Short Communication
Association Between Thalassemia and Leucocytic DNA Damage: A Pilot Study

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Abstract: Objectives: Thalassemia with frequent blood transfusion was considered under oxidative stress because of the chance of iron overload. Patients with thalassemia trait with no blood transfusion might also suffered from oxidative stress because of increased iron metabolism. This pilot study was to investigate if patients of alpha or beta thalassemia trait and received no blood transfusion were suffered from oxidative stress in term of DNA damage in peripheral leucocytes. Method: Comet assay was used to measure DNA damage of 20 normal subjects, 8 alpha and 12 beta thalassemia patients who did not received blood transfusion. The baseline and UV-mediated DNA damages of peripheral white blood cells were measured. The degree of DNA damage was quantified by visual scoring under light microscope after staining with Giemsa stain. Results: The mean (± standard deviation) comet score for normal baseline samples was 8.3 ± 6.1 whereas 105.4 ± 15.7 and 69.8 ± 20.3 for alpha and beta thalassemia respectively. While the comet scores were 71.9 ± 19.6, 193.1 ± 21.8 and 211.8 ± 51.6 for normal, alpha and beta thalassemia samples respectively in UV-treated samples. Results showed that both alpha and beta thalassemia patients had higher leucocytic DNA damage in baseline and oxidative stressed samples. Conclusion: Our data suggested thalassemia patients were under oxidative stress even no iron over loaded through transfusion.

INTRODUCTION

DNA damage can be caused by reactive species, radiation, and other chemicals (De Boer et al., 2002; Jackson and Bartek, 2009). Some of the damages are reversible by the body's repair mechanism. Different checkpoints are present within the cell cycle to ensure the intactness of the cell development. Oxidative DNA damage could be induced by natural metabolic by-products in our body or from the environment (Cadet & Davies, 2017). Reactive oxygen species (ROS) are common cause of DNA damage. They can be the natural byproduct of oxygen metabolism inside our body. When ROS level in the body are high, they could lead to significant damage to macromolecules and the cell structure. One of the endogenous sources of ROS is from the ATP generation process. ROS are also produced by immune cells such as neutrophils and eosinophils as the weapon of the immune system. Exogenous sources of ROS include but not limited to tobacco, ionizing radiation, pollutants and drugs (Kryston et al., 2011). The oxidative imbalance increases damage of DNA which may cause genomic instability which is the concern of cancer and ageing (Petr et al., 2020). Chronic diseases, such as hemochromatosis and diabetes, are suggested to relate to oxidative stress and associated with DNA damage (Galaris & Pantopouos, 2008; Szeto & Yeung, 2021).

Thalassemia is an inherited blood disorder in which the body produces abnormal hemoglobin. Defective gene is found in chromosome 16 in alpha-thalassemia and chromosome 11 for beta thalassemia. Thalassemia patients tend to have smaller red blood cells (RBCs), higher RBC count and higher red cell distribution width (RDW). The body tries to produce more RBCs to maintain the oxygen supply, though most of the RBCs would be microcytic due to the defective or partially defective hemoglobin. Low hemoglobin level is caused by the higher RBCs destruction rate. Most of the patients only inherit the trait of thalassemia with no significant clinical sign. The absence or reduced amount of beta and/or alpha globin gene expression would cause a decreased functionality of the hemoglobin inside the RBCs. Severity depends on the number of missing or defective globin gene. Patients with severe anemia would require frequent blood transfusion which could possibly lead to iron overload which could cause DNA fragmentation (Shaw et al., 2017). Nevertheless, thalassemia patients with no regular blood transfusion still have higher rate of hemoglobin degradation. The iron absorption through duodenum is higher than normal (Hershko et al., 1998). The ineffective erythropoiesis may contribute to oxidative stress (Rifkind & Mohanty, 2014). The linkage between thalassemia with no blood transfusion and leukocytic DNA damage has not been established. The aim of the study was to investigate the association between subjects with thalassemia trait but no blood transfusion and the DNA damage of leucocytes.

MATERIALS AND METHODS

Forty EDTA specimens were collected based on the laboratory findings in hematology and clinical chemistry. Convenient specimens of normal subject were included. Since age is positively associated with DNA damage of peripheral white blood cells (unpublished results), average age of normal subject were kept higher or equal to the thalassemia group. Specimens with Hb H inclusion bodies seen in a supravital stained blood film were classified as alpha thalassemia. Although HbA2 level greater than 4.0% is generally considered at as beta thalassemia carrier (Hoffbrand et al., 2016), HbA2 higher than 5.0% was included in the current study. All patients did not receive blood transfusion. Specimens were stored in -20°C until comet assay was performed.

Table 1: Demographics of normal subjects and thalassemia patients

	Normal subjects (n=20)	Alpha-thalassemia (n=8)	Beta-thalassemia (n=12)
Age (years)	56.1 ± 3.7	48.1 ± 24.7	48.4 ±19.3
Gender (M/F)	16/4	5/3	7/5

Comet assay was used to assess the leukocyte DNA damage under oxidative stress mediated by UV light. This was to determine the resistance of DNA against exogenous stress. The procedure was modified based on previous study (Szeto et al., 2015). Microscope slides was pre-coated with 1% standard agarose to enhance gel attachment. One percent low melting point agarose (Type XI, Sigma-Aldrich, St. Louis, Missouri,

US) was prepared in phosphate buffered saline (PBS) and being kept at 40°C water bath to maintain molten state. Sixteen μL of EDTA whole blood was mixed gently with 340 μL of low melting point agarose in the 1.5 mL microtube. Eighty-five μL of mixture was transferred immediately onto the slide followed by covering with 20 x 20 mm cover slip at room temperature for solidification. Each microscope slides can accommodate 2 gels and 2 slides were prepared for each sample. One slide was irradiated with UV while the other one with no treatment. The coverslip was removed from the gels and irradiated under the UV-B light (280-320 nm) for 2 minutes at 6 cm distance.

Lysis solution was used to remove cell membrane and organelles of leukocytes. It was prepared by adding 0.35 mL of Triton X-100 (Sigma-Aldrich, St. Louis, Missouri, US) and 3.5 mL of dimethyl sulfoxide (DMSO, Sigma-Aldrich St. Louis, Missouri, US) to 35 mL stock solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, pH = 10). The solution was well mixed in a staining jar before use. The slides with coverslip removed were placed into a staining jar and kept at 4°C for one hour wrapped with aluminium foil to avoid undesirable light contact. Electrophoresis solution (0.3 M NaOH, 1 mM EDTA) was prepared for alkaline treatment of the slides. The slides were placed into another jar containing 40 mL of electrophoresis solution at 4°C and left for 10 minutes with two changes for DNA unwinding and alkaline-liable site expression. The slides were placed gently on the platform of the electrophoresis tank. Electrophoresis was performed at 30V constant voltage and 0.3A current for 30 minutes in a cooled environment, ice and water were prepared for keeping the electrophoresis tank in a cold condition during the electrophoresis. After electrophoresis, slides were transferred to another staining jar containing distilled water for 5 minutes. The electrophoresis solution was removed with two changes of distilled water and slides were air dried at room temperature.

Giemsa stain was used to visualize the nucleus of leukocyte. The slides were stained in a working solution of Giemsa stain prepared from a stock solution. Five percent of Giemsa stain was prepared in a pH 7.2 buffer. The slides were gently placed into a mailer or staining jar for 30 minutes. The slides were removed from the stain after 30 minutes and rinsed with distilled water gently to avoid physical damage to the slides. They were air dried at room temperature and kept in the dark before scoring.

The DNA damage was quantified by visual scoring. One hundred cells were scored per gel under light microscope at 400X magnification. The nuclei were differentiated into 5 categories from 0 (no damage, the nucleus is intact) to 4 (the nucleus was severely damage with a clear comet tail clearly seen under the microscope). The score of each gel would range from 0-400 arbitrary units. The calculations were done as follow:
Total score = (0 x no. of cells scored 0) + (1 x no. of cells scored 1) + (2 x no. of cells scored 2) + (3 x no. of cells scored 3) + (4 x no. of cells scored 4).

RESULTS
The comet scores of alpha and beta thalassemia patients were higher than normal subjects in both baseline and UV treated samples (p<0.001, ANOVA). The mean (± standard deviation) comet score for normal baseline samples was 8.3 ± 6.1 (n=20) whereas 105.4 ± 15.7 and 69.8 ± 20.3 for alpha and beta thalassemia respectively (Figure 1a). For the UV-treated samples, the comet scores were 71.9 ± 19.6, 193.1 ± 21.8 and 211.8 ± 51.6 for normal, alpha and beta thalassemia samples respectively (Figure 1b).

DISCUSSION
Patients with Thalassemia usually present abnormal RBC indexes while white blood cell (WBC) count and morphology are not affected. A complete blood picture is the first line test to identify anemia patients with the potential of thalassemia. Certain types of hemoglobin are usually elevated in thalassemia patients, i.e. hemoglobin A2 in beta thalassemia and hemoglobin

H in alpha thalassemia. Hemoglobin pattern analysis and hemoglobin H inclusion bodies detection are the follow-up test for the diagnosis (Brancaleoni et al., 2016).
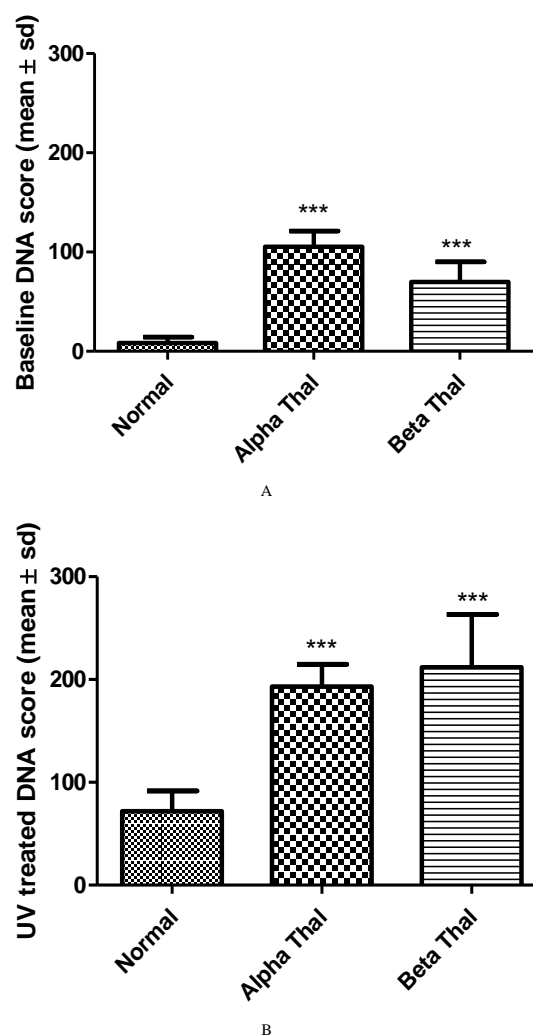

Figure 1) DNA damage in healthy subject, alpha and beta thalassemia patients. a) basal DNA damage b) UV-irradiated DNA damage. Error bars represent 1 SD. The means of thalassemia patients were significantly higher than normal subjects.

Treatment to thalassemia patients depends on the severity of anemia. Blood transfusion is the most common treatment for thalassemia major patients. To prevent iron overload due to repeated blood transfusion, iron chelation is needed to avoid damage to cells of liver or heart. Iron deposited at vital organs enhances Fenton and Haber-Weiss reactions which leading to ROS generation. ROS in turn damage cellular components and complications resulted (Burkitt, 2003). It has been shown that thalassemia patient with blood transfusion are under oxidative stress since iron and haem are oxidizing molecules (Voskou et al., 2015). Nevertheless, thalassemia trait patients who do not receive transfusion also demonstrated oxidative stress exemplified by increased DNA damage of leucocytes in the current study. It has been suggested non-transfusion dependent thalassemia patients also develop iron overload but at later stage than transfusion dependent thalassemia patients (Taher & Saliba, 2017). Complications are not the same as those developed in patients received transfusion. Overload of iron because of increased iron absorption in intestine of thalassemia patients is likely the cause (Taher et al., 2010).

CONCLUSION
To conclude, the current pilot study demonstrated both alpha and beta Thalassemia patients were under oxidative stress with DNA damage in white blood cell even received no blood

transfusion. In general, thalassemia patients are associated with higher oxidative stress in terms of DNA damage irrespective transfusion history.

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