Use of rapid biomarking technique to estimate oxidative stress in course dependent children with sickle cell disease in Saudi Arabia

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Oxidative stress, rapid assay, hydroxyurea treatment.

ABSTRACT

Sickle cell disease is characterized by a chain of polymerization reactions in the deoxygenated phase, manifesting in debilitating conditions like inflammation, painful vaso-occlusive crisis and disruption of the bodily defense systems. This vaso-occlusion accompanied by cell adhesion and ischemia-reperfusion injury is linked to a vicious pathway resulting in oxidative stress and an enhanced free radical generation. In children with sickle cell disease, the antioxidant defense system is continuously challenged, resulting in a compromised immunity, and a host of complications. An early, easy, and rapid technique to assess the oxidative stress would help in early therapeutic interventions. As prevalence of sickle cell disease is high in Saudi Arabia, the need for early interventions in children with the problem is of dire necessity. Currently hydroxyurea is the only drug of choice administered. We therefore utilized the free oxygen radical transference (FORT), and free oxygen radical defense (FORD) measurements in children with sickle cell disease on hydroxyurea therapy and compared them with patients not taking the drug. Though patients of both the groups exhibited oxidative stress, the values of free radical transference were considerably higher in the group which did not undertake any treatment as compared to those on hydroxyurea therapy. No appreciable changes were noticed in the FORD values representing the antioxidant capacity. Our results show that the technique is feasible for quick measurements of oxidative stress, and intervention with hydroxyurea therapy benefits in decreasing it. Its incorporation in screening practices would help understand the disease stage better.

Abbreviations- SCD-sickle cell disease, FORT- free oxygen radical transference, FORD- free oxygen radical defense

استخدام تقنية مؤشرات حيوية سريعة لتقدير الإجهاد التأكسدي في الأطفال المصابين بمرض فقر الدم المنجلي المعتمدين على العلاج المنتظم في المملكة العربية السعودية

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الكلمات الدالة

مرض فقر الدم المنجلي ، انتقال جذور الأكسجين الحرة ،دفاع جذور الأكسجين الحرة.

المُستلخص

مرض فقر الدم المنجلي يتميز بسلسلة من تفاعلات البلمرة في مرحلة نزع الاكسجين التي تظهر كظروف منهكة مثل الالتهاب، وأزمة انسداد الأوعية الدموية المؤلمة وتعطيل أنظمة الدفاع الجسدي. يرتبط أزمة انسداد الأوعية الدموية بالتصاق الخلايا والاصابة بنقص التروية الذي يؤدي الى الإجهاد التأكسدي وزيادة في توليد الجذور الحرة. ان نظام دفاع مضادات الاكسدة عند الأطفال المصابين بمرض فقر الدم المنجلي في تحدي مستمر الذي يؤدي الى جهاز مناعي ضعيف ومجموعة من المضاعفات. ان من شأن تقنية مبكرة وسهلة وسريعة لتقييم الإجهاد التأكسدي أن تساعد في التدخلات العلاجية المبكرة. بما أن انتشار مرض فقر الدم المنجلي مرتفع في المملكة العربية السعودية، فإن الحاجة إلى التدخل المبكر في الأطفال الذين يعانون من هذه المشكلة هي ضرورة ملحة. حاليا هيدروكسي يوريا هو الدواء الوحيد المختار. لذلك استخدمنا قياسات انتقال جذور الأكسجين الحرة ودفاع جذور الأكسجين الحرة عند الأطفال المصابين بمرض فقر الدم المنجلي الذين هم على علاج هيدروكسي يوريا ومقارنتها مع المرضى الذين لا يتناولون الدواء. مع أن المرضى في كلا المجموعتين أظهروا الإجهاد التأكسدي إلا أن قيم انتقال جذور الأكسجين الحرة كانت أعلى بشكل كبير في المجموعة التي لم تقم بأخذ العلاج بالمقارنة مع تلك المجموعة التي على علاج هيدروكسي يوريا. لم تُلاحظ أي تغيرات ملموسة في قيم دفاع جذور الأكسجين الحرة التي تمثل قدرة مضادات الأكسدة. تُظهر النتائج التي توصلنا إليها أن التقنية مجدية للقياسات السريعة للإجهاد التأكسدي و استخدام فوائد علاج الهيدروكسي يوريا في خفضه. وسيساعد إدماجها في ممارسات الفحص على فهم مرحلة المرض بشكل أفضل.

Introduction

Sickle cell disease is a typical genetic disease affecting people in many countries around the globe. A prevalence of 24 per 10,000 has been noted in Saudi children above 5 yrs. according to a study [Al-Qurashi, et al.2008].Oxidative stress has a pivotal role in sickle cell disease pathogenesis [Erica, et al.2012; Ama Moor, et al.2016; Jyoti Titus,2004]. The overall redox status is considerably altered in patients with sickle cell disease leading to dysfunctions in the DNA, lipid, protein, and antioxidant fractions of the cellular components, ultimately causing tissue damage [Chan, et al. 1999]. Chronic inflammation, recurrent ischemic reperfusion injury and excessive levels of cell-free hemoglobin serving as a catalyst for oxidative reactions, are causative of this oxidative stress [Erfan Nur, et al.2011].

Cell membrane peroxidation increases endothelial toxicity causing an upregulation of adhesion molecules such as VCAM-1 and ICAM-1 which contribute towards vaso-occlusion [Klings and Farber,2001].The resultant phenotypic expression of inflammation, vaso-occlusion, and microvascular injury to the organs arrives through a complex chain of cellular reactions [Teixeira, et al.2011]. Sickle cells are known to spontaneously produce twice the amount of reactive oxygen species as compared to normal red blood cells [Hebbel, et al.1982]. Oxidative as well as nitrosative stress was found to be greater in sickle cell anemia patients when compared with controls [Renoux, et al. 2018]. Moreover an overload of pro-oxidant reactive products may be responsible for the concomitant decrease in the total antioxidant defense capacity [Okorie, et al. 2018; Chiu, et al. 1982].

Hydroxyurea (HU) is the most common treatment given to sickle cell patients. HU increases the synthesis of fetal hemoglobin (Hb F) thereby reducing the frequency of vaso-occlusive crisis, pain and the resultant hospitalizations. Researches verify that increases in HbF expression not only causes a reduction in the Hb polymerization, oxidative stress in SCD, and the sickling process, but also contributes towards the body's antioxidant defenses resulting in reduced cell damage [Torres, et al. 2012]. The release of nitric oxide from HU exhibits beneficial effects locally on the endothelium by reducing the vaso-occlusive process as well as vascular dysfunction [Heeney and Ware, et al. 2010].An early and quick diagnosis of oxidative stress would help in alleviating the complications experienced by these patients. The FORT and FORD tests (CR3000, Callegari, Catellani, Italy) measuring the free radical generation and antioxidant capacity are efficient and rapid with ease of performance.

FORT measures hyproperoxides which represent a fairly stable free radical index comprising of products of lipid, peptide and amino-acid oxidation. Moreover FORT measurements have been shown to be reliable and reproducible, and quicker means of assessing oxidative stress [Garelnabi, et al.2008]. Higher FORT values represent higher generation of ROS denoting higher levels of oxidative stress. Principally FORT is a colorimetric test that measures the catalytic breakdown of ROOH hydro peroxides in any biological sample to the RO and ROO radical derivatives by Fe3+/Fe2+ metal ions. The FORD test (CR3000, Callegari, Catellani, Italy) measures the presence of antioxidants present in any biological sample using a colorimetric assay and is expressed as Trolox equivalents. The significant antioxidant contributors measured by FORD are those of the SH-group namely GSH, apart from albumin, bilirubin, certain plasma proteins and vitamin C [Palmieri & Sblendorio, 2007]. The assay is quick and can be performed within a span of six to seven minutes [Kamhieh Milz and Salama, 2014].

We therefore measured the oxidative stress in pediatric patients with SCD on HU therapy using FORT and FORD measurements and compared them with those in patients not undergoing HU treatment.

Materials and Methods

The present study was conducted at the King Fahd Medical Research Center (KFMRC), King Abdulaziz University, Kingdom of Saudi Arabia. We studied eighty six patients with sickle cell disease from different hospitals who were enrolled

at the Nutritional Center of KFMRC. No phenotype bias was considered.

Ethical clearance--This study was conducted in accordance with the ethical norms and safety guidelines after approval by the King Abdulaziz University institutional ethical committee. All parents of sickle cell disease children submitted a signed consent form before commencement of the study.

Study population--A total of 252 sickle cell disease pediatric patients irrespective of their phenotypes were enrolled in the program study from Sep 2016-June 2017 at the King Fahd Medical Research Center, KAU, Jeddah. Among the patients enrolled, 86 consenting patients, have been included in the study. They consisted of 42males and 44 females. They were divided into two groups: one receiving HU treatment (n=37), and a second untreated group (n=49) which was not subjected to HU treatment.

Criteria for sample selection:

- Prevalence in the given population
- Practicality (Due to non- compliance, blood from normal healthy pediatric patients was not taken. It is a matter of great concern for the parents of healthy normal children who are unwilling to donate their child's blood for research purposes).
- Systematic sampling of the target study population—children with sickle cell disease aged 5-16 years from KAU Hospital follow up clinic/ other hospital in Jeddah or Makkah / day care / during diet counselling at KFMRC were enrolled for the study.

Blood sampling

Patients' blood collection was performed during the steady state and they did not receive any transfusion at least three months before commencement of this study. Venous blood samples (0.5 ml) were initially collected in heparinized tubes.

Assessment of free radical species (FORT assay)

Free radicals generated were measured using the FORT method (CR3000, Callegari, Catellani, Italy). These derivative radicals transform into a radical cation at 37°C on addition of an amine chromogen CrNH2. The resultant colored solution is read colorimetrically at 505nm. Briefly, 20µL heparinized blood samples were immediately mixed with the reagent, centrifuged and analyzed colorimetrically Results are expressed in FORT units which are representative of H2 O2 concentrations present, the linearity of which ranges from 1.22 to 4.56 mmol /L of H2 O2. One FORT unit represents 0.26 mmol/L of H2 O2. Classification represents values above 400 FORT units (above 3.04 mmol/L H2 O2) as high oxidative stress, intermediate between 230-400 FORT units (1.75-3.04 mmol/L H2 O2) , and values below 230 FORT units (1.75 mmol/L H2 O2) as reduced stress [Kamhieh Milz and Salama, 2014].

Assessment of free oxygen radical defense capacity (FORD assay)

Basically it is the sample's capacity to reduce a cation radical. The chromogen 4-amino-N, N-diethyl aniline sulfate forms a stable colored product in the presence of FeCl3 as an oxidant in an acidic atmosphere which is read at 505nm. The antioxidant present in the biological sample quenches the color and decolorizes it due to the reduction of the radical cation. The absorbance observed is proportional to the concentration of antioxidant in the given sample and is expressed as Trolox equivalents with linearity ranging from 0.25 to 3.0 mmolL-1 Trolox. Normal values of FORD range between 1.07-1.53 mmol/L of Trolox. [Gaman, et al.2014] Classification represents values above 1.53 mmol/L Trolox as good antioxidant capacity, normal between 1.07 and 1.53 mmol/L Trolox, and values below 1.07 as reduced antioxidant capacity.

Statistical analysis- Results have been expressed as means ±the standard deviations. Analysis was performed using the t-test, and p values of < 0.05 considered significant.

Results

The demographics of patients enrolled in the study are represented in table1.The average hemoglobin value for the children was 7.577 ± 0.86 SD. The freshly collected heparinized blood samples were investigated for FORT and FORD assays. When the HU treated and untreated groups were compared for their oxidant and pro oxidant parameters, the oxygen transference capacity (FORT) value showed a high significance with p value < 0.001. There was apparently no significant difference in the FORD values between the groups when compared over this age group (5-16years) with a probability value of 0.976 as seen in table 2. The FORD values denoting the antioxidant defense were considerably less for all the patients assessed. All values observed for FORT were around 4, which is well beyond the normal range (intermediate range for FORT being 1.75-3.04 mmol/L H2 O2). Values of FORD in all patients were around 1 which is on the lower side of the normal range, (normal range for FORD being 1.07-1.53 mmol/L Trolox).

Table1-Clinical characteristics of sickle cell disease patients

	Male-42		Female-44	
Group /Age Range	(5-10)	(11-16)	(5-10)	(11-16)
HU treated Group	N=13	N=5	N=13	N=6
HU untreated Group	N=16	N=8	N=10	N=15

Table2- Oxidant (FORT) and antioxidant (FORD) parameters in sickle cell disease affected children in hydroxyurea treated and untreated group

Parameters	N	Hydroxyurea treatment group (37)	No treatment group (49)	Probability 'p'
FORT	86	3.60 ± 0.69 SD	4.06 ± 0.48 SD	< 0.001*
FORD	86	0.50 ± 1.17 SD	0.63 ± 1.18 SD	NS or 0.976

Slight differences in gender were observed regarding the FORT values (tables3 &4) with 3.72 ± 0.61SD for the hydroxyurea treated males and 4.11 ± 0.49 SD for the untreated males with a p value of < 0.05.Females exhibited FORT values 3.50 ± 0.75 SD for the HU treated and 4.02 ± 0.47 SD for the untreated group with a significant p value of < 0.01. Females exhibited more oxidative stress as compared to males in terms of the lower p value. The FORD values in males between the groups did not show any significance with a p value of 0.601 (table 3). FORD values in females too did not show any significance with a p value of 0.976(table 4). When compared for gender differences, the p values for FORD in males was distinctly lower as compared to females. The method is quick, reliable, and easily reproducible, with great usefulness in early interventions and field studies.

Table 3: Oxidant (FORT) and antioxidant (FORD) parameters in male sickle cell disease affected children, in hydroxyurea treated and untreated group

Parameters	N	Hydroxyurea treatment group (37)	No treatment group (49)	Probability 'p'
FORT	42	3.72 ± 0.61 SD	4.11 ± 0.49 SD	< 0.05*
FORD	42	0.52 ± 1.22 SD	0.52 ± 1.13 SD	NS or 0.601

Table 4: Oxidant (FORT) and antioxidant (FORD) parameters in female sickle cell disease children, in hydroxyurea treated and untreated group

Parameters	N	Hydroxyurea treatment group (37)	No treatment group (49)	Probability 'p'
FORT	44	3.50 ± 0.75 SD	4.02 ± 0.47 SD	< 0.01*
FORD	44	0.48 ± 1.13 SD	0.73 ± 1.22 SD	NS or 0.976

Discussion

SCD patients are known to require greater oxygen consumption. Availability of low oxygen leads to the increased formation of the deoxygenated HbS polymer. This and an enhanced free radical generation could possibly be the two mechanisms causing damage to the RBC [Ramasamy Jagadeeswaran, et al.2017]. Nitric oxide (NO) is known to be a potent vasodilator, and an antioxidant. Endothelial nitric oxide synthase is rendered inactive in sickle cell disease, thereby reducing the blood levels of nitric oxide (NO). This inhibition of NO is causative of endothelial dysfunction in at least 50% of the patients [Rusanova et al, 2010].The catalase mediated NO production by HU, renders it to act as an antioxidant thereby reducing oxidative stress [Jimming Huang, et al. 2003]. Increase in HbF levels by HU administration appears to reduce lipid peroxidation and seems to be influenced by the NO mechanistic pathway. As of now HU remains the drug of choice in SCD, which is contributory to alleviating the oxidative stress in these patients [Wood, et al .2008]. In accordance with other studies, we found that sickle cell disease children, demonstrate significantly higher intensities of oxidative stress ,and treatment with hydroxyurea decreased the oxidative stress significantly thereby exhibiting lower FORT values. In our study, we observed decreased oxidative stress in patients undertaking HU treatment as compared to those not taking any treatment. This is seen by the decreased FORT values and is suggestive of the free radical quenching ability of HU [Torres, et al .2012]. In our study, though FORT values were increased in all patients, females showed slightly higher figures with a lower p value. This could partly be due to the cultural sensitivity and societal pressures experienced by females to the understanding of disease leading to relatively higher depression. Also the expression of pain is more vocalized by males as compared to females who seem not only more tolerant but also upset due to gender discrimination. In our study, FORD values of both genders were low in both the groups irrespective of the treatment given. Nevertheless females exhibited a higher antioxidant defense (FORD) as compared to males which is in accordance with previous studies. In females, the presence of estrogen imparting antioxidant protection for expression of NO synthase activity has been suggested [Vicky Jocelyne Ama Moor, et al.2016] .HU has also been shown to increase levels of the potent antioxidant glutathione, which contributes towards reduction of oxidative stress [Teixeira, Neto, et al. 2011]. Nevertheless a few studies infer that HU may not be very effective, in increasing antioxidant defense as noticed by some researches [Hundekar, et al.2010] Some researchers also suggest only a partially effective role of HU in containing oxidative stress [Gizi ,et al. 2011]. Therefore interventions aiming at increasing the antioxidant potential could possibly benefit such patients [Fasola, et al .2007, Okorie, et al. 2018].

The assays of FORT and FORD are quite feasible in terms of performance, reliability and the short time period required .This allows for a quick interpretation of stress to help take prospective measures in inhibiting oxidative damage and allow recommendations. It may also help in the study of preventive healthcare to quickly understand distal and proximate causes. Most importantly in the determination of the pre analytic step before the administration of optimum HU dosage to avoid adverse effects. Also expanding the findings of this study would help in clinical dispensing of appropriate amounts of optimum amount of antioxidants at the required time of the disease.

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