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Ranges for Reticulocyte Fractions and Reticulocyte Hemoglobin of Mongolian Donors

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Objectives: The goal of this study was to determine the performance of reticulocyte fractions and reticulocyte hemoglobin for early detection of iron deficiency and iron deficiency anemia among Mongolian blood donors. **Methods:** Blood samples were collected before and after blood donations and analyzed using a Sysmex XN- 2000 analyzer. Iron levels (Umol/L) and Ferritin levels (μmol/L) were measured using a COBAS E601 (ROCHE, Japan). **Results:** Anemia was diagnosed in 3.2% all of the donors. In donors with anemia the results were: HGB 10.6±0.6 g/dl, serum ferritin 5.5 (4; 9) μmol, serum iron 5.7 (4; 7) μmol and Ret-He 26.5 (23; 28) pg. The mean value of reticulocyte was 1.2±0.3 and 1.1±0.3 within a reference range of 0.5-2%. The mean values of IRF before and after blood donations were 6.3±2.6 and 5.9±2.7 respectively with range of 1.1 - 15.9%. There were no clear differences in reticulocyte count and its fractions among donors before and after blood donation. The mean Ret-He was 32.1+1.7 pg before blood donation; 31.2+2.2 pg 28 days after donation and 31.7+ 1.6 pg within 2-3 months after the first blood donation. There are significant differences in RET-He between the before and after blood donations. **Conclusion:** IRF and Ret-He are very useful parameters for estimating hematopoiesis, monitoring for iron treatment and early detection of iron deficiency.

Keywords: Reticulocytes, Anemia Iron-Deficiency, Immature Reticulocyte Fraction, Reticulocyte Hemoglobin.

Introduction

Frequent blood donation can lead to pre-clinical iron deficiency and iron deficiency anemia. With each donation men lose 242 ±17 mg and women lose 217 ±11 mg of iron [1]. A healthy individual can donate blood as often as four times a year, i.e., at three month intervals as iron stores will replete within this period [2]. In 2015, our study showed that 34.1% of regular blood donors were not able to donate blood due to low levels of hemoglobin [3]. For a safe blood supply, it is necessary to strictly monitor blood donor health. Traditionally, donor hemoglobin is assessed using the copper sulfate specific gravity method. In recent years, quantitative methods that measure hematocrit and hemoglobin have been increasingly used in many transfusion centers. Various technological solutions in hematology analyzers enabled us to determine additional hematologic parameters including;

reticulocyte, reticulocyte fraction and reticulocyte hemoglobin. Reticulocytes are the transitional cells from erythroblasts to mature erythrocytes. By removing the endoplasmatic reticulum, the reticulocyte develops into a mature red blood cell typically within 4 days. Reticulocyte remains in the bone marrow for an additional 3 days and only 1 day in the peripheral bloodstream [4]. In automated flowcytometry, reticulocytes have been classified morphologically into three maturational stages: low fluorescent reticulocytes (LFR), middle fluorescent reticulocytes (MFR) and high fluorescent reticulocytes (HFR) [5]. Immature reticulocyte fraction (IRF) is defined as the ratio of immature reticulocytes to the total number of reticulocytes. IRF represents the sum of medium and highly fluorescent reticulocytes, whereas LFR represents immature reticulocytes. IRF is larger with the greatest light scatter properties due to their high level of RNA. IRF normally constitutes less than 5% of total number of reticulocytes as it is released into peripheral blood during the period of erythropoeitic stimulation [6]. If IRF detected excess of 5%, it is considered bone marrow recovery [7]. This information is important in various clinical situations such as anemia, bone marrow regeneration following the transplantation of hematopoietic stem cells and after chemotherapy [8]. Here, we concentrated on the effect of blood donation on erythropoeitic intensity by using reticulocyte fraction.

It seems reasonable to confirm adequate iron reserves in blood donors for a safe blood supply. Recently, many papers reported that direct measurement of hemoglobin content in RBC precursors, particularly in reticulocytes (Ret-He) might have greater sensitivity and specificity for diagnosing iron deficiency than traditional iron measurements [9,10]. Here, we concentrated whether the Ret-He would be clinically informative about iron deficiency in blood donor.

The goal of this study was to determine the performance of reticulocyte fractions and reticulocyte hemoglobin for early detection of iron deficiency and iron deficiency anemia among blood donors of Mongolia.

Materials and Methods

1. Study Population

A total of 92 blood donors (47 female, and 45 male), aged 17-57 were involved in the study. They visited the Mongolian National Center of Blood Transfusion during the period of December 2015 to March 2016. We selected only regular blood donors who gave blood more than three times a year.

2. Data collection

Venous blood samples collected in ethylenediaminetetraacetic acid K3 (EDTA-K3) and were analyzed in a Sysmex XN- 2000 analyzer (Sysmex, Kobe, Japan). The Ret-He and red cell parameters were obtained by flow cytometry technology using fluorocellTM RET reagent (BN337-547, Sysmex, Japan) specific for RNA/DNA. A sample volume of a whole blood specimen was introduced into the analyzer where a portion of it was automatically diluted into a 1:200 solution with CELLPACK DFL. Fluorocell RET was then added. The labeled sample was introduced into the sheath flow detector where forward scattered light and side fluorescence were measured allowing the Reticulocyte, Ret-He, IRF, HFR, MFR, LFR and RBC counts.

Iron levels (Umol/L) and Ferritin levels (μmol/L) were measured using a biochemical analyzer COBAS Integra E601 (ROCHE, Japan). Iron levels were detected using protocol based on the FerroZine method without deproteinization. The Enzymun-Test Ferritin method was used to determine Ferittin level.

3. Statistical analysis

Data was entered, coded and analyzed using the SPSS version 20 program. The normality of the distribution was tested by the Kolmogorov–Smirnov method. Abnormal distributed parameters were transformed into normal distribution by Log-transformation. The differences between groups (gender, age and time/date of donation) were measured by a repeated measure ANOVA test. We analyzed correlations between IRF and RET, Ret-He and iron resources using the Pearson correlation coefficient.

4. Ethical statement

Ethical approval and clearance were obtained from the Ethical Review Board of the Mongolian National University of Medical Sciences on November 26, 2015. Each blood donor signed a consent form before participating in the study.

Results

Reticulocyte counts and its fraction were evaluated in 92 donors at the Mongolian National Center of Blood Transfusion from December 2015 to March 2016. There were 47 (51%) females

and 45 (49%) males with ages between 17 to 57 (mean age 30.2 years). Anemia associated with each donation was diagnosed in 3.2% all of the donors. In donors with anemia the results were: HGB 10.6±0.6 g/dl, serum ferritin 5.5 (4; 9) µmol, serum iron 5.7 (4; 7) µmol and Ret-He 26.5 (23; 28) pg. In donors without anemia the results were: HGB 14.9±1.6 g/dl, serum ferritin 58 (30; 118) µmol, serum iron 13 (9; 19) µmol and Ret-He 32 (31; 33) pg. We took three (3) blood samples at different intervals from each donor: 1) before blood donation; 2) on the 28th day after blood donation and; 3) 2-3 months after the initial blood donation.

The mean value of reticulocyte was 1.2±0.3 and 1.1±0.3 with a reference range of 0.5-2%. The mean values of IRF before and after blood donation were 6.3±2.6 and 5.9±2.7 respectively with range of 1.1 - 15.9% (Table1). There were no clear differences in reticulocyte and reticulocyte fractions except in IRF among donors before and after blood donation. There was tendency of increased IRF on the 28th day after blood donation. These data suggested hematopoeitic intensity is very stable in the healthy donor population. Analysis by repeated measure

Table 1. Reference interval and mean for reticulocyte and its fractions before and after blood donation

	Mean±SD					
	Count	Normal Range by Sysmex %	Before blood donation	28th day	2-3 months after blood donation	p- value
RET	92	0.5-2	1.2±0.3	1.2±0.4	1.1±0.3	0.1
LFR	92	86.5-98.5	93.6±2.6	93.4±4	93.81±3.9	0.8
MFR	92	1.5-11.3	5.7±2.2	5.±2.9	5.4±2.3	0.5
HFR	92	0-1.4	0.6±0.5	0.6±1.3	0.5±0.4	0.5
IRF	92	1.1-15.9	6.3±2.6	6.5±4	5.9±2.7	0.6

RET- reticulocyte, LFR - low fluorescence reticulocytes, MFR - medium fluorescence reticulocytes, HFR - High fluorescence reticulocytes, IRF - Immature reticulocyte fraction.

Table 2a. RET and IRF characteristics of Mongolian donors by gender

		Total		Male		Female		p-value
		Count	Mean±SD	Count	Mean±SD	Count	Mean±SD	
RET	Before blood donation	92	1.20±0.33	45	1.19±0.29	47	1.20±0.38	0,123
	28th day after blood donation	92	1.22±0.39	45	1.25+0.41	47	1.19±0.38	0,502
	2-3 months after blood donation	92	1.13±0.33	45	1.16±0.36	47	1.01±0.31	0,269
IRF	Before blood donation	92	6.3±2.7	45	5.8±2.2	47	6.8±3.0	0,983
	28th day after blood donation	92	6.6±4	45	6.6±4	47	6.5±4	0,474
	2-3 months after blood donation	92	5.9±2.7	45	5.7±2.7	47	6.2±2.8	0,422

Table 2b. RET and IRF characteristics of Mongolian donors by age groups

		Age								p-value
		16-25		26-35		36-45		46 <		
		Count	Mean±SD	Count	Mean±SD	Count	Mean±SD	Count	Mean±SD	
RET	Before blood donation	41	1.1±0.33	27	1.2±0.25	10	1.3±0.33	14	1.2±0.38	0.15
	28th day after blood donation	41	1.2±0.35	27	1.2±0.42	10	1,3±0.5	14	1.1±0.34	0.84
	2-3 months after blood donation	41	1.1±0.33	27	1.1±0.36	10	1,2±0,33	14	1.05±0.28	0.53
IRF	Before blood donation	41	5.9±2.9	27	6.1±2.19	10	6.8±1.58	14	7.5±3.20	0.14
	28th day after blood donation	41	6.3±4.3	27	6.4±2.82	10	7.0±3.8	14	6.9±5.27	0.5
	2-3 months after blood donation	41	6±2.4	27	5.4±2.5	10	6.8±3.17	14	6.1±3.57	0.76

Table 3. Reference interval and mean of Ret-He before, 28 days after blood donation and 2-3 months after blood donation.

	Mean±SD				p-value
	Normal Range by SYSMEX	Before blood donation	28th day after blood donation	2-3 months after blood donation	
Ret-He (pg)	28-35	32.1±1.7	31.2±2.2	31.7±1.6	0.04
RBC (10^6u/l)	3.7-5.5	5.16±0.53	5.07±0.5	5.08±0.55	0.42
HGB (g/l)	14.9±1.6	14.9±1.6	14.5±1.7	14.6±1.7	0.57

ANOVA shows that RET, IRF does not have a significant effect before and after blood donation (p>0.05)

We checked whether the mean of IRF is different by gender and age. Interestingly, IRF was higher in females than males. IRF was 5.8±2.2 in males and 6.8±3.0 in females with a range of 1.5-13.7% and 1.1-15.9% respectively. However, significant differences were not observed between gender, age and blood donation interval (p>0.05) (Table 2a, 2b). Table 2b shows the tendency of increased IRF due to aging.

We also observed that IRF presented a direct correlation (Spearman Rank –order Coefficient = 0.40) with reticulocyte count (Figure 1).

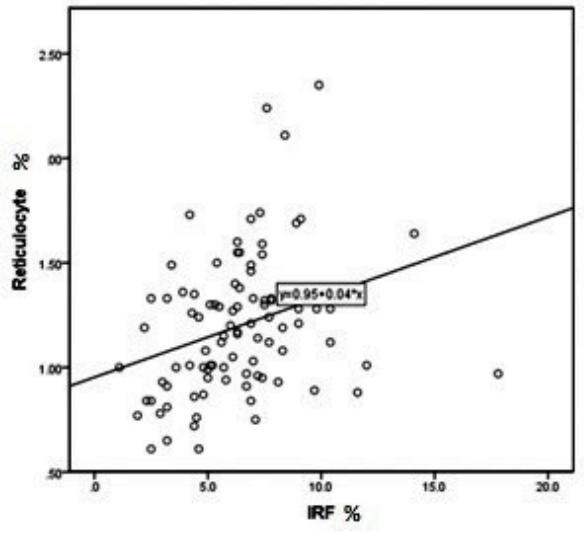

Figure 1. Correlation between reticulocyte count and IRF. There was direct correlation between reticulocyte count and immature reticulocyte fraction (Spearman Rank – order Coefficient r=0.4, p = 0.001)

Ret-He - reticulocyte hemoglobin, RBC - red blood cell, HGB - hemoglobin

The mean Ret-He was 32.1+1.7 pg in before blood donation, 31.2+2.2 pg at the 28th day of donation and 31.7+ 1.6 pg 2-3 months after blood donation (Table3).

Ret-He is useful in the evaluation of hematopoiesis and it reflects the synthesis of hemoglobin in marrow precursors. Therefore, we analyzed the correlations of Ret-He with hemoglobin and Ret-He with serum iron and ferritin (Figure 2). There was direct correlation between reticulocyte hemoglobin

Table 4. Reference interval of Ret-He, Iron and Ferritin

	Median			
	Count	**RET-HE (pg)**	**Iron (Umol/l)**	**Ferritin (µmol/l)**
male	45	32 (31;33)	13 (9;19)	54 (19;135)
female	47	32 (31;33)	13 (10;17)	65 (31;110)

Ret-He - reticulocyte hemoglobin

Ret–He was 32pg in both genders. Ferritin were 54 µmol/l in males, and 65 µmol/l in females (Table 4).

and hemoglobin (r = 0.6, p = 0.001) (Figure 2A). It was observed that a direct correlation between Ret-He and serum iron (r = 0.5, p = 0.001) (Figure 2B) exits. Correlation between Ret-He and

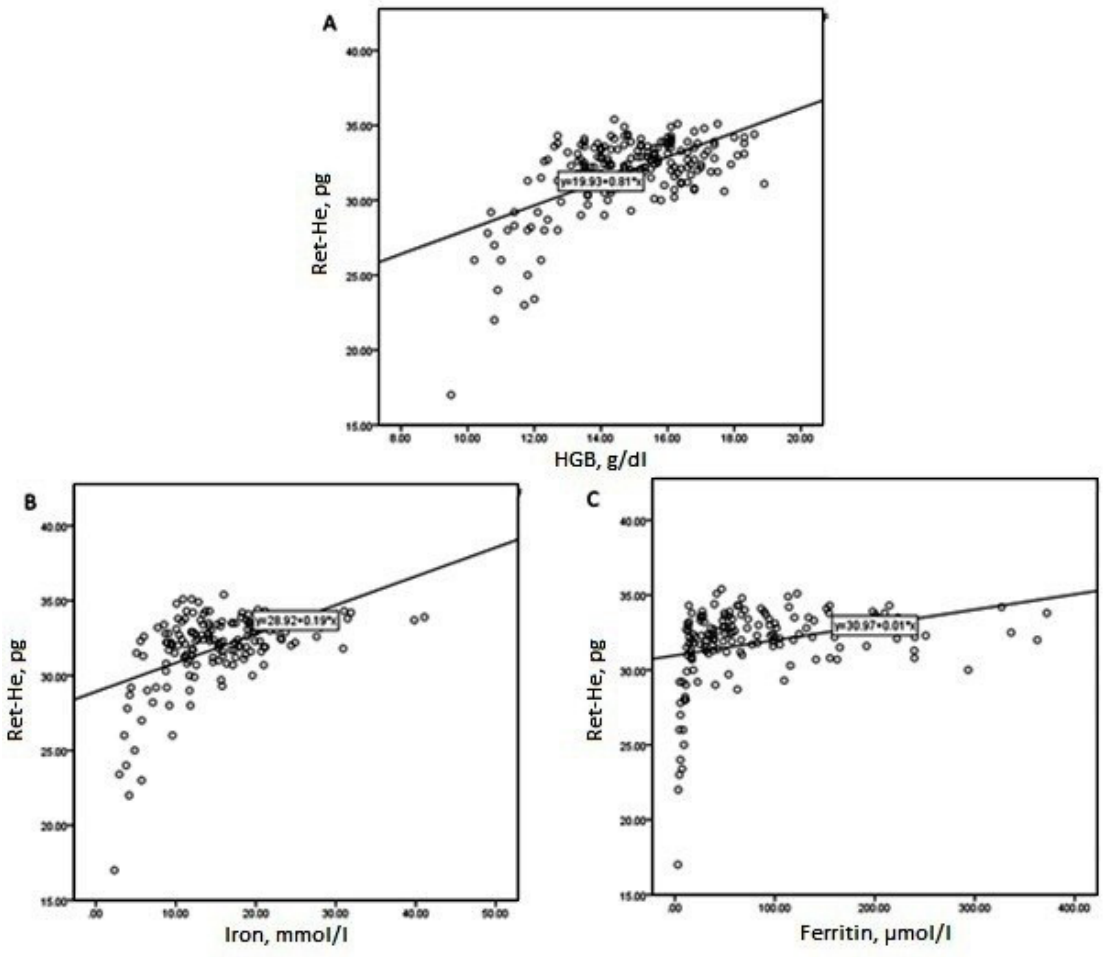

Figure 2. Correlation between reticulocyte hemoglobin and iron resources.
A. There was a direct correlation between reticulocyte hemoglobin and hemoglobin (r = 0.6, p = 0.001). B. Correlation reticulocyte hemoglobin and serum iron Ret-He (r = 0.5, p = 0.001). C. Correlation between Ret-He and ferritin (r=0.3. p=0.001)

ferritin (r=0.3. p=0.001) was observed (Figure 2C).

Discussion

The result of our study showed blood donor's hematopoietic intensity is stable in healthy donors. However, there is a difference between before and after blood donations in RET-He which showed RET-He decreases after blood donation.

The mean IRF was 6.3% and mean Ret-He was 32 pg in Mongolian blood donors. The reference range for the IRF was similar to that of Iuri Vicente, Camargo Morkis et al. who obtained a median of 5.35% (range 1.6-12.1%) [11]. The reference range for the Ret-He was similar to that of C. Brugnara, B. Schiller et al. who obtained a mean of 32.47 pg (range 28-35 pg). They [and us] used the same methodology. These studies were conducted with the use of a Sysmex analyzer. Our study showed that after blood donation, IRF increased. It means blood donation stimulates erythtopoesis. An elevated proportion of immature reticulocytes is an early indicator of increased erythropoietic response in the bone marrow and may precede a measurable increase in the absolute reticulocyte count. [12]. A recent survey done by Linda M et al. showed that 40% of hematologist-oncologists (8/20) and 33% of nephrologists (2/6) would like the reticulocyte reports to include the IRF. Other medical practitioners never used this parameter in their daily practice. Linda M et al. reported: There are several possible reasons why IRF has not been embraced widely by clinicians. First, IRF is available only in laboratories that have automated reticulocyte analyzers. Second, the reference ranges are not standardized across different analyzers. These reasons are similar to our country Mongolia. These new parameters are not widely used by laboratories and clinics. Also, not all laboratories that use automated analyzers, detect reticulocyte and IRF [13]. Several studies have demonstrated the value of these indices in the context of hematopoietic stem cell transplantation as indicators of hematopoiesis, in transfusion assessment and the anticipation of successful engraftment. Our study showed an iron deficiency in 3.2% of all subject donors. The decrease in the Ret-He result of donors with anemia suggests that we can determine iron deficiency early. Researcher Fishbane et al. (1997) study suggested that if the value of Ret-He is less than 26pg, it appears to be an early indicator of functional iron deficiency. Ret-He is useful in the evaluation of hematopoesis with respect to the incorporation of iron [14-16]. Measurement of reticulocyte hemoglobin content in peripheral blood samples is useful for diagnosis of iron deficiency in adults [9]. As Ret-He level increase HGB level also increased which suggests that it can be a treatment of monitoring iron deficiency anemia. Ret-He provides an early measure of the response to iron therapy increasing within 2-4 days of initiation of intravenous iron therapy [17]. Ret-He content is a recent addition to an expanding list of biomarkers that can be used to differentiate iron deficiency from other causes of anemia [18]. Therefore, Ret-He is an inexpensive and quick way to determine iron deficiency in bone marrow. Also, compared to other biochemical markers (iron, ferritin) Ret-He is a more stable measurement. Iron, Ferritin and other parameters are used as the markers of iron, but they are susceptible to circadian variation or physiological variation (especially inflammation) and they may not be able to reflect the accurate kinetic change of iron. Clinically, a threshold value of 20 μg/L (ng/mL) has proven useful in the detection of prelatent iron deficiency. Latent iron deficiency is defined as a fall below the 12μg/L (ng/mL) ferritin threshold [19]. If the depressed ferritin level is accompanied by hypochromic microcytal anemia, then manifest iron deficiency is present [20]. Ret-He as compared to ferritin level quickly reflects the actual iron supply to erythrocytes, in real time. Thus, Ret-He should be used in practical terms to detect iron deficiency early. In Mongolia, these new parameters are not widely used by laboratories and clinics. Therefore, IRF and Ret-He are very useful parameters for estimating hematopoiesis, monitoring for iron treatment and early detection of iron deficiency in clinical practice. We are introducing new parameters and suggesting use in daily practice.

Our study had some limitations. First, there was a sample size limitation. Our donor size significantly dropped after the third blood donation because donors did not donate on time. Secondly, there were no references or comparison data because this kind of study was conducted for the first time in Mongolia. However, despite the small sample size our study indicated a reasonable correlation between red cells parameters and biochemical parameters.

In the future, to confirm correlation between number of blood donation and early iron deficiency another study must involve more donors to assure a larger sample group. We recommend that in the future, researchers should focus on donor's lifestyle, diet and blood donation frequency to determine how it affects erythropoesis and iron stores.

Conflict of Interest

The authors state no conflict of interest.

Acknowledgements

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