Original Article

Study on Oxidative Stress in Thalassemic Red Blood Cells

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Abstract: *Thalassemia red blood cells (RBCs) are susceptible to oxidative stress, which play a major role in hemolysis. In order to evaluate the oxidative stress in RBCs, we measured the mean fluorescent intensity of dichlorofluorescein (DCF) in RBCs after incubation either with or without 2 mM H_2O_2 by flow cytometry. The subjects were 20 healthy adults, 10 α-thalassemia or Hemoglobin (Hb) H patients, 12 un-splenectomized and 11 splenectomized β-thal/Hb E patients. For unoxidized RBCs, significantly higher fluorescent intensity of DCF were observed in thalassemic RBCs, especially in Hb H disease (10.5$\pm$7.0), when compared with healthy subjects (2.2$\pm$0.4) ($p < 0.001$), un-splenectomized β-thal/Hb E (4.0$\pm$1.5) ($p = 0.008$), but no statistical significant difference was found when compared with splenectomized β-thal/Hb E (7.1$\pm$3.8) ($p > 0.05$). Flow cytometric analysis of H_2O_2 - oxidized RBCs in all groups showed significantly higher DCF intensity than unoxidized RBCs ($p < 0.001$). The H_2O_2 - oxidized RBCs of Hb H disease (300.1$\pm$191.2) were higher than un-splenectomized (56.8$\pm$50.4) and splenectomized β-thal/Hb E (79.2$\pm$51.7) ($p < 0.001$, 0.005, respectively). There were no significant difference between un-splenectomized and splenectomized β-thal/Hb E ($p > 0.05$). These findings suggest that there are higher oxidative stress in thalassemic RBCs than healthy subjects and Hb H RBCs seem to have higher oxidative stress than β-thal/Hb E.*

Key Words : ● *Oxidative stress* ● *Thalassemia* ● *Flow cytometry*

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Thalassemia (thal) is a genetic disorders caused by a partial or complete deficiency of α- or β-globin chain synthesis[1]. In Thailand, α- and β-thalassemia and abnormal Hb E are common[2-4]. The pathophysiology of diseases related to the degree of anemia is caused by both intramedullary hemolysis and red blood cells (RBCs) destruction in peripheral blood.

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Thalassemic RBCs are generally prone to hemolysis and have shortened life span compared with normal RBCs. Rapid iron turn over and tissue deposition of excess iron are also found. The pathologic of RBCs is thought to be the direct consequence of the excess unpaired globin chains, β-chains in the case of α-thalassemia (hemoglobin (Hb) H disease) and α-chains in the case of β-thalassemia. There has been accumulating evidence showing that α- and β-thalassemic RBCs have different abnormalities resulting from the deleterious effects of excess globin chains which attributed to increased oxidative stress[5-11]. The mechanisms facilitating oxidative damage are multifactorials and still unclear. The degree of oxidative stress was indirectly measured by the alteration of antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and catalase[12-14] or the products of lipid peroxidation such as malonyldialdehyde (MDA) content[14-17]. However, these methods are complicated and in some cases can be subjective. There are also several direct procedures for measuring oxidative stress, including pulse radiolysis, electron spin resonance spectroscopy and chemiluminescence. Again, those methods are expensive, tedious and complicated. Detection of oxidative stress in various types of cells using 2',7'-dichlorofluorescein diacetate (DCFH) has been widely used[18,19]. Intracellular oxidation of nonfluorescent DCFH to highly fluorescent 2',7'-dichlorofluorescein (DCF) provided a signal which can be detected on a single cell basis, by flow cytometry. This method is an indirect measure of reactive oxygen species production and offers several advantages, mainly the ability to quantitate a large number of cells. Recently, Amer et al[20], used flow cytometric analysis to show that increasing mean fluorescent intensity of DCF in the RBCs was due to the effect of oxidative stress on the β-thalassemic RBCs which were incubated either with or without H_2O_2. This study reports an attempt to evaluate the oxidative status of various thalassemic RBCs incubated either with or without 2 mM H_2O_2 by using flow cytometric technique.

Materials and Methods

Venous blood were collected in K_2EDTA from healthy volunteers subjects (n = 20), Hb H disease (n = 10), patients with splenectomized β-thal/Hb E (n = 11) and un-splenectomized (n = 12) β-thal/Hb E patients. Their ages range between 20-52 years. The diagnosis of thalassemia was performed on the basis of clinical and laboratory findings as previously described[2,3]. All patients are in steady state and have not received blood transfusion for at least 3 months before blood collection.

For the preparation of stimulated or unstimulated RBCs with 2 mM H_2O_2[20], 2 μL of blood samples of both healthy subjects and thalassemic patients was diluted with 9 mL of phosphate buffered saline (PBS) (Sigma, St. Louis, USA). Then, 60 μL of 20 mM DCFH was added to 3 mL of blood suspension and incubated in 5% CO_2 at 37°C for 15 min. RBCs

were washed 2 times with 3 mL of PBS by centrifugation at 2,000 rpm for 5 min. Then, RBCs was resuspended in 1 mL of PBS. 500 μL of the cell suspension were left at room temperature with or without freshly prepared 2 mM H_2O_2. H_2O_2, an oxidizing agent, used to react with cellular iron which will eventually lead to Fenton reaction and the generation of free radicals.

Two thousand RBCs were analyzed by a Fluorescence Activated Cell Sorter (FACScan, Becton Dickinson, San Jose, USA) and the arithmetic mean fluorescence intensity of DCF, detected with green 520 nm fluorescence, was derived by CellQuest software (Becton Dickinson, San Jose, USA) (Fig.1). A 488 nm argon laser beam was used for excitation.

Data were analyzed with SPSS for Windows, release 7.5. Difference were considered statistically significant at $p < 0.05$. For comparison of the mean fluorescence intensity of DCF in RBCs, the nonparametric tests were used. Comparisons between unoxidized and oxidized RBCs with 2 mM H_2O_2 was performed with Wilcoxon-signed ranks test. The data obtained from thalassemia and healthy subjects were evaluate by using Mann-Whitney U test.

Results

The mean fluorescence intensity of DCF in RBCs unoxidized or oxidized with 2 mM H_2O_2 in each group are shown in Table 1. Unoxidized Hb H RBCs were significantly higher than healthy subjects ($p < 0.001$) and un-splenectomized β-thal/Hb E ($p = 0.008$), but no statistical significant difference was found when compared with splenectomized β-thal/Hb E ($p > 0.05$). However, significant differences were observed when compared between splenectomized β-thal/HbE and un-splenectomized β-thal/HbE ($p = 0.029$) and also healthy subjects ($p < 0.001$). In all groups of oxidized RBCs with 2 mM H_2O_2, the mean fluorescence intensity of DCF were significantly higher than unoxidized RBCs ($p < 0.001$). Oxidized RBCs of Hb H disease were higher than un-splenectomized and splenectomized β-thal/Hb E ($p < 0.001$, 0.005, respectively). However, there were no statistically significant differences between healthy subjects and β-thal/Hb E patients ($p > 0.05$).

Discussion

Prasartkaew et al,[12] Ong-Ajyooth et al,[14] who studied antioxidants enzymes in the RBCs of Hb H diseases and β-thal/Hb E, showed a significant elevation of SOD and GSH-Px activities and also the level of both enzymes in Hb H diseases were higher than β-thal/Hb E. Their results reflect the status of oxidative stress within the thalassemic RBCs, due to the increased production of reactive oxygen species such as superoxide anion and H_2O_2. Our finding are consistent with the previous reports. Vatanavichan et al,[15] found that both MDA level and non-heme iron in Hb H diseases and β-thal/Hb E were significantly higher than control and suggested that in thalassemic RBCs, the lipid is more susceptible to autoxidation either by higher

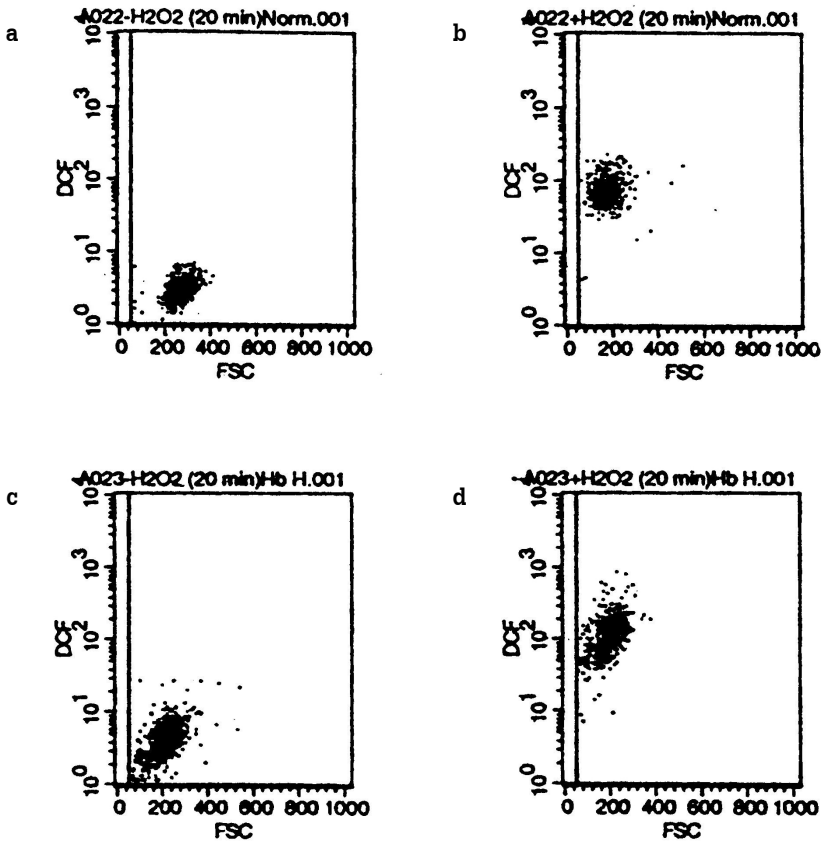

Fig. 1 Flow cytometric analysis of mean fluorescent intensity of DCF in red blood cells unoxidized and oxidized with 2 mM H_2O_2 of healthy subjects (a, b) and Hb H disease (c, d). Red blood cells were incubated with 0.4 mM DCFH and then washed (a, c) or oxidized with 2 mM H_2O_2 (b, d). Dot-plots of 2,000 red blood cells with intensity of the DCF fluorescence and forward scatter light (FSC) are shown. The population had an arithmetic mean fluorescent intensity of 2.99 (a), 78.14 (b), 4.67 (c), and 131.27 (d), respectively.

Table 1 The mean fluorescent intensity of DCF of red blood cells from healthy subjects and thalassemic patients.

Subjects	Unoxidized with 2 mM H_2O_2		Oxidized with 2 mM H_2O_2	
	Mean±SD	Min - Max	Mean±SD	Min - Max
Healthy (n = 20)	2.2±0.4	1.5 - 3.0	73.0±46.7	18.8 - 202.5
Hb H disease (n = 10)	10.5±7.0	3.5 - 26.8	300.0±191.2	73.5 - 620.7
β-thal/Hb E (n = 12)	4.0±1.5	2.2 - 6.8	56.8±50.4	14.9 - 205.7
β-thal/Hb E (S) (n = 11)	7.1±3.8	3.0 - 13.3	79.2±51.7	19.0 - 173.1

β-thal/Hb E = β-thalassemia/Hb E (Un-splenectomy); β-thal/Hb E (S) = β-thalassemia/Hb E (Splenectomy)

lipid content of cell membrane, inadequate peroxide defensive mechanism or more oxygen free radicals. Increased non-heme iron content may be cause of increased membrane lipid peroxidation, since they could generate more superoxide radicals[21-23]. Excessive globin chain which were expected in thalassemic RBCs, have also been shown to generate superoxide[24,25].

RBCs of patients with Hb H diseases contain excessive β-globin chains which are unstable and can be oxidized to form intracellular precipitate and become attached to cell membrane which cause local oxidative damage and membrane dysfunction[6,7,10,26]. The amount of excess α-globin chains was found to correlate directly with the extent of hemolysis in β-thalassemia[26]. Unpaired α-globin chains are more unstable than unpaired β-globin chains and therefore they precipitate earlier, while still in the nucleus and cytoplasm of young nucleated RBCs and prior to react with specific sites on the membrane which lead to defective erythroid maturation and short RBCs survivals[28]. In the present study, we have used flow cytometric assays to monitor reactive oxygen species production by RBCs. A wide application of this method has been previously used to study the oxidative burst of neutrophils induced by different stimuli[18]. Intracellular DCFH was also oxidized by reagent H_2O_2. However, the fluorescent intensity of DCF in both H_2O_2 -stimulated and unstimulated RBCs of Hb H disease are significantly higher than healthy subjects and β-thal/Hb E patients.

Oxidative damage in thalassemic RBCs is complex and multifactorial. Actual proof of excessive free radicals production in RBCs is still warranted. However, it is difficult to measure such a active radicals. There is still a need for additional data in order to complete the understanding of the oxidative status in thalassemic RBCs.

In summary, measurement of the mean fluorescent intensity of DCF, using flow cytometer, can be used to detect oxidative status in RBCs. Our findings are preliminary data that reflect oxidative status in thalassemic RBCs, and provide supportive evidence that oxidative stress in thalassemic RBCs are higher than healthy subjects and that Hb H Rbcs are more severe than β-thal/Hb E. Further studies are needed to understand the cause of oxidative stress in order to prevent the oxidative damage.

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การศึกษา Oxidative Stress ในเม็ดเลือดแดงธาลัสซีเมีย

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บทคัดย่อ: *เม็ดเลือดแดงธาลัสซีเมียมีความไวรับต่อภาวะ oxidative stress ซึ่งมีบทบาทสำคัญต่อการทำลายเม็ดเลือดแดง เพื่อที่จะประเมินภาวะ oxidative stress ในเม็ดเลือดแดง การศึกษาครั้งนี้จึงได้วัดค่าเฉลี่ยการเรืองแสงฟลูออเรสเซนซ์ของสาร dichlorofluorescein (DFC) ในเม็ดเลือดแดงที่ถูกไม่กระตุ้นและถูกกระตุ้นด้วย 2mM H_2O_2 โดยวิธีโฟลไซโตเมตรี ตัวอย่างตรวจประกอบด้วยผู้ใหญ่ที่มีสุขภาพดี 20 ราย ผู้ป่วยอัลฟ่า-ธาลัสซีเมียหรือโรคฮีโมโกลบินเอช (10 ราย) เบต้า-ธาลัสซีเมีย/ฮีโมโกลบิน อี ไม่ตัดม้าม (12 ราย) และ เบต้า-ธาลัสซีเมีย/ฮีโมโกลบิน อี ตัดม้าม (11 ราย) ผลการศึกษาพบว่า ค่าเฉลี่ยการเรืองแสงฟลูออเรสเซนซ์ของสาร DCF ในเม็ดเลือดแดงที่ไม่ถูกออกซิไดส์ ของผู้ป่วยโรคฮีโมโกลบิน เอช (10.5±7.0) มีค่าสูงอย่างมีนัยสำคัญทางสถิติเมื่อเทียบกับผู้มีสุขภาพดี (2.2±0.4) ($p < 0.001$) และ เบต้า-ธาลัสซีเมีย/ฮีโมโกลบิน อี ไม่ตัดม้าม (4.0±1.5) ($p = 0.008$) แต่ไม่แตกต่างจากเบต้า-ธาลัสซีเมีย/ฮีโมโกลบิน อี ตัดม้าม (7.1±3.8) ($p > 0.05$) การวิเคราะห์โดยโฟลไซโตเมตรีของเม็ดเลือดแดงกลุ่มที่ถูกออกซิไดส์ด้วย H_2O_2 จะมีค่าเฉลี่ยการเรืองแสงฟลูออเรสเซนซ์ของสาร DCF สูงกว่ากลุ่มที่ไม่ถูกออกซิไดส์อย่างมีนัยสำคัญทางสถิติ ($p < 0.001$) เม็ดเลือดแดงของโรคฮีโมโกลบิน เอช (300.1±191.2) ที่ถูกออกซิไดส์ด้วย H_2O_2 มีค่าเฉลี่ยสูงกว่าเบต้า-ธาลัสซีเมีย/ฮีโมโกลบิน อี ไม่ตัดม้าม (56.8±50.4) และเบต้า-ธาลัสซีเมีย/ฮีโมโกลบิน อี ตัดม้าม (79.2±51.7) ($p < 0.001$, 0.005 ตามลำดับ) แต่ในผู้ป่วยเบต้า-ธาลัสซีเมีย/ ฮีโมโกลบิน อี ไม่ตัดม้าม และตัดม้ามไม่มีความแตกต่างกันอย่างมีนัยสำคัญทางสถิติ ($p > 0.05$) ผลการศึกษาแสดงให้เห็นว่าในเม็ดเลือดแดงธาลัสซีเมียมีภาวะ oxidative stress สูงกว่าผู้มีสุขภาพดี และในเม็ดเลือดแดงฮีโมโกลบิน เอช มี oxidative stress สูงกว่าผู้ป่วยเบต้า-ธาลัสซีเมีย/ฮีโมโกลบิน อี*

Key Words : ● *Oxidative stress* ● *Thalassemia* ● *Flow cytometry*
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คติธรรมนำกำยัดจุดอ่อน

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