

The differences of lactate dehydrogenase and activin A levels among thalassemia major and non-thalassemia



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ABSTRACT

Background: Indonesia is located in the global thalassemia belt region, with major thalassemia prevalence that increase every year. Ineffective erythropoiesis (IE) is a condition that is commonly found in major thalassemia. An increase in lactate dehydrogenase (LDH) level and Activin A level was found in major thalassemia patient, that is caused by ineffective erythropoiesis (IE) and other factors. However until now, there is still no known studies that compare LDH level and Activin A level in major thalassemia and non-thalassemia. The aim of the study is to evaluate difference between LDH level and Activin A level in major thalassemia and non-thalassemia.

Methods: An observational analytical study with cross sectional design was conducted in March – September 2020, which consist of 25 major thalassemia patients in Dr. R. Soedjati Grobogan Public Hospital and Dr. R. Soetrasno Rembang Public Hospital, and 25 healthy population with equivalent age. LDH levels were measured using photometry and Activin A levels were measured using ELISA. Differences between LDH levels and Activin A levels in major thalassemia and non-thalassemia were analyzed using Independent Sample T test, which $p < 0.05$ was considered significant.

Results: There is a significant difference ($p=0.00$) between LDH levels in major thalassemia (524.48 ± 167.44 U/L) and non-thalassemia (294.48 ± 131.24 U/L). There is a significant difference ($p = 0.04$) between Activin A levels in major thalassemia (118.75 ± 45.47 pg/ml) and non-thalassemia (95.66 ± 26.26 pg/ml).

Conclusion: Hypoxia due to IE and the formation of ROS due to repeated transfusions causes an increase in LDH levels and Activin A levels in thalassemia major patients, indicated by a significant difference between LDH levels and Activin A levels in thalassemia major and non-thalassemia.

Keywords: Activin A, LDH, major thalassemia, ineffective erythropoiesis.

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INTRODUCTION

Thalassemia is a hereditary blood disorder, that occurs when hemoglobin is not produced adequately by the body.¹ Clinically thalassemia can be classified into major, intermedia, and minor.² Individuals with thalassemia major have severe anemia and hepatosplenomegaly, they usually experience clinical problems at the age of 6 months to 24 months, without proper treatment, usually children with thalassemia major will experience developmental failure and short life span.³

Indonesia is located in the global thalassaemic belt, where Indonesia has a high frequency of thalassemia genes, which is around 3 – 10% for the beta thalassemia

gene. Data from the Thalassemia Center, Department of Child Health, Medical Faculty, Indonesia University-Dr. Cipto Mangunkusumo National Central Hospital stated that up to May 2014 there were 1,723 beta thalassemia major patients, with an increase of 75 – 100 people/year.⁴

In beta thalassemia patients, there exist a disruption in beta globin chains production, which causes excessive free alpha globin chains, whereas in alpha thalassemia patients, the disruption occurs in alpha globin chains production.⁵ When the capacity of free globin chain stabilizing protein is exceeded, this free globin chain will undergo auto-oxidation, formation of

alpha-hemichrome, and reactive oxygen species (ROS). This will cause a series of reactions that lead to hemolysis and ineffective erythropoiesis (IE).⁶ IE is a condition in which there exist a abnormal maturation of erythroid progenitors, accompanied by increased abnormal erythroblast destruction. In thalassemia major, IE accompanied by dysfunction of hemoglobin causes inadequate tissue oxygenation.⁷

Lactate dehydrogenase (LDH) is an enzyme that catalyzes pyruvate conversion to lactate and NADH to NAD⁺, which occurs during glycolysis in hypoxic state.⁸ Ghulam, et al. stated that lactic acid in beta thalassemia major patients increases due

to increased anaerobic glycolysis, which is caused by inadequate tissue oxygenation. This indicates that thalassemia major patients also experienced an increase in LDH as an enzyme that catalyzes the formation of lactic acid.⁹ Toren et al. also stated that in patients with beta thalassemia major, there exist an increase in serum LDH levels, which was due to IE.⁷

LDH has long been associated as a marker of intravascular hemolysis, in which serum LDH levels will increase in intravascular hemolysis, such as in hemolytic anemia.¹⁰ Iron overload as a complication of repeated blood transfusions has also been found to increase LDH levels due to excessive ROS generation.¹¹ Prolonged high levels of lactic acid can cause lactic acidosis which is a life threatening condition.¹²

Hypoxia in thalassemia major patients cause activation of HIF2 α , where this molecule will increase erythropoietin (Epo) production from the kidneys. This is also accompanied by an increase in the expression of Gdf11 in response to ROS, where GDF11 cause a decrease in erythrocyte progenitor cell differentiation.^{13,14} Overexpression of Gdf11 in beta thalassemia patients lead to increased oxidative stress and alpha globin precipitation. In addition, Gdf11 expression itself is also triggered by oxidative stress, causing an autocrine amplification loop.¹⁵ Jones, et al. stated that overexpression of Gdf11 increases circulating levels of Activin A.¹⁶

Activin A is a pleiotropic cytokine belonging to transforming growth factor (TGF)- β superfamily.¹⁷ Activin A was found to act as a commitment factor, where Activin A made the progenitor commit to differentiation or apoptosis, depending on the presence of Epo as an anti-apoptosis.¹⁸ However, some literature also states that Activin A has an erythropoiesis inhibitor effect, so until now the mechanism underlying the effect of Activin A on erythropoiesis is still unclear.¹⁹ An increase in Epo accompanied by Smad2/3 activation by Activin A and Gdf11 can lead to increase erythroid cell proliferation and decreased erythroid cell maturation, which can exacerbate IE.^{13,19,20} Voskaridou et al. stated that there

is a positive correlation between serum Activin A levels and hemolysis markers in thalassemia major patients.²¹ Blood transfusion in thalassemia major, can cause iron overload resulting in formation of ROS, which then increase Gdf11 expression, followed by increased levels of Activin A.²² Overexpression of Activin A has been found associated with several cancers that can be life-threatening.²³

Although several studies have examined LDH levels and Activin A levels in thalassemia major patients, until now there exist no known studies that compared the differences in LDH levels and Activin A levels in thalassemia major and non-thalassemic patients. The hypothesis of this study is that there are differences in LDH levels and Activin A levels in thalassemia major and non-thalassemia. The aim of this study is to prove the differences in LDH levels and Activin A levels in thalassemia major and non-thalassemia.

Researchers hope that this study can provide evidence of LDH and Activin A as markers to prevent complications of thalassemia, such as lactic acidosis and cancer. In addition, it is also hoped that this study can provide evidence to make Activin A a therapeutic target in improving the clinical symptoms of thalassemia major patients.

METHODS

Research Subject

This study is an analytic observational study with cross sectional approach. The research took place between March and September 2020 and the samples were taken by consecutive sampling. Sample population were routinely transfused thalassemia patients at dr. R. Soedjati, Grobogan Public Hospital and dr. R. Soetrasno, Rembang Public Hospital, as well as healthy populations of the same age, who checked themselves in the growth and development clinic at dr. R. Soedjati, Grobogan Public Hospital and dr. R. Soetrasno, Rembang Public Hospital. Laboratory examination of LDH levels and Activin A levels were each carried out in the Iodine Deficiency Disorder laboratory, Diponegoro University Medical Faculty and Diponegoro National Hospital laboratory, Semarang.

This study recruited 50 subjects consisting of 25 thalassemia major patients routinely transfused at dr. R. Soedjati, Grobogan Public Hospital and dr. R. Soetrasno, Rembang Public Hospital and 25 healthy populations with equal age, that have met the inclusion and exclusion criteria. The inclusion criteria of this study were patients with thalassemia major and non-thalassemia, both men and women with an age range of 2-20 years, thalassemia major patients who had received more than 10 transfusions, and patients willing to become study respondents. The exclusion criteria of this study were patients with leukocytosis and fever, and patients with alcohol intake.

Research Data

Data taken in this study are primary data obtained directly from research subjects. The primary data obtained came from venous blood taken from thalassemia major and non-thalassemia patients at Dr. R. Soedjati, Grobogan Public Hospital and dr. R Soetrasno, Rembang Public Hospital.

Data Analysis

Data were collected from interviews, physical examinations, and laboratory examinations for LDH and Activin A levels. Coding, entry, editing of the data were done using computer software. Data analysis includes descriptive analysis (frequency and mean), and hypothesis testing. Furthermore, each numerical data was analyzed statistically by performing a normality test using the Shapiro-Wilk test because the sample size was ≤ 50 . LDH levels were normally distributed, while the data distribution of Activin A levels was not normal, so the data was transformed first. LDH levels and Activin A levels were normally distributed ($p > 0.05$), then a comparative test was carried out using the independent sample T test, the results were considered significant if $p < 0.05$.

The analysis of confounding variables on LDH levels was carried out by first using the normality test, namely using the Shapiro-Wilk test because the sample size was ≤ 50 . LDH levels were not normally distributed, so data transformation was carried out. After data transformation, LDH levels were normally distributed ($p > 0.05$), so that the analysis was continued

by using the independent sample T test, the results were considered significant if $p < 0.05$.

RESULTS

Research Subject Characteristics

Based on table 1, it can be seen that thalassemia major patients have a balanced gender distribution between 12 men (48%) and 13 women (52%). In non-thalassemia, the majority of the gender were women as many as 16 subjects (64%). The mean age of thalassemia major study subjects (11.52 ± 3.85 years) was higher when compared to non-thalassemics (10.80 ± 4.03 years).

Table 1. Research subject characteristics distribution.

Variable	Mean \pm SD	n, %	
Age (years)			
- Thalassemia major	$11,52 \pm 3,85$		
- Non-thalassemia	$10,80 \pm 4,03$		
Gender		Male	Female
- Thalassemia major		n= 12 (48%)	n= 13 (52%)
- Non-thalassemia		n= 9 (36%)	n= 16 (64%)

Table 2. LDH levels and Activin A levels distribution and comparative test among thalassemia major and non-thalassemia.

Variable	Mean \pm SD	p^*
LDH levels (U/L)		
Thalassemia major	524.48 ± 167.44	0.00
Non-Thalassemia	294.48 ± 131.24	
Activin A levels (pg/ml)		
Thalassemia major	118.75 ± 45.47	0.04
Non-Thalassemia	95.66 ± 26.26	

p^* , independent sample t test (significant)

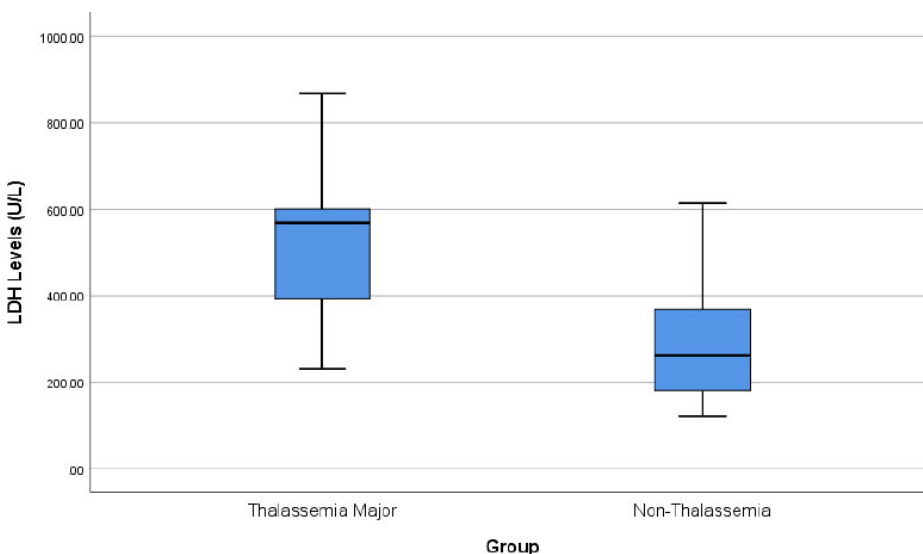


Figure 1. LDH levels box plot graph in thalassemia major and non-thalassemia.

LDH levels and Activin A levels distribution analysis

The mean LDH level in thalassemia major patients was 524.48 ± 167.44 U/L. Meanwhile, LDH levels in non-thalassemia have a mean of 294.48 ± 131.24 U/L. The mean level of Activin A in thalassemia major patients was 118.75 ± 45.47 pg/ml. Meanwhile, the Activin A level in non-thalassemia had a mean value of 95.66 ± 26.26 pg/ml. Normality test results using Shapiro-Wilk were normal ($p > 0.05$), so the comparative analysis of the data was performed using independent sample t test. The results of the independent sample t test showed a significant difference

between LDH levels in thalassemia major and non-thalassemia ($p = 0.00$) (Table 2 & Figure 1).

Normality test using Shapiro-Wilk showed an abnormal distribution ($p = 0.01$ and 0.51), after data transformation was carried out, the data distribution became normal ($p = 0.76$ and 0.75), so the comparative analysis of the data was carried out using the independent sample T test. The results of the independent sample t test showed a significant difference-between Activin A levels in thalassemia major and non-thalassemia ($p = 0.04$) (Table 2 and Figure 2).

Confounding Variable Analysis on LDH Levels

LDH levels in 25 subjects who took vitamin C supplements had a mean of 442.56 ± 184.89 U/L. Meanwhile, LDH levels in 25 subjects who did not take vitamin C supplements had a mean of 376.40 ± 190.64 U/L. Normality test using Shapiro-Wilk showed an abnormal distribution ($p = 0.84$ and 0.02), after data transformation was carried out, the data distribution became normal ($p = 0.51$ and 0.12), so the comparative analysis of the data was carried out using the independent sample T test. The results of the independent sample T test showed no significant difference between LDH levels of patients who took vitamin C supplements and patients who did not take vitamin C supplements ($p = 0.16$) (Table 3 and Figure 2).

DISCUSSION

LDH Levels in Thalassemia Major and Non-Thalassemia

In this study it was found that the mean LDH level in thalassemia major was 524.48 ± 167.44 U/L, while in non-thalassemia the mean LDH level was 294.48 ± 131.24 U/L. Statistical analysis showed that the differences in LDH levels between thalassemia major and non-thalassemia were significant ($p = 0.00$). These results prove that this research hypothesis, which is there are differences in LDH levels in thalassemia major and non-thalassemia, is in accordance with the results of the study.

Oda et al. also stated similar results, where there was a significant increase in LDH levels in beta thalassemia patients.¹⁰

Research by Galila et al. showed that patients with beta thalassemia major had significantly higher levels of LDH, when compared to patients with sickle cell disease and beta thalassemia intermedia.²⁴

Hemolytic anemia in patients with thalassemia major, often causes an increase in LDH levels, which is a marker of intravascular hemolysis.²⁵ Some studies suggest that the increase in LDH levels

in thalassemia major is due to IE.⁷ IE itself is a condition in which an increase in erythroid cells fails to produce an increase in erythrocytes. As a result, iron absorption continues to increase in response to stress and that iron is stored in the organs. Repeated transfusion in thalassemia major was also found to suppress endogenous erythropoiesis. Both these increase iron absorption and repeated transfusion are the primary causes of iron overload in thalassemia major, which cause exacerbation of IE.²⁶ Hemolysis accompanied by IE in thalassemia major lead to anaerobic glycolysis, which is the cause of increased LDH levels in thalassemia major.⁹

In this study, there was a confounding variable on LDH levels, which is vitamin C supplements intake. Statistical analysis showed that there was no significant difference between the LDH levels of subjects who took vitamin C supplements and subjects who did not take vitamin C supplements in this study ($p= 0.16$). Several studies have stated conflicting concerns regarding the effect of vitamin C supplements intake on LDH levels. Shreef et al. stated that there was a significant reduction in LDH levels in the group who consumed vitamin C supplementation with a dose of 500 mg for 90 days.²⁷ However, other studies have stated that after giving vitamin C with a loading dose of 3.0 grams / day, there is an increase in LDH levels.²⁸

Besides vitamin C supplements intake, there are several factors that can affect LDH levels, such as alcohol intake, intense physical activity, tumors, and drugs (anesthesia, aspirin, narcotics, and procainamide).^{29,30,31,32} Intense physical activity can increase LDH levels by 30-50%, where serum LDH is often used as an indicator of muscle damage after resistance exercise and may indicate the status of muscle cell membranes.^{30,33} Excessive alcohol intake can cause damage to the liver, where as a result serum LDH levels will be impaired.³⁴

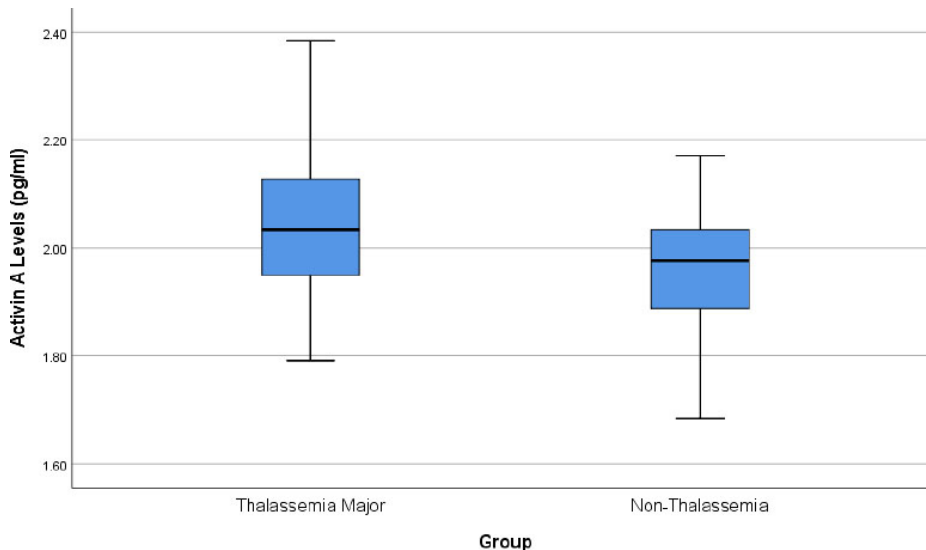


Figure 2. Activin A levels box plot graph in thalassemia major and non-thalassemia.

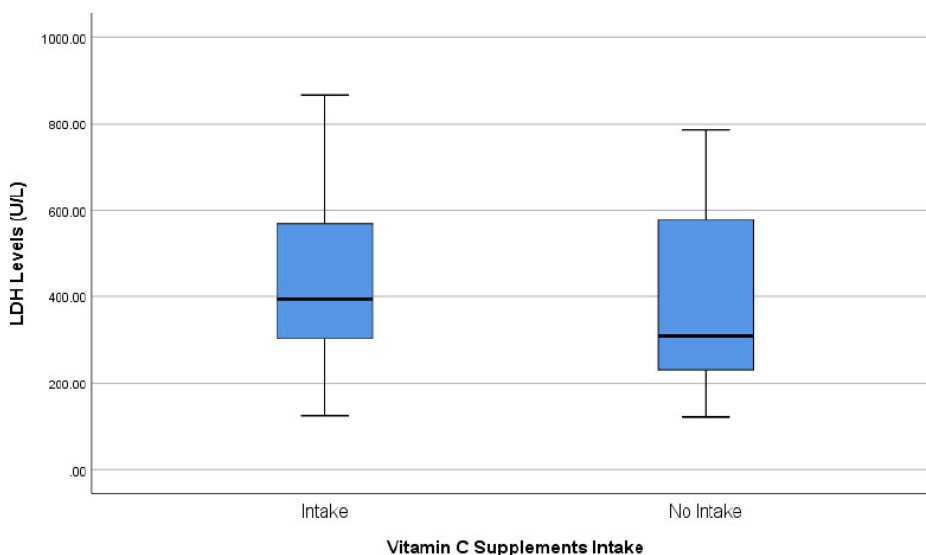


Figure 3. LDH levels box plot graph in subjects who took vitamin C supplements and subjects who did not take vitamin C supplements.

Table 3. LDH levels distribution and comparative test in subjects who took vitamin C supplements and subjects who did not take vitamin C supplements.

Variable	Mean ± SD	<i>p</i>
LDH Levels (U/L)		0.16
Taking vitamin C supplements (25 subjects)	442.56 ± 184.89	
Not taking vitamin C supplements (25 subjects)	376.40 ± 190.64	

p, independent sample t test

Activin A levels in thalassemia major dan non-thalassemia

In this study it was found that the mean level of Activin A in thalassemia major was 118.75 ± 45.47 pg/ml, while in non-

thalassemia the mean Activin A level was 95.66 ± 26.26 pg/ml. Statistical analysis showed that the differences in Activin A levels between thalassemia major and non-thalassemia were significant ($p=0.04$). These results prove that this research hypothesis, which is there are differences in Activin A levels in thalassemia major and non-thalassemia, is in accordance with the results of the study. Voskaridou et al. also stated the same thing, in which it was found that there was a significant increase in Activin A levels of thalassemia major with $p=0.04$.²¹

When the free globin chain in thalassemia major exceeds globin chain stabilizing protein capacity, the free globin chain will undergo auto-oxidation and produce ROS.⁶ These ROS will then activate Gdf11, which in turn will increase Activin A.¹⁶ Gdf11 and Activin A will then activate SMAD2/3, which causes a decrease in erythroid cell maturation. This will then exacerbate IE in thalassemia major patients, as a result these patients will undergo hypoxia.^{16,22} Hypoxia will then increase the production of Epo from the kidneys, whose function is to increase the surviving erythroid progenitor and its proliferation.¹⁴ However, due to impaired erythroid cell maturation by Gdf11 and Activin A, IE in thalassemia major will then experience amplification.²²

Hypoxia also causes a decrease in hepcidin expression, which results in an increase in free iron for erythrocyte production. This condition is exacerbated by hemolysis of erythrocytes that enter the body through repeated blood transfusions in thalassemia major patients. Through the Fenton reaction free iron will be converted into ROS, which will stimulate Gdf11 and increase levels of Activin A.^{20,22}

There are several factors that can affect Activin A levels, such as age and pregnancy that greatly affect Activin A levels. Some studies showed that with increasing age both men and women have increased levels of Activin A, especially in the last decade of life.^{35,36} Some studies have also suggested that Activin A levels and follistatin serum increased significantly during pregnancy and Activin A levels decreased in the presence of *nonviable*

trophoblast, in which the placenta is the main source of Activin A.^{37,38,39,40}

CONCLUSIONS

Hypoxia as a result of IE in thalassemia major patients, causes an increase in LDH levels and Activin A levels. This is indicated by a significant difference between LDH levels and Activin A levels in thalassemia major and non-thalassemia. In addition, the high *free iron* as a result of repeated transfusions in thalassemia major patients also affects the increase in LDH levels and Activin A levels, namely through the formation of ROS.

Further research needs to consider analyzing IE levels and tissue oxygenation effect on LDH levels and Activin A levels. Gdf11 levels also need further analysis to see their effect on Activin A levels. LDH and Activin A can be used as markers to prevent complications of thalassemia, such as lactic acidosis and cancer. In addition, Activin A can be used as a therapeutic target in improving the clinical symptoms of thalassemia major patients.

CONFLICT OF INTEREST

All author declares there are no conflict of interest regarding publication of this article.

AUTHOR CONTRIBUTION

All author had contributed equally in manuscript preparation and agreed to final version of manuscript for publication.

ETHICAL CONSIDERATION

This research has receive ethical clearance from the Health Research Ethics Commission, Faculty of Medicine, Universitas Diponegoro, Semarang Number 102/EC/KEPK/FK-UNDIP/VI/2020. All research subjects were asked for written informed consent prior to this research. Before asking for approval, research subjects were explained in advance about the objectives, benefits, and research procedures. Research subjects have the right to refuse, and will not be given any consequences. The identity of each research subject is kept secret and all

costs for this study are the responsibility of the researcher.

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