were significantly decreased when compared to normal (83.33 ± 6.25% ; p-value = 0.0012 for IDA and 78.48 ± 5.66 %; p-value = 0.0006 for G6PD def. respectively as compared with 91.24 ± 3.05% in normal), whereas, the M and H reticulocyte percentages were significantly increased in IDA (p-value = 0.0061 for MRet and = 0.0005 for HRet respectively). Significant increase in MRet was also found in G6PD def. patients (p-value = <0.0001). However, although higher than normal, the HRet value was not statistically significantly different from that of normal. The combination of % MRet and % HRet (MRet + HRet%), which represented immature reticulocytes also showed significant increase in both IDA and G6PD def. patients (p-value = 0.0006 and = 0.0003 for IDA and G6PD def. respectively, Table 3, Fig. 2).

Reticulocyte indicies analysis

Upon comparison of the reticulocyte indicies (Table 3), significant decrease in MCVr was found in IDA patients (p-value = <0.0001), whereas, the opposite was demonstrated in G6PD def. patients (p-value = <0.0001). In addition, signifi-

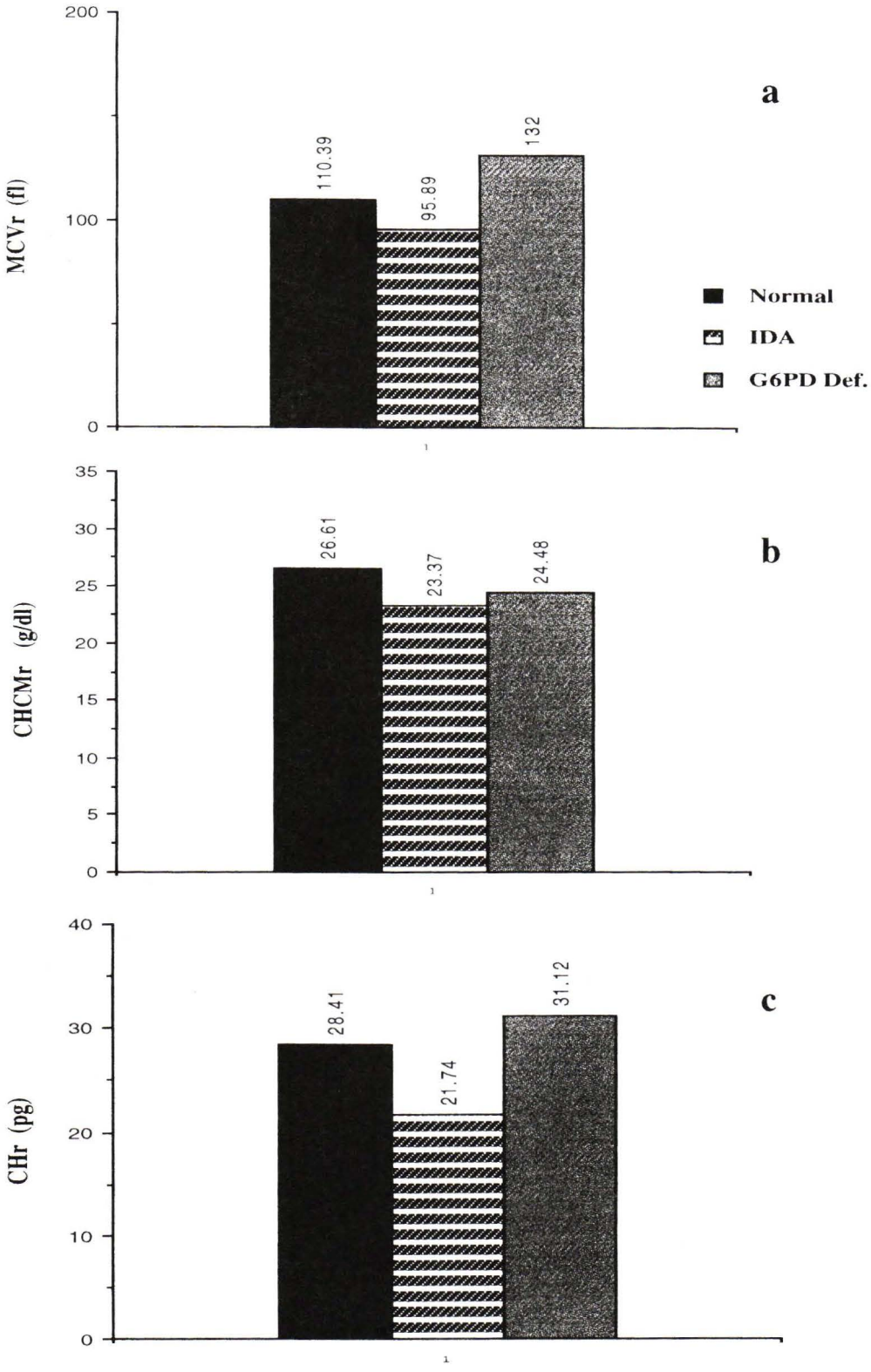

Fig. 3. Comparison between the MCVr (a), CHCMr (b) and CHr (c) in normal, IDA and G6PD def. patients.

cant decreases in CHCMr and CHr values were also detected in IDA patients (p-value = <0.0001 for both CHCMr and CHr). For G6PD def. patients, decreased level of CHCMr and increased level of CHr were also detected, but not statistically significantly different from those found in normal subjects (Table 3; Fig. 3a, b and c).

DISCUSSION

Iron deficiency anemia is the most common nutritional deficiency anemia, and is the most common cause of anemia in children and adult women[20]. G6PD deficient red blood cells will hemolyze under oxidative stress, such as after infection or certain drugs intake. Anemia will result in compensatory increased erythropoiesis in the bone marrow[21]. The IDA patients and G6PD def. showed an elevated percentage of reticulocytes indicating that accelerated compensatory erythropoiesis leads to a prominent reticulocytosis in the peripheral blood[22,23].

The reticulocyte maturation represented by L, M and H Reticulocytes from the H*3 RTX hematological analyzer is very valuable as the marker for active erythropoiesis of the bone marrow[7,13,24,25]. The most mature reticulocytes are counted as L Reticulocyte because of their low cytoplasmic RNA thus stained low fluorescence had the normal percentage of 91.24 ± 3.05 (mean ± SD). The less mature, M and H Reticulocytes are normally less present in the blood circulation in the mean percentage of 6.78 ± 2.55 and 1.59 ± 1.34 respectively. In IDA and G6PD def. patients, L Reticulocyte percentage was decreased but M and H reticulocyte percentages were increased. In addition, the MRet. plus HRet. percentage was found to be significantly increased in both groups. These results reflected the active and compensatory erythropoiesis in bone marrow leading to a greater release of immature reticulocytes in the blood circulation[26], while the mature ones decreased comparable to the anemic status of the patients. The MCVr of the IDA patients was significantly reduced. This indicates that their reticulocytes were smaller than the normal reticulocytes and the CHCMr was also decreased which corresponded to the significantly decreased CHr level indicating that the IDA's reticulocyte contained a low level of hemoglobin content. In G6PD def., there was no significant change in CHCMr and CHr but a significant increase in MCVr was detected. The reticulocyte analysis is useful in evaluating patients with anemia, especially anemias caused by increased red cell loss which can be separated from those caused by decreased red cell production.

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REFERENCES

1. Deiss A, Kurth D. Circulating reticulocytes in normal adults as determined by the new methylene blue methods. Am J Clin Pathol 1970; 53: 481-4.
2. Peebles DA, Hochberg A, Clarke TD. Analysis of manual reticulocyte counting. Am J Clin Pathol 1981; 76: 713-7.
3. Inoue T, Tatsumi N. Evaluation of erythropoiesis by new methylene blue staining to establish reticulocyte maturity in bone marrow aspirates and peripheral blood. Acta Cytologica 1991; 35: 479-80.
4. Kojima K, Niri M, Setoguchi K, et al. An automated optoelectronic reticulocyte counter. Am J Clin Pathol 1989; 92: 57-61.
5. Davis B, Bigelow N. Clinical flow cytometric reticulocyte analysis. Pathobiology 1990; 58: 99-106.
6. Tichelli A, Gratwohl A, Driessen A, et al. Evaluation of the Sysmex R-1000® : An automated reticulocyte analyzer. Am J Clin Pathol 1990; 93: 70-8.
7. Tanke HJ, Rothbarth PH, Vossen JMJJ, et al. Flow cytometry of reticulocytes applied to clinical hematology. Blood 1983; 61: 1091-7
8. Khuhapinant A, Bunyaratavej A, Tatsumi N, et al. Number and maturation of reticulocytes in various genotypes of thalassemia as assessed by flow cytometry. Acta Haematol 1994; 91: 119-25.
9. Watanabe K, Kawai Y, Takeuchi K, et al. Reticulocyte maturity as an indicator for estimating qualitative abnormality of erythropoiesis. J Clin Pathol 1994; 47: 736-9.
10. d' Onofrio G, Chirillo R, Zini G, et al. Simultaneous measurement of reticulocyte and red blood cell indicies in healthy subjects and patients with microcytic and macrocytic anemia. Blood 1995; 85: 818-23.
11. Tatsumi N, Kojima K, Tsuda I, et al. Reticulocyte count used to assess recombinant human erythropoietin sensitivity in hemodialysis patients. Contrib Nephrol 1990; 82: 41-8.
12. Kanold J, Bezou MJ, Coulet M, et al. Evaluation of erythropoietic/ hematopoietic reconstitution after BMT by highly fluorescent reticulocyte counts compares favorably with traditional peripheral blood cell counting. Bone Marrow Transplant 1993; 91: 119-25.
13. Tanke HJ, van Vianen PH, Emiliani FMF, et al. Changes in erythropoiesis due to radiation or chemotherapy as studied by flow cytometric determination of peripheral blood reticulocytes. Histochemistry 1986; 84: 544-8.
14. World Health Organization control of nutritional anemia with special reference to iron deficiency. Report of an IAEA, USAID, WHO Joint Meeting 1975. WHO technical report series no. 580.
15. Mohandas N, Kim YR, Tycko DH, et al. Accurate and independent measurement of volume and hemoglobin concentration of individual red cells by laser light scattering. Blood 1986; 68: 506-13.
16. Bollinger PB, Drewinko B, Brailas CD, et al. The Technicon H*1™ an automated hematology analyzer for today and tomorrow. Am J Clin Pathol 1987; 87: 71-9.
17. Buttarello M, Bulian P, Venudo A, et al. Laboratory evaluation of the Mile H*3 automated reticulocyte counter a comparative study with manual reference method and Sysmex R-1000. Arch Pathol Lab Med 1995; 119: 1141-8.
18. Brugnara C, Zelmanovic D, Sorette M, et al. Reticulocyte hemoglobin an integrated parameter for evaluation of erythropoietic activity. Am J Clin Pathol 1991; 108: 133-42.
19. Brugnara C, Hipp MJ, Irving PT, et al. Automated reticulocyte counting and measurement of reticulocyte cellular indicies evaluation of the Miles H*3 blood analyzer. Am J Clin Pathol 1994; 102 : 623-32.
20. Brugnara C, Zurakowski D, DiGanzio J, et al. Reticulocyte hemoglobin content to diagnose iron deficiency in children. JAMA 1999; 281: 2225-30.
21. Crosby WH, Finch CA, Haurani FI, et al. Modern methods for quantitation of red blood cell production and destruction. Erythrokinetics. Blood 1961; 18: 225-35.
22. Hillman RS. Characteristics of marrow production and reticulocyte maturation in normal man in response to anemia. J Clin Invest 1969; 48: 443-53.
23. Crouch JY, Kaplow LS. Relationship of reticulocyte age to polychromasia, shift cells, and shift reticulocytes. Arch Pathol Lab Med 1985; 109: 325-9.
24. Davis BH, Bigelow N, Ball ED, et al. Utility of flow cytometric reticulocyte quantification as a predictor of engraftment in autologous bone marrow transplantation. Am J Hematol 1989; 32: 81-7.
25. Davis BH, Bigelow NC. Flow cytometric reticulocyte quantitation using thiazole orange provides clinically useful reticulocyte maturity index. Arch Pathol Lab Med 1989; 113: 684-9.
26. CheChang C, Kass L. Clinical significance of immature reticulocyte fraction determined by automated reticulocyte counting. Am J Clin Pathol 1997; 108: 69-73.

การวิเคราะห์เม็ดเลือดแดงอ่อนในเลือดผู้ป่วยโรคโลหิตจางจากการขาดเหล็กและโลหิตจางที่มีสาเหตุมาจากการทำลายผิดปกติ

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จากการศึกษาวิเคราะห์เม็ดเลือดแดงอ่อนในกระแสเลือดของผู้ป่วยโรคโลหิตจาง จากการขาดเหล็กจำนวน 15 ราย โลหิตจางที่เกิดจากการแตกทำลายผิดปกติที่เกิดจากการขาดเอ็นซัยม์ G6PD และร่วมกับภาวะที่เป็นพาหะของโลหิตจาง ที่เกิดจากความผิดปกติของการสร้างฮีโมโกลบินจำนวน 13 ราย เปรียบเทียบกับคนปกติจำนวน 25 ราย พบว่าในโรคโลหิตจางทั้งสองประเภทดังกล่าวจะมีจำนวนของเม็ดเลือดแดงอ่อนในกระแสเลือดสูงกว่าคนปกติ แต่การพัฒนาไปเป็นเม็ดเลือดแดงแก่จะช้ากว่าปกติ และจากการวิเคราะห์ปริมาณของสารฮีโมโกลบินในเม็ดเลือดแดงอ่อนเหล่านี้พบว่า จะมีการลดลงในโรคโลหิตจางที่เกิดจากการขาดเหล็กอย่างมีนัยสำคัญทางสถิติซึ่งแตกต่างจากในโรคโลหิตจางจากการแตกทำลายผิดปกติ และนอกจากนี้ยังพบว่าค่าเฉลี่ยของปริมาตรของเม็ดเลือดแดงอ่อนในโรคโลหิตจางที่เกิดจากการขาดเหล็กจะมีขนาดเล็กกว่าเม็ดเลือดแดงอ่อนในคนปกติ แต่พบว่าในโรคโลหิตจางที่เกิดจากการแตกทำลายผิดปกติจะมีปริมาตรที่ใหญ่กว่าปกติ จากการศึกษานี้ทำให้สรุปได้ว่ามีความผิดปกติที่แตกต่างกันระหว่างการสร้างเม็ดเลือดแดงอ่อนในภาวะโลหิตจางที่มีสาเหตุต่างกัน

คำสำคัญ : การวิเคราะห์เม็ดเลือดแดงอ่อน, โลหิตจางจากการขาดเหล็ก, โลหิตจางจากการขาดเอ็นซัยม์ จี6พีดี

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