

Oxidative Stress In Glucose 6-Phosphate Dehydrogenase Deficiency In Children

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Abstract

The present study was conducted to evaluate the changes occurring in children affected with glucose 6-phosphate dehydrogenase deficiency (G6PD), this study included 60 children, patients and healthy, 40, children affected with G6PD deficiency and 20 children were used as control group. Their ages ranged between 1 year to 13 years and divided into four groups, first group 1-3 years, second group > 3-5 years, third group > 5-10 years, and fourth group > 10-13 years.

The results of packed cell volume (PCV), RBCs count and hemoglobin concentration (Hb) showed significant decrease ($P < 0.05$) in all ages groups when comparison with control. Levels of total serum bilirubin, and direct serum bilirubin, reticulocyte recorded significant increase ($P < 0.01$) in all age groups. The present study also showed significant decrease ($P < 0.01$) in the levels of reduced glutathione and catalase activity. The changes these changes which are above illustrated may be attributed to high levels of RBCs destruction due to the oxidative stress.

الخلاصة

صممت هذه الدراسة لمعرفة بعض التغيرات الحاصلة في بعض المعايير الدموية والكيموحيوية للأطفال المصابين بنقص انزيم نازع الهيدروجين ٦-فوسفات. اشتملت الدراسة ٦٠ طفلاً، (٤٠) طفل كانوا مصابين و (٢٠) طفل اصحاء استخدموا كمجموعة سيطرة تراوحت اعمار جميع الاطفال بين سنة الى ١٣ سنة، وقسموا الى اربعة مجاميع عمرية وكالاتي: ($1 < 3$ سنوات، $3 < 5$ سنوات، $5 < 10$ سنوات، $10 < 13$ سنة). بينت النتائج حصول انخفاض معنوي ($P < 0.05$) في قيم حجم الخلايا المضغوطة وعدد كريات الدم الحمر وتركيز الهيموغلوبين في جميع الاطفال المصابين. في حين بينت قيم كل من الخلايا الشبكية والبيرويين الكلي والمباشر ارتفاع معنوي بمستوى $P < 0.05$ و $P < 0.01$ على التوالي.

سجلت قيم كل من الكلوتاثيون المختزل وانزيم الكاتليز انخفاض معنوي بمستوى ($P < 0.01$) في الاطفال الاصحاء. ان النتائج المستحصلة من هذه الدراسة يمكن اعزاؤها الى التكسر العالي الحاصل لكريات الدم الحمر بسبب الاجهاد التأكسدي.

Introduction

In the early 1950 it was known that certain antimalarial drugs caused hemolysis in certain susceptible individual. The use of primaquine as an antimalarial treatment for soldiers in the Korean war enabled investigators to study the effects of the drugs in controlled situations (Pittiglio and Sacher, 1987).

Glucose-6-phosphate dehydrogenase (G6PD) is the most common human enzyme defect, being present in more than 400 million people world wide. African, Middle Eastern and South Asian people are affected the most along with those who are mixed with any of the above (Campbellini and Fiorelli, 2008).

The side effect of this disease is that it confers protection against malaria in particular the form of malaria caused by plasmodium faciparum, the most deadly form of malaria. One theory to explain this is that cells infected with plasmodium parasite are cleared more rapidly by the spleen. This phenomenon might give G6PD deficiency carriers an evolutionary advantage by increasing their fitness in malarial endemic environments (Metha *et al.*, 2000). G6PD deficiency is transmitted by a mutant gene located on the long arm of the chromosome, on band xq28. G6PD is an enzyme in the pentose phosphate pathway. This enzyme converts glucose-6 phosphate into phosphogluconoacton and is the rate-limiting enzyme of this metabolic pathway that supplies reducing energy to cells by maintaining the level of the co-enzyme nicotinamide adenine dinucleotide phosphate (NADPH).

The NADPH in turn maintains the supply of reduced glutathione in the cells that is used to inhibit free radicals that cause oxidative damage (Johanson *et al.*, 2000).

The G6PD/NADPH pathway is the only source of reduced glutathione in red blood cells (erythrocytes). The role of red cells as oxygen carriers puts them at substantial risk of damage from oxidizing free radicals except for the protective effect of G6PD/NADPH/ glutathione (Corran *et al.*, 2008).

Materials and Methods

-The subjects for the study

The present study was applied over 10 months period from March to October 2011. The number of subjects was 60 children, patients and healthy children; 40 children affected with glucose 6-phosphate dehydrogenase deficiency and 20 children were used as control group. Their ages ranged between 1 year to 13 years and divided into four groups; first group $1 \leq 3$ years, second group $> 3-5$ years, third group $> 5-10$ years; and fourth group $> 10-13$ years (Gill and O'brien, 1988).

-Methods

The blood was collected in pediatric hospital at time between 9-11 a.m by using venipuncture needles with gauge 23.

A-Blood parameters

1-Red cells count (RBCs count)

The blood samples were diluted with formal citrate solution (1% formalin with 38 g/L of trisodium citrate. A twenty microliter of blood was added into 4 mililiters of diluting fluid, and after mixing by a mechanical mixture; the Neubaur hemocytometer chamber was loaded and examined under the microscope. (Dacie and Lewis, 1995).

2-Determination of packed cell volume (PCV)

method of microhematocrit was used. A heparinized capillary tubes were used. The tubes were filled to approximately three quarters of its length and then the unmarked end is closed with clay and put in microhematocrit centrifuge (Dacie and Lewis, 1995).

3- Estimation of hemoglobin (Hb)

Cyano methemoglobin method was used to determinate Hb. The principle of this method was based on Drabkin's cyanide ferricyanide solution (Markarek, 1974).

4-Total white blood cells count (WBCs count):

The sample of blood (20 micro liters) was added into 0.4 ml of Turk's solution (1ml of glacial acetic acid, 2ml of gentian violet, and 100ml of distilled water). The mixture was mixed in a mechanical mixture. The nebulizer chamber was used to count the total WBCs (Dacie and Lewis, 1995).

5- Determination of reticulocytes

The sample of blood was added into new methylene blue staining solution (0.5 gm new methylene blue, 1.6 gram of potassium oxalate, and 100 ml of distilled water). Equal amounts of blood and staining solution were added in test tube and then mixed. A thin smear was prepared from blood mixture smear was examined under oil in consecutive immersion field (Pittiglio and Sacher, 1987).

6- Estimation of reduced glutathione

Reduced glutathione (GSH) was estimated by using the method of Ellman's (Ellman, 1959). 1ml of serum was mixed with 0.5 ml of Ellman's reagent (19.8 mg of 5,5'-dithiobis nitro-benzoic acid [DTNB] in 100 ml of 0.1% solution of Sodium nitrate) and 3.0 ml of phosphate buffer (0.2M, pH 8.0). The absorbance of mixture was read at 412 nm.

7- Assay of catalase

Catalase (CAT) was determined colorimetrically at 620nm and expressed as μmol of H_2O_2 consumed Min/Mg/Hb as described by (Shina, 1972). The reaction of mixture (1.5) ml contained 10ml of hemolysate, and 0.4ml of 2mole H_2O_2 . The reaction was stopped by addition of 2.0ml of dichromate-acetic acid reagent (5% potassium dichromate and glacial acetic acid were mixed in 1:3 ratio).

8- Detection of G6PD activity

Fluorescent spot test is the most specific method. The principle of this method is in the use of small amounts of blood (10 μl or dry blood on a special paper). G6PD enzyme in the appropriate environment cause the change of NADP to NADPH. This NADPH produce fluorescent under the UV light by wave length of 365 nm. (Missiou *et al.*, 1991).

9- Measurement of total serum bilirubin

Working solution was prepared by mixing of 20 R1 volume (Sulfanilic acid, hydrochloric acid, and dimethyl sulfoxide) with 1R3 reagent (Sodium nitrate). The mixture incubated exactly for 5 minutes at 37°C and read at 555nm (according to procedure recommended by biomerixu company).

10- Measurement of direct serum bilirubin

Working solution was prepared by mixing of 20 volume R2 (Sulfanilic acid and hydrochloric acid) with 1 volume R3 (Sodium nitrate). The mixture was mixed well and incubated exactly for 5 minutes at 37°C and then read at 555nm (according to procedure recommended by biomerixu company).

11- Determination of in direct serum bilirubin

The result of indirect serum bilirubin were obtained by the subtraction of values of direct bilirubin from the values of total bilirubin (Walter and Gerard, 1970).

12- Statistical analysis

All data were expressed as means \pm SE. SPSS program was used in this study. The statistical significance was evaluated by student's t-test (Daniel, 1999).

Results

The results which are obtained from all groups of patients and illustrated in table (1) showed a significant decrease ($P<0.05$) in the levels of RBCs, PCV, and Hb, and reticulocytes. At the same time, the levels of total WBCs showed insignificant ($P>0.05$) decrease in all patient group when compared with healthy children.

Values which are illustrated in table (2) showed significant increase ($P<0.01$) in the levels of total serum bilirubin and indirect serum bilirubin and showed significant decrease ($P<0.01$) in the levels of reduced glutathione and catalase in all patient groups in a comparison with control healthy groups.

Table (1):- Means of Packed cell volume (PCV), red blood cells count (RBCs) hemoglobin concentration (Hb), Total white blood cells count (WBCs), and reticulocytes in children affected with glucose 6-phosphate dehydrogenase defficiency (G6PD).

Age (Years)	RBCs cellx10 ⁶ /mm ³		Hb concentration g/dL		PCV%		Total WBCs cellx10 ³ /mm ³		Reticulocytes %	
	Patient group	Control groups	Patient group	Control groups	Patient group	Control groups	Patient group	Control groups	Patient group	Control groups
1-≤3	*4.513±0.31	5.31±0.104	*11.52±0.43	13.45±0.35	*35.21±0.45	40.21±0.712	7.521±0.35	6.5±0.221	*1.51±0.35	1±0.2
>3-5	*3.91±0.55	4.71±0.2	*10.9±0.41	12.56±0.45	*33.21±0.21	41.5±0.21	6.23±0.34	5.61±0.62	*1.5±0.72	1.1±0.1
>5-10	*4.31±0.51	4.9±0.91	*11.6±0.67	13.1±0.51	*30.11±0.51	40.11±0.52	5.91±0.21	6.31±0.211	*2.11±0.41	1±0
>10-14	*3.95±0.41	4.6±0.21	*10.52±0.33	13.0±0.23	*32.5±0.4	42.51±0.34	4.52±0.45	5.5±0.721	*1.8±0.5	1±0.42

-values are given as means ± SE

-values with asterisk (*) are significantly at p<0.05

Table (2):- Means of total serum bilirubin, direct serum bilirubin, indirect serum bilirubin, reduced glutathione, and catalase in children affected with glucose 6-phosphate dehydrogenase defficiency.

Age (Years)	Total serum bilirubin mg/dL		Direct serum bilirubin mg/dL		Indirect serum bilirubin mg/dL		Reduced glutathione mg/dL		Catalase U/mg/Hb	
	Patient groups	Control groups	Patient group	Control groups	Patient group	Control groups	Patient group	Control groups	Patient group	Control groups
1-≤3	**8.92±0.59	2.91±0.02	0.32±0.01	0.25±0.12	**12.5±0.5	1.3±0.05	**20.1±0.98	30.5±0.61	**30±0.621	50.6±0.421
>3-5	**10.5±0.4	1.21±0.05	0.42±0.3	0.39±0.05	**11.34±0.5	2.1±0.16	**23.78±0.81	35±0.77	**35.01±0.67	45.5±0.521
>5-10	**11.16±0.62	2.51±0.23	0.34±0.32	0.29±0.02	**13.41±0.91	1.5±0.07	**45.01±0.23	40.5±0.789	**31±0.56	55.9±0.51
>10-14	**11.0±0.56	3.51±0.5	0.45±0.5	0.46±0.5	**10±0.512	3.51±0.01	**21±0.81	39.5±0.72	**25.5±0.116	60.6±0.66

-values are given as means ± SE

-values with two asterisk (**) are significantly at p<0.01

Discussion

The results which are illustrated in table (1) showed a significant decrease ($P < 0.05$) in the levels of PCV, Hb, and RBCs count affected in children with G6PD deficiency. In healthy person, a red blood cells survives 90 to 120 days in the circulation, so about 1% of human red blood cells break down each day. The spleen (part of the reticuloendothelial system) is the main organ that removes old and damaged RBCs from circulation. In healthy individuals, the break down and removal of RBCs from the circulation is matched by the production of new RBCs in the bone marrow (Bilto, 1998). The G6PD/NADPH pathway is the only source of reduced glutathione in red blood cells. The role of red cells as oxygen carriers puts them at substantial risk of damage from oxidizing free radicals except for the protective effect of G6PD/NADPH/glutathione (Beutter, 2008).

People with G6PD deficiency are therefore at risk of hemolytic anemia instates of oxidative stress. Oxidative stress can result from infection and from chemical exposure to medication and certain food. Broad beans, e.g., favabeans, contain high levels of vicine, convicine and isouramil, all of which are oxidants. (Metha *et al.*, 2000). As a results of destruction of RBCs, body homeostatic mechanism acts to adjust the level of oxygen in the blood, cells which are responsible on secretion of erythropoietin in kidneys, tend to release a adequate amount of this hormone to stimulate bone marrow, and in turn elevate the number of RBCs. This compensatory mechanism is in sufficient to adjust the destructed number of RBCs in the circulation. So that, the levels of immature RBCs (reticulocytes) are increased in the circulation (Rees *et al.*, 1999).

The resulting oxidation of hemoglobin lead to progressive precipitation of irreversibly denatured hemoglobin (Heinz bodies). The cells lack normal deformability when sulfhydryl groups are oxidized and consequently encounter difficulties navigating in the microcirculation. Premature destructions of the cells results when they undergo intravascular lyses or when they are sequestered and destroyed in the liver and spleen (Nkhoma *et al.*, 2009).

The results which are presented in table (2) showed a significant increase in the levels of total serum bilirubin and direct serum bilirubin in all age groups affected with G6PD deficiency. In conditions where the rate of RBCs break down is increased, the body initially compensates by producing more RBCs, however, break down of RBCs can exceed the rate that body can make RBCs, and so anemia can develop. Billirubin, a break down product of hemoglobin, can accumulate in the blood, causing jaundice, and be excreted in the urine causing the urine to become a dark brown color (Schwoebel and Schuschke, 1997).

Results which are obtained from this study pointed out a significant decrease ($P < 0.01$) in the levels of catalase and reduced glutathione.

Reactive oxygen species (ROS) have been reported to induce oxidative damage to membrane of lipid, proteins, and DNA, and might in cell death by necrosis or apoptosis (Gamaley and Klubin, 1999). Both glutathione peroxidases and catalase are major defences against harmful effects of (ROS) in cells, both have a high capacity to degrade exogenous hydrogen peroxide (H_2O_2). (Bjorkman and Ekholm, 1995).

Our results agreed with studies of (Kilic *et al.*, 2004) and Turgut *et al.*, (2004) they found that during cellular oxidative stress. NADPH is critical for maintaining glutathione in its reduced form (GSH), which is essential for detoxification of reactive oxygen species and lipid peroxidation. In children with G6PD deficiency and severe hyperbilirubinemia, NADPH was reduced that lead to decrease in GSH.

The decrease concentration of glutathione supports the hypothesis that jaundice is an important causative factor in the pathogenesis of lipid peroxidation. These data reveal that antioxidant defense mechanisms might be impaired in jaundice patients (Mason, 1996). When all remaining reduced glutathione is consumed, enzymes and other proteins (including hemoglobin) are subsequently damaged by oxidants, leading to protein deposition in the cells especially hemoglobin (Heinz bodies). Damaged red cells are phagocytosed and sequestered by spleen. The hemoglobin is metabolized to bilirubin (causing jaundice at high concentration). (Macdonald, 1995).

Other studies showed there is a negative correlation between total serum bilirubin and catalase in G6PD deficient children. This may be due to reduce NADPH which is known to maintain catalase activity and because G6PD deficiency lead to increase accumulation of H₂O₂ in RBCs and hemolysis occurrence. A production of the hemolytic process is bilirubin that lead to increase bilirubin and decrease catalase. This was the explanation of the negative correlation between total serum bilirubin and catalase (Carroll *et al.*, 2006).

Results which are obtained from this study may be attributed to high level of destruction of RBCs because of deficient of G6PD.

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