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Hyperferritinemia and oxidative stress in the kidney of beta thalassemia major

Dian Ariningrum,[1*] Purwanto Adhipireno,[2] Lisyani Budipradigdo Suromo[2]

ABSTRACT

Background: Thalassemia is a genetic disorder which until now has become a global health problem. Regular blood transfusions in managemen of BTM result in iron overload. Free iron in the form of intracellular ferrous (Fe^{2+}), in the presence of oxygen, will initiate the formation of reactive oxygen species (ROS), produce another free radical superoxide that will oxidize the lipid and protein compounds of the cell membrane. F_2-IsoPs is the best, most stable and more accurate marker of lipid peroxidation in vivo, found urine. This study aimed to determine the association between hyperferritinemia and lipid peroxidation in kidney reflected as elevated u-F_2IsoPs as controlling several factors affecting oxidative stress in the kidney.

Methods: This cross sectional study was conducted between May and June 2016. Thirty subjects of BTM admitted to pediatrics unit of Dr. Moewardi Hospital Surakarta who met the inclusion and exclusion criteria were enrolled. The data were analyzed to calculate prevalence ratio and 95%CI for each variable, followed by multivariate analysis with logistic regression.

Results: The prevalence of lipid peroxidation in kidney, characterized with elevated u-F_2IsoPs of > 2 ng/mg urine creatinine, was 67%. Age, hyperferritinemia status and duration of illness did not associate with lipid peroxidation in the kidney. Transfusion volume of ≥25 unit/year showed statistically significant association with lipid peroxidation (PR 24.10; 95%CI 2.16-268.42; p=0.010).

Conclusion: Transfusion volume, rather than hyperferritinemia, independently associated to oxidative stress and lipid peroxidation of the kidney in BTM.

Keywords: ferritin, lipid peroxidation, urinary F_2-Isoprostane, thalassemia

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[1]Clinical Pathology Department, Medical Faculty, Sebelas Maret University/ Dr. Moewardi Hospital, Surakarta, Central Java, Indonesia
[2]Clinical Pathology Department, Medical Faculty, Diponegoro University, Semarang, Central Java, Indonesia
Department of Dermatology and Venereology, General Hospital of Sumatera Utara University, DR. Mansyur No. 66, Medan, 20154
Clinical Pathology Department, Dr. Moewardi Hospital at Surakarta, Central Java, Indonesia

[*]Correspondence to:
Dian Ariningrum, Clinical Pathology Department, Medical Faculty, Sebelas Maret University/ Dr. Moewardi Hospital, Surakarta, Central Java, Indonesia;
dianariningrum@staff.uns.ac.id

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INTRODUCTION

Thalassemia is a genetic disorder which until now has become a global health problem. World Health Organization (WHO) in 1994 stated that ± 4.5% of the world's population were carriers of thalassemia (heterozygous form), and 80-90 million were beta thalassemia carriers.[1] The distribution of thalassemia starts from the Mediterranean, Middle East, India, Burma, and Southern China, Thailand, the Malayan Peninsula, the Pacific Islands, and Indonesia. Those areas are commonly referred to as the 'thalassemia belt' because the average prevalence of thalassemia reaches 2.5-15%.[2]

Management of beta thalassemia major (BTM) includes continuous blood transfusion with all its consequences. Regular blood transfusions result in iron overload in various organs, such as the liver, heart, kidneys, pancreas, and others. Free iron in the form of intracellular ferrous (Fe^{2+}), in the presence of oxygen, will initiate the formation of reactive oxygen species (ROS), produce another free radical superoxide that will oxidize the lipid and protein compounds of the cell membrane causing cell damage and death.[3,4,5] The increase of lipid peroxidation can be assessed by measuring lipid peroxidation products in the blood, urine, cerebrospinal fluids, and other biological fluids, one of the markers is F_2-Isoprostane (F2-IsoPs).[6]

At present, F_2-IsoPs is the best, most stable and more accurate marker of lipid peroxidation in vivo. F_2-IsoPs are found in almost all biological fluids, but blood (plasma or serum) and urine are the most common samples used as they are easy to obtain, least invasive, and provide accurate and precise oxidative stress indices.[7,8]

Medical interventions provided were related to the effects of recurrent transfusion in TBM patients, namely iron/ chelation therapy. Transfusion, iron chelation with antioxidants will improve the survival of TBM patients until their thirties.[8,9,10]

Iron chelation requires strict monitoring of kidney function due to its nephrotoxic effects. Many studies reported renal tubular dysfunction and decreased glomerular filtration rate (GFR) in BTM patients receiving iron chelators. Bakr et al. (2014) stated that 68% of BTM patients receiving deferoxamine suffered from proteinuria and decreased GFR.[11,12]

The success of thalassemia therapy and the improvement of life expectancy cause new problems, that is the emergence of various complications, including impaired kidney function due to

iron overload. Bakr et al. stated the occurrence of proteinuria, renal tubular dysfunction and decreased glomerular filtration rate (GFR) in BTM patients despite the administration of iron chelation therapy.[12]

Little information is available about oxidative stress, lipid peroxidation and impaired renal function in BTM. The purpose of this study was to determine the relation between hyperferritinemia and u-F2 IsoPs as a marker of oxidative stress and lipid peroxidation in the kidney of BTM patients by controlling the factors affecting kidney disorders.

MATERIAL AND METHODS

This study was an observational analytical with a cross-sectional approach, conducted at the Clinical Pathology Department of the Dr. Moewardi Hospital in Surakarta between May-June 2016. The subjects of this study were pediatric patients diagnosed with BTM visiting Pediatric Outpatient Clinic and carried out laboratory tests at the clinical pathology laboratory of the Dr. Moewardi Hospital. The research subjects were taken consecutively based on inclusion and exclusion criteria.

Inclusion criteria include ages 5-18 years; diagnosed with BTM by pediatrician based on history taking, physical examination and laboratory results of Hb electrophoresis; received 10–15 times of transfusions for more than 1 year; received the same iron chelation therapy, namely Deferasirox (DFX) and did not suffer from urinary infection (UTI). The parents agreed that their child would take part in the study by signing informed consent. History of liver and kidney disease in addition to the effects of BTM therapy, patients with acute infection/ inflammation; conditions that increase oxidative stress such as DM, asthma, allergies, rheumatoid arthritis, obesity and smokers and BTM patients receiving non-steroidal anti-inflammatory drugs (NSAIDs) of cyclooxygenase inhibitors were excluded from the study. The research subjects were 30 children.

Serum creatinine levels were examined enzymatically by using ILAB 650 chemistry analyzer. The estimated glomerular filtration rate (eGFR) is calculated using the Schwartz formula.

The blood and urine F_2IsoPs were measured by enzyme-linked immunoassay (ELISA) with reagents from the Cayman Chemical 8-IsoPs EIA. The samples were a serum and random urine. The venous blood was put into two tubes with a clot activator, then was centrifuged for 10-15 minutes at the speed of 6000 rotations per minute (rpm) to obtain the serum. Serum samples for F_2-IsoPs were paced into a tube added with 0.005% butylated hydroxytoluene (BHT) as a free radical scavenger, and stored at -80°C.[14,15]

Urine samples for u-F_2IsoPs were transferred into two 5 ml polypropylene tubes which had been given 20 µl BHT and 20 µl indomethacin, exactly 2 ml of urine per tube, then the sample was divided into four aliquots each of 0.5 ml and immediately stored at -80°C. Storage of blood and urine samples for F_2IsoPs examination was carried out in an accredited reference laboratory until the time of analysis. The analysis of serum and urine F_2IsoPs were carried out in the same laboratory. A normal level of u-F_2IsoPs is <2 ng/ mg urine creatinine.[13,14] Urinary creatinine measurement to correct the u-F_2IsoPs was performed enzymatically using ILAB 650 chemistry analyzer.

The subject's data were obtained from medical records, the history taking from the parents as well as registers of thalassemia obtained from POPTI, an organization of thalassemia patient's parents at Surakarta.

Hyperferritinemia was defined as a mean ferritin level of more than 2500 ng/ml in the last year. Episodes of hyperferritinemia were the frequency of a patient experiencing hyperferritinemia in the last year, categorized as >4 times and <4 times annually.

The data distribution was assessed by the Saphiro-Wilk test. The characteristics data were presented as mean + standard deviation (SD) if it is normally distributed, and median (min-max) if it is not normally distributed. Comparative analysis was done to assess differences between groups based on u-F_2IsoPs levels using independent-t test if normally distributed and Mann Whitney test if the data is not normally distributed. Bivariate analysis was conducted to find out the relationship between hyperferritinemia and other variables that might influence lipid peroxidation in the kidneys using a 2x2 table to get the prevalence ratios (PR) and 95% confidence of intervals (95%CI). Multivariate analysis was carried out by analyzing the influence of other variables on the relationship between hyperferritinemia and lipid peroxidation in the kidneys. The analysis was processed using SPSS 23.0 computer program, p-value was considered significant if <0.05.

RESULTS

The research subjects were comprised of 15 boys (50%) and 15 girls (50%) with the mean age of 12.2± 3.58 years. The mean u-F_2IsoPs is 2.36 ± 1.11 ng/mg urine creatinine. Increased lipid peroxidation in the kidney, characterized by u-F_2IsoPs level of >2 ng/mg urine creatinine, occurred in 20 subjects (66.67%). The group with u-F_2IsoPs level of >2 ng/mg urine creatinine significantly had older age compared to the group with u-F_2IsoPs of <2 ng/mg urine creatinine (13.1 ± 3.34 years vs.

10.4 ± 3.5; p=0.001). The mean serum creatinine and ferritin levels between the two groups were not significantly different (p=0.086 and p=0.309). All subjects had an estimated glomerular filtration rate (eGFR) of more than 120 mL/min/1.73 m^2, but the eGFR of group with u-F_2IsoPs level of > 2 ng/mg urine creatinine was significantly higher than the group with u-F2IsoPs level of <2 ng/mg urine creatinine (220.5 mL/min/1.73 m^2 vs. 167 mL/min/ 1.73 m2; p= 0.001). Other variables that differed significantly between the two groups were duration of illness (p=0.01) and volume of

Table 1 Baseline characteristics of the respondents

Variables	Total (n=30)	BTM with lipid peroxidation (uF_2IsoPs > 2 ng/mg urine creatinine) [n = 20 (66.67%)]	BTM without lipid peroxidation (uF_2IsoPs < 2 ng/mg urine creatinine) [n = 10 (33.33%)]	p
Age (years)	12.2 ± 3.58	13.1 ± 3.34	10.4 ± 3.5	0.001*
Sex				0.001*
Male	15 (50%)	12 (40%)	3 (10%)	
Female	15 (50%)	8 (26.67%)	7 (23.33%)	
Serum Creatinine (mg/dl)	0.3 (0.2-0.5)**	0.3 (0.2-0.5)**	0.3 (0.2-0.5)**	0.086
eGFR (ml/min/1,73 m^2)	189.5 (121-330)**	220.5 (134-330)**	167 (134-330)**	0.001*
Serum Ferritin (ng/ml)	5685.24 ± 2642.50	5918.16 ± 2558.74	5219.42 ± 2883.86	0.309
Duration of illness (months)	97.6 ± 46.29	104.4 ±50.33	84 ± 35.33	0.01*
Transfusion volume (unit/year)	40.4 ± 19.9	47.8 ± 19.0	25.6 ± 12.4	0.002*
u-F_2IsoPs (ng/mg urine creatinine)	2.37 (0.331-4.813)**	2.83 (2.014-4.813)**	1.12 (0.331-1.995)**	0.001*

eGFR estimated Glomerular Filtration Rate
*significant if p<0.05
**data not normally distributed, expressed in median (min-max), Mann-Whitney U-test

Table 2 Bivariate analysis of hyperferritinemia and other variables with lipid peroxidation in BTM

Variables	u-F_2IsoPs (ng/mg urine creatinine)		PR (95%CI)	p
	> 2	<2		
Age				
< 12 years	8 (26.67%)	6 (20%)		
≥ 12 years	12 (40%)	4 (133.33%)	1.313 (0.769-2.240)	0.301
Sex				
Female	8 (26.67%)	7 (23.33%)		
Male	12 (40%)	3 (10%)	1.500 (0.877-2.566)	0.121*
Hyperferritinemia				
< 2,500 ng/ml	7 (23.33%)	4 (13.33%)		
≥ 2,500 ng/ml	23 (76.67%)	16 (53.34%)	1,217 (0,607-2,442)	0,542
Episode of hyperferritinemia				
< 4 x per year	16 (53.34%)	10 (33.34%)		
≥ 4 x per year	4 (13.33%)	0 (0.00%)	1.625 (1.199-2.202)	0.129*
Transfusion volume				
< 25 unit/ year	3 (10.00%)	8 (26.67%)		
≥ 25 unit/ year	17 (56.67%)	2 (6.66%)	3.281 (1.235-8.717)	0.0001*
Duration of illness				
< 72 months	6 (20%)	3 (10%)		
≥ 72 months	14 (46.67%)	7 (23.33%)	1.000 (0.576-1.737)	1.000

PR: Prevalence Ratio, CI: Confidence of Interval
* significant if p<0.25

Table 3 Multivariate analysis of hyperferritinemia and other variables affecting lipid peroxidation in BTM

Variable	PR	95%CI	P
Model 1			
Episode of hyperferritinemia ≥ 4 x/year	0.00	0.00-∞	0.999
Transfusion volume ≥ 25 unit/year	24.10	2.16-268.42	0.010*
Male	8.09	0.73-89.67	0.088
Model 2			
Transfusion volume ≥ 25 unit/year	34.41	3.12-379.22	0.004*
Male	6.84	0.61-76.39	0.118

PR: Prevalence Ratio, CI: Confidence of Interval
* significant if p<0.05

transfusion (p=0.002). The full description of the characteristics of the research subject can be seen in table 1.

The eGFR variable was not included in the bivariate analysis because all subjects experienced an increase in eGFR. Hyperferritinemia variable included in the bivariate analysis was the mean of ferritin level of ≥ 2500 ng/ml and the hyperferritinemia episodes of ≥ 4 times per year.

Bivariate analysis between hyperferritinemia as well as other variables and levels of u-F_2IsoPs (Table 2), demonstrated that sex, episodes of hyperferritinemia and transfusion volume are associated to the lipid peroxidation in the kidneys, with a prevalence ratio (PR) of 1.500 (95%CI: 0.877-2.566), p=0.121; PR=1.625 (95%CI: 1.199-2.202), p=0.129 and PR=3.281 (95%CI: 1.235-8.717) p=0.0001, respectively. These three variables were included in the subsequent analysis process. Age, mean of hyperferritinemia and duration of illness was not related to lipid peroxidation in the kidneys, with a p=0.301; p=0.542 and p=1.000, respectively.

Table 3 demonstrates the multivariate analysis of sex, episodes of hyperferritinemia and volume of transfusion with lipid peroxidation in the kidneys.

The results of multivariate analysis showed, in Model 2, after adjustment for sex, transfusion volume of ≥25 units/year was independently and significantly associated with lipid peroxidation in the kidneys with PR=34.41 (95%CI: 3.12-379.22; p=0.004). Adjustment of male sex and episodes of hyperferritinemia of ≥4 times per year (Model 1), transfusion volume of ≥25 units/year still showed a statistically significant relationship with lipid peroxidation (PR=24.10; 95%CI: 2.16- 268.42; p=0.010).

DISCUSSION

In this study, 66.67% of subjects experienced an increased u-F_2IsoPs. Previous studies reported an increase in biomarkers of oxidative stress such as MDA (malondialdehyde) and TBARS (thiobarbituric acid reactive substances) which are byproducts of lipid peroxidation.[15] Matayatsuk et al. (2007) reported an increase in urinary F_2IsoPs in BTM patients compared to healthy controls with an average of 3.38 ± 2.15 ng/mg urine creatinine vs. 0.86 ± 0.55 ng/mg urine creatinine.[16] The finding is consistent with the occurrence of oxidative stress and lipid peroxidation in the kidneys of thalassemia patients.

Serum creatinine was normal in all our study subjects (median 0.3 mg/dl; median 0.2-0.5 mg/dl). The eGFR for all subjects in both groups was more than 120 mL/min/1.73 m^2, but the eGFR of group with u-F_2IsoPs >2 ng/mg urine creatinine was significantly higher than the group with u-F_2IsoPs of <2 ng/mg urine creatinine. There is a strong and significant positive correlation between u-F2IsoPs and eGFR (r=0.628; p=0.001, data not shown), indicating that there is an increase in the value of eGFR along with an increase in u-F_2IsoPs.

The eGFR results were at a median of 271.3 ml/min/1.73m^2 with a minimum value of 199.8 ml/min/1.73 m^2 and a maximum value of 476.0 ml / min/1.73 m^2 indicating glomerular hyperfiltration which is the initial stage of renal glomerular damage, as researched by Ziyadeh et al. (2012).[17] Deveci et al. (2016) found that there were glomerular and tubular defects in 68.8%, 40% of patients had glomerular hyperfiltration with GFR values of >130 ml/min/1.73 m^2, none of the study subjects had a decrease GFR.[18,19] A retrospective study by Lai et al (2012) on 81 adult BTM patients (aged 28.5 +2.7 years) stated that only 18.5% of subjects experienced a decrease in eGFR of <90 ml/min/1.73 m^2.[20] Brenner et al. (1996) argued that a long-term (≥1 year) glomerular hyperfiltration condition without therapy for underlying disease would result in podocyte stress and renal glomerular damage followed by albuminuria, irreversible changes in glomerular structure, glomerulosclerosis, decreased GFR and eventually chronic kidney disease.[21]

Our bivariate analysis demonstrated that age, hyperferritinemia status and the duration of illness were not associated with the process of lipid peroxidation in the kidneys. A study by Lai et al. (2012), in most adult BTM patients, eGFR tends to be normal after suffering BTM for ten years.[20]

The bivariate analysis revealed that hyperferritinemia of > 2500 ng/ml was not associated with the lipid peroxidation in the kidneys, as well as episodes of hyperferritinemia of ≥4 times per year in our multivariate analysis. Ferritin levels cannot describe the severity of kidney hemosiderosis and iron chelation therapy alone is not enough to reduce iron accumulation in the kidneys. It is still believed that kidney damage is caused by iron overload, but cannot be assessed only by serum ferritin levels.[20] This condition is because ferritin is also an acute-phase reactant that increases in inflammatory conditions, infections, and malignancies, and the intact ferritin molecule is a very stable, inert protein and non-toxic.[22] Many studies state that ferritin levels and the trend of ferritin levels are not predictors of liver iron content (LIC). [23]

Cabantchik (2014) proposed that in transfusional iron overload, the iron component that plays a role in oxidative stress is labile plasma iron (LPI) which depicts the labile fraction of plasma non-transferrin-bound iron (NTBI). Labile plasma iron is redox-active iron which is an exchangeable form, so it is the target of chelation. Technical constraints are still a major problem in measuring this labile fraction.[24]

In our study, the transfusion volume of ≥25 units year was independently and significantly associated with lipid peroxidation in the kidney with PR 34.41 (95% CI: 3.12-379.22; p=0.004). One unit of packed red cell (PRC) contains 200-250 mg of iron. Based on the transfusion algorithm for BTM, 100-200 ml PRC per kilogram bodyweight is transfused per year, and this is equivalent to 116-232 mg iron per kilogram body weight per year or 0.32-0.64 mg per kilogram body weight per day.[25] The volume of blood transfused is a predictor of iron overload and organ dysfunction.

This study has some limitations as it is cross-sectional research so that it is not possible to find the definitive cause and effect relationship. We also did not measure the reference value of u-F_2IsoPs levels in the pediatric population so that the normal level of u-F_2IsoPs in this population was unknown. This research is a local study in the Surakarta and surrounding areas, so further multicenter research is needed with a larger number of subjects. Hopefully, this study will provide an idea for further research so that a predictive model of oxidative stress to detect early kidney function disturbance in BTM patients can be made.

CONCLUSION

In this study, hyperferritinemia was not associated with oxidative stress and lipid peroxidation in the kidneys. After adjustments for sex and episodes of hyperferritinemia, the volume of transfusion remains independently associated to the occurrence of lipid peroxidation which is characterized by an increase in the level of u-F_2IsoPs of > 2 ng/mg urine creatinine. Further research needs to be done with a larger number of subjects and case-control design by considering attention to the factors affecting the outcome such as the severity of anemia. The increased urine F_2IsoPs levels as a marker of oxidative stress in the kidneys have not been able to determine whether the oxidative stress originates from damage to the glomerulus or renal tubules, so further research is needed using specific markers of glomerular or tubular.

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CONFLICT OF INTEREST

The author reports no conflicts of interest in this work. This paper has not been published in other journals, but presented in Annual Scientific Meeting of Clinical Pathology and Laboratory Medicine Association in Bali, November 1st, 2018.

ETHICAL CLEARANCE

The study was approved by the research ethics committee of Dr. Moewardi Hospital in Surakarta.

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AUTHOR CONTRIBUTION

All of authors are equally contributed from the manuscript preparation, statistical analysis, until study reports to be published.

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