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Immature Reticulocyte Fraction in Thalassemia Major Receiving Single and Combination Iron Chelation Therapy

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ABSTRACT

Background: Ineffective erythropoiesis in thalassemia major causes anemia and requires regular blood transfusions. However, routine transfusions cause iron accumulation, which can be removed through iron chelation therapy (single or combination). To monitor erythropoietic activity non-invasively, Immature Reticulocyte Fraction (IRF) and reticulocyte count have been proven effective, eliminating the need for invasive bone marrow examination.
Objective: This study aimed to determine the differences in IRF percentage and reticulocyte count between thalassemia major patients receiving single and combination iron chelation therapy.
Methods: An observational analytical study with a cross-sectional design was conducted at the Paediatric Haematology and Oncology Clinic of Dr. Kariadi General Hospital, Semarang, from November 2023 to January 2024. Subjects were thalassemia patients aged ≤ 18 years and receiving more than 10 regular transfusions. IRF and reticulocyte count were examined using fluorescence flow cytometry on whole blood samples with EDTA anticoagulant.
Results: A total of 71 thalassemia major patients were enrolled, consisting of 40 males (56%) and 31 females (44%). The mean IRF percentage was 11.62±5.4% in thalassemia major patients receiving single iron chelation therapy and 17.01±10.0% ($p<0.05$) in the group that was administered combination therapy. Mean reticulocyte count was 0.106±0.08 x106/µL in thalassemia major patients receiving single iron chelation therapy and 0.110±0.08 x106/µL in the group administered combination therapy ($p>0.05$).
Conclusion: There is a difference in IRF between thalassemia major patients, with high erythropoietic activity in the group receiving combination iron chelation therapy.
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1. Introduction

Thalassemia is a hereditary hemolytic disease caused by mutations in the globin gene that disrupt globin chain synthesis. This disease is characterized by decreased or absent synthesis of the α, β, and/or other globin chains that form the normal structure of the main hemoglobin molecule in adults. Furthermore, decreased synthesis of α and β chains causes the inability of the body to form red blood cells properly, leading to anemia in childhood that persists throughout life [1,2]. Based on clinical abnormalities, thalassemia is divided into major, intermediate, and minor. Thalassemia major is the most severe form, requiring regular blood transfusions.[1],[3].

As the hereditary hemolytic disease with the highest prevalence, thalassemia is a very serious health problem, accounting for the deaths of children yearly. According to data from the World Health Organization (WHO) in 2020, the global incidence of thalassemia reached approximately 54.348 million people, or 7% of the world's population. The highest incidence of 21.7 million people (40%) occurred in Asian countries. Data from 2021 showed that the global prevalence of thalassemia was estimated to be 156.74 million people, or approximately 20% of the total global population. Based on the data from the Indonesian Thalassemia Foundation, there has been a continuous increase in cases. Since 2012, there have been 4,896 cases,

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which have steadily increased to 10,973 as of June 2021. Among the total cases, 30% are in Central Java, ranking second nationally after West Java. In terms of financing, the 2020 BPJS Kesehatan data recorded a consistent rise in healthcare cost burden from 2014 to 2020. Thalassemia ranks fifth among non-communicable diseases, after heart disease, kidney failure, cancer, and stroke, with an estimated cost of IDR 2.78 trillion in 2020 [3–6]. Kariadi General Hospital in Semarang serves as a referral hospital for thalassemia patients, where the demand for blood supplies for therapy is extremely high.

Thalassemia major is the most severe clinical condition, which occurs because the hemoglobin-coding gene on two chromosome alleles is defective. This causes ineffective erythropoiesis, leading to anemia and the need for regular blood transfusions. Patients often require blood transfusions from the first year of development, typically between 6 and 24 months of age, which continues during the lifetime. However, regular blood transfusions (>10x) cause iron accumulation in the body, which affects several organs, suggesting the need for single or combination iron chelation therapy [7],[8]. Iron, which the body cannot naturally excrete, requires the assistance of chelators to facilitate its excretion. The three types of iron chelators currently in use are deferoxamine (DFO), deferiprone (DFP), and deferasirox (DFX). Iron chelation therapy is expected to reduce accumulation by binding iron and increasing hepcidin, thereby affecting erythropoiesis [3],[7].

Erythropoietic activity can be assessed through peripheral blood reticulocyte examination, making it easier to perform than invasive bone marrow examination. Among various markers, Immature Reticulocyte Fraction (IRF) is a sensitive early indicator for evaluating erythropoietic regeneration through hematological analysis. In thalassemia, an imbalance in the production of α and β globin chains leads to a decreased survival of erythrocytes and their precursors. Ineffective erythropoiesis in thalassemia patients is characterized by an increase in erythroid precursors and progenitor cells, leading to ineffective erythropoiesis and anemia. In this context, administration of single or combination iron chelation to thalassemia major patients is performed to reduce ferritin levels, which are expected to affect the quality of erythropoiesis. Previous studies have shown that high transferrin and hepcidin levels can decrease iron bioavailability for erythroblasts, causing an increase in erythroid precursors in rats with β-thalassemia [9],[10],[11]. Therefore, iron chelation therapy is expected to improve anemia in thalassemia major patients [12,13]

Studies on the differences in the percentage of IRF and reticulocyte count between thalassemia major patients receiving single and combination iron chelation therapy are still limited. Therefore, this study aimed to provide an overview of the percentage of IRF and reticulocyte count in thalassemia major patients, offering insight into the erythropoietic status

2. Methods

This analytical observational study with a cross-sectional design was conducted from November 2023 to January 2024 at the Pediatric Haematology and Oncology Clinic of Dr. Kariadi General Hospital, Semarang. The inclusion criteria were patients aged ≤ 18 years who received more than 10 regular transfusions. The exclusion criteria were patients with a history of splenectomy and renal failure. Data, including age, gender, and laboratory parameters, were collected from the electronic medical records (EMRs) of Dr. Kariadi General Hospital, Semarang. A total sample of 245 patients was obtained, where 174 were excluded due to incomplete data, and 71 patients were included in the study sample.

Data were obtained through electronic medical records. Haemoglobin levels, erythrocyte count, IRF percentage, and reticulocyte count were measured at Dr. Kariadi General Hospital using a Sysmex XN-1000 haematology analyzer. In comparison, ferritin was measured using patients' serum through the Vidas Enzyme-Linked Fluorescent Assay (ELFA) method.

Data were processed using SPSS 23, and a normality test was conducted using the Kolmogorov-Smirnov test. Analysis of differences was performed using the independent t-test and the Mann-Whitney test. Statistical test results were considered significant when the p-value was <0.05.

3. Result

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Table 1. Characteristics of Patient's Data

Variables	Total n = 71	Iron Chelation Therapy		p
		Single n = 44 (62%)	Combination n = 27 (38%)	
Male	40 (56.3%)	21(47.7%)	19 (70.3%)	0.062[§]
Female	31 (43.6%)	23 (52.2%)	8 (29.6%)	
Age#	11 (4-17)	10 (5-16)	13 (4-17)	0.024[§]
Hemoglobin# (g/dL)	7.46 (4-10.1)	7.36 (4-10.1)	7.62 (5-9.7)	0.27[§]
Erythrocytes# (10^6/uL)	3.27 (1.61-4.25)	3.32 (1.61 – 4.29)	3.32 (2.20 – 4.35)	0.72§

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Iron chelation				
Deferasirox		32 (72.7%)		
Deferipron		12 (27.2%)		
Transfusion frequency				0.81$
Every 2 weeks	23 (31.9%)	12 (27.2%)	11 (40.7%)	
Every 3 weeks	15 (20.8%)	7 (15.9%)	8 (29.6%)	
Every 4 weeks	33 (45.8%)	25 (56.8%)	8 (29.6%)	
Ferritin#§ (µg/dL)	3262 (338–13999)	2111 (338-6760)	3264 (976-13999)	0.024§

SD, standard deviation; min, minimum; max, maximum; #, non-normal data distribution; §Mann-Whitney test; $Chi -quare

Data were analyzed for normality using the Kolmogorov-Smirnov test. Analysis continued with a test for differences in IRF percentage and reticulocyte count, between the two groups. The values obtained were p = 0.015123456and p = 0.83, respectively, as shown in Table 2.

In this study, a significant difference was observed between the IRF percentage in single and combination iron chelation therapy groups. Additionally, the Cohen's d analysis showed a value of 0.72, indicating a substantial difference between single and combination iron chelation therapy groups, with clinical significance. The mean reticulocyte count in the combination was descriptively higher than in the single iron chelation therapy group. However, there was no significant difference between the two groups.

Table 2. Difference test of IRF percentage and reticulocyte count in thalassemia major patients with single and combination iron chelation therapy

Variables	**Total n = 71**	**Iron Chelation Therapy**		**p**
		Single n = 44 (62%)	Combi-nation n = 27 (38%)	
IRF (%)^	13.6 ± 7.92	11.62 ± 5.4	17.01 ± 10.0	0.015*
Reticulocyte (106/uL)^	0.107 ± 0.07	0.106 ± 0.08	0.110 ±0.08	0.83

§Mann-Whitney test; ^Independent t-test ; *p< 0,05

4. Discussion

This study aimed to determine the differences in IRF percentage and reticulocyte count between pediatric thalassemia major patients receiving single and combination iron chelation therapy.

Patients with thalassaemia major are at high risk of developing iron overload from an early age. This progressive accumulation of iron affects various organs. Therefore, iron chelation therapy is an essential component of management. The administration of combination iron chelation therapy in children is based on several clinical considerations: it is administered if serum ferritin levels are >2500 ng/mL and have persisted for at least 3 months, or if monotherapy is insufficient to optimally reduce iron load, or if deferoxamine (DFO) cannot be used. [7],[13]

This approach leverages the differences in pharmacokinetic characteristics and tissue distribution of the two drugs, enabling more comprehensive iron mobilization and excretion. Iron distribution in thalassemia patients is not homogeneous. In the early phase, iron accumulation predominantly occurs in the liver, but over time it can involve the heart and other organs. Deferasirox is known to be effective in reducing hepatic iron burden, whereas deferiprone has superior ability to eliminate iron from cardiac tissue. Combination therapy of oral iron chelators—deferiprone (DFP) with deferasirox (DFX)—is an efficacious and safe modality to reduce serum ferritin in multi-transfused thalassemic children. [13]

This approach exploits the differences in the pharmacokinetic characteristics and tissue distribution of the two drugs, thereby enabling more comprehensive mobilisation and excretion of iron. Iron distribution in thalassaemia patients is not homogeneous. In the early stages, iron accumulation predominantly occurs in the liver, but over time it can involve the heart and other organs. Deferasirox is known to be effective in reducing hepatic iron load, whilst deferiprone is more effective at eliminating iron from cardiac tissue. The combination of the two in paediatric patients at risk of, or with evidence of, vital organ involvement is expected to provide a synergistic effect in reducing ferritin levels. [13]

The results found a significant difference in IRF percentage between thalassemia major patients receiving single and combination iron chelation therapy. The IRF percentage was obtained from the sum of the percentages of Medium-fluorescence reticulocyte (MFR) or semi-mature reticulocyte and High-fluorescence reticulocyte (HFR) or immature reticulocytes. Meanwhile, the percentage of Low-fluorescence reticulocyte (LFR) or mature reticulocyte was obtained by subtracting 100% from the IRF percentage. This suggested that a higher IRF percentage correlated with a lower LFR. An increase in IRF represents a rise in immature reticulocytes that have recently been released from the bone marrow. This shows an erythropoietic response from the bone marrow, characterized by the accelerated release of immature reticulocytes due to anemia. Therefore, IRF is a better indicator of bone marrow function compared to other reticulocyte parameters [14]. Andriyani et al. (2021) showed that the IRF percentage in transfusion-dependent thalassemia patients with severe anemia was significantly higher than in moderate cases. Furthermore, thalassemia major patients showed low hepcidin expression and high ferroportin expression, causing enhanced iron absorption through the gastrointestinal tract. Transfusion iron overload and increased gastrointestinal iron absorption are causes of secondary hemosiderosis in thalassemia patients [1],[15]. The Cohen's d analysis in this study also confirms that the effect of combination therapy on increasing IRF has a

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clinically strong impact compared to single therapy.

Chelation therapy aims to eliminate and prevent iron overload by clearing excess iron and maintaining natural levels. Therefore, the administration of iron chelation can reduce the toxic effects of iron overload. Based on the mechanism of operation, iron chelation enters cells, binds free iron, and removes it from the body [15]. The combination of DFP and DFX is more effective in clearing the heart and liver of iron siderosis. This is due to the synergistic effect as well as the ability of DFX and DFP to enter cells and chelate intracellular iron [16]. Karami et al. (2017) showed a significant difference in liver iron concentration (LIC) in β-thalassemia major patients before and after combination iron chelation therapy (DFP and DFX) [17].

Iron restriction with combination therapy can reduce TSat and erythroid iron intake, thereby limiting heme synthesis. This shows the potential to limit hemichrome production, which hinders the toxicity associated with excess α-globin chains. Reducing hemichrome formation can also enhance the quality of the RBCs produced, thereby increasing lifespan and the number of circulating RBCs. Additional beneficial effects of iron restriction include low hypoxia and EPO synthesis, with increased extramedullary erythropoiesis and the erythroid progenitor/RBC ratio [12]. Deferiprone (DFP) increases hepcidin expression, altering iron distribution by moving it out of parenchymal cells and into circulating transferrin, enhancing iron-mediated signaling to hepcidin expression. Specifically, DFP binds iron when there is an overload and removes excess from the body. It also has antioxidant effects, preventing free radicals, ferroptosis, and cuproptosis, thereby inhibiting tissue damage caused by excess ferritin accumulation [18]. In comparison, DFX binds iron at a 2:1 ratio, reducing toxicity by binding trivalent iron with high affinity to a stable form that is excreted in the urine [19].

In this study, reticulocyte count in the combination iron chelation therapy group was descriptively higher. There wasn't a statistically significant difference between the two groups. This was because reticulocyte count represented the total number of reticulocytes, while IRF percentage was calculated by summing the MFR and HFR percentage. Immature Reticulocyte Fraction (IRF) is a more sensitive indicator because it assesses cell maturation quality. IRF detects an increased release of very young reticulocytes, which are rich in RNA, into the circulation, thereby reflecting the dynamic response of the bone marrow to iron clearance induced by combination therapy. The principle of reticulocyte examination is flow cytometry. RNA/DNA in reticulocyte is stained, then the stained cells are detected and counted based on a two-dimensional distribution, namely, forward scattered light and side fluorescent light. [1],[20].

The majority of subjects were male (56.3%) and aged over 5 years (94.3%). Based on age, these results were in line with data from a previous study showing that the average age of thalassemia patients was 11.1±3.71 years.[21] However, this result was in contradiction with previous studies that reported a higher number of female patients compared to male patients in thalassemia.[22] According to data from the Indonesian Thalassemia Foundation (FKHTI) in 2014, the number of patients was 6,439, with 3,219 males and 3,220 females [23].

A total of 44 patients (62%) received single iron chelation therapy, with 32 given DFX and 12 had DFP. In line with the results, approximately 31.9%, 20.58%, and 45% of patients received transfusions every 2, 3, and 4 weeks. Similarly, a previous study reported that thalassemia patients started transfusions at an average age of 3.78 years, with a frequency of approximately once a month (87.5%) [24]. Median ferritin levels were higher in the group receiving combination iron chelation therapy compared to the single. These results varied compared to another report, where ferritin levels in combination with iron chelation therapy were significantly lower than in single therapy [7]. The variation was because the determination of ferritin levels in this study was conducted without considering the duration of therapy and previous levels. Additionally, combination therapy is applied as an escalation step for patients with massive iron overload who do not respond optimally to monotherapy. This is also influenced by iron accumulation due to long-term transfusion history, the gradual kinetics of iron mobilization from tissues to circulation, regimen complexity that reduces patient compliance, and the role of ferritin as an acute-phase reactant elevated by chronic inflammation or organ damage. [8, 12, 16, 18].

A limitation of this study include the small sample size, particularly in the combination iron chelation therapy, the use of a cross-sectional study design, the inability to consider the duration of iron chelation therapy as well as ferritin levels before and after iron chelation. Therefore, the amount of iron released to influence ineffective erythropoiesis in thalassemia major patients is not determined during the analysis.

5. Conclusion

This study shows that there is a significant difference in IRF percentage among thalassemia major patients receiving single and combination iron chelation therapy ($p<0.001$). However, reticulocyte count does not show a significant difference between the two groups. This suggests that erythropoietic activity in thalassemia major patients receiving combination iron chelation therapy is higher. Moreover, further studies are needed to periodically determine IRF percentage and reticulocyte count in thalassemia major patients receiving single and combination iron chelation therapy, as well as to measure ferritin levels before and after administration.

Ethical Approval

The ethical approval was received from the Research Ethics Committee of Dr. Kariadi General Hospital, Semarang, under number 1697/EC/KEPK-RSDK/2024.

Conflicts of Interest

The authors declare no conflict of interest.

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Author Contributions
APA: conceptualization, methodology, formal analysis, investigation, resources, data curation, writing-original draft, visualization. NSW: conceptualization, methodology, formal analysis, investigation, resources, data curation, writing-original draft, visualization, supervision, project administration, funding acquisition.

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