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Evaluation of DNA damage and Antioxidant Status with G6PD-deficient in Iraqi Patients

Asmaa Mohammed Saud*
Department of Biotechnology, College of Science, University of Baghdad, Baghdad, Iraq

Abstract

Thirty blood samples of clinically glucose-6-phosphate dehydrogenase (G6PD)-deficient patients were collected from pediatrics hospital and Al- Kadhumia teaching hospital in Baghdad, Iraq. .Besides that, blood samples were taken from 30 apparently healthy individuals (without Hemoglobinopathy disorders) as a control group. The apoptotic response shown by G6PD-deficient patients was evaluated using neutral method of Comet assay. Comet assay result was demonstrated as comet index which represent a mean of the scored that calculate from the ratio of cell diameters (L/W) of total cells in each sample. These results revealed that lymphocytes exhibited an increase apoptosis percentage (44.5%) compare with percentage of control (7%). This revealed that there was DNA damage in G6PD-deficient patients. On the other hand, repeated blood transfusion in G6PD-deficient patients may lead to oxidative tissue injury by secondary iron overload. The results indicated that there was a significant decrease in catalase activity levels ($P<0.05$), whereas the lipid peroxidation end product (MDA) levels and both G-Px, SOD activity levels were significantly increased in all G6PD-deficient patients ($P < 0.05$) as compared with the control group. MDA values showed a significant positive correlation with G-Px levels ($r = + 0.756$), while it was observed negative correlated with catalase levels ($r = - 0.352$). These data indicates an increase in free radical generation and thus antioxidant defense mechanisms is impaired in peroxidation associated with a significant elevation in MDA levels in the erythrocytes of the G6PD deficiency than that found in the control group which demonstrate the presence of an increased oxidative stress due to reduction in NADPH which is generated in erythrocytes. This suggests that oxidative stress and reduced antioxidant defense mechanism play an important role in pathogenesis and DNA damage of G6PD-deficient patients.

Keywords, G6PD deficiency, DNA Damage, Comet assay, Oxidative stress, Antioxidant, Oxidative damage

تقييم تلف DNA وحالة مضادات الاكسدة لمرضى أنيميا الفول العراقيين

اسماء محمد سعود

قسم التقنيات الاحيائية، كلية العلوم، جامعة بغداد، بغداد، العراق

الخلاصة

جمعت عينات الدم من 30 مصاب سريريا بمرض أنيميا الفول من مركزين للمرض في بغداد– العراق : (مستشفى الطفل والكاظمية التعليمي) ، ومن 30 شخص من الأصحاء ظاهريا وغير مصابين بإمراض الدم الوراثية (مجموعة سيطرة). وقيمت الاستجابة للموت الخلوي في المرضى باستخدام الطريقة المتعادلة لفحص المذنب (comet assay) . وأوضحت النتائج مدى التلف الحاصل لدنا الخلايا اللمفاوية لمرضى الثلاسيميا بفحص المذنب (comet assay) والذي سمي comet index حيث تم حسابه من نسبة الطول إلى عرض النوية. وبينت النتائج إن نسبة التلف الحاصل بألدنا هي 44.5 % مقارنة بالنسبة المئوية لمعاملة السيطرة

*Email: asmagenetic@yahoo.com

(7%) حيث بين الاختبار حدوث تلف في الـ DNA لمرضى أنيميا الفول ..من ناحية أخرى، فأن نقل الدم المتكرر في مرضى أنيميا الفول قد يؤدي إلى تلف الانسجة التاكسدي نتيجة تراكم الحديد الثانوي . وأظهرت النتائج أنخفاض معنوي ($P<0.05$) في مستوى فعالية انزيم الكاتليز بينما كان هناك ارتفاعا ملحوظا في مستوى أكسدة الدهون ومضادات الأكسدة الإنزيمية عند مقارنتها بالإفراد الأصحاء. وقد لوحظ وجود علاقة موجبة ذو دلالة إحصائية ($r=0.756$) بين مستوى أكسدة الدهون و مستوى انزيم glutathion peroxydase (GPx) بينما لوحظ علاقة سالبة ذو دلالة إحصائية ($r= -0.352$) مع مستوى انزيم الكاتليز Catalase. وتشير هذه البيانات إلى ارتفاع في توليد الجذور الحرة وانخفاض آليات الدفاع المضادة للأكسدة المرافقه مع ارتفاع معنوي ($P<0.05$) في مستويات أكسدة الدهون في كريات الدم الحمر لمرضى أنيميا الفول من تلك الموجودة في مجاميع السيطرة ،التي تثبت وجود ارتفاع في الإجهاد التأكسدي نتيجة انخفاض في NADPH المتولدة في خلايا الدم الحمر. هذا ربما يشير إلى أن الاجهاد التاكسدي وانخفاض آلية الدفاع المضادة للأكسدة قد تلعب دورا هاما في أمراضية وتلف DNA لمرضى أنيميا الفول .

Introduction

Glucose-6-phosphate dehydrogenase (G6PD) is a housekeeping enzyme that catalyzes the first and rate-limiting step in the pentose phosphate pathway. Its key role in metabolism is to provide reducing power in the cytoplasm in the form of NADPH. This role is particularly important in red blood cells where NADPH serves as an electron donor for detoxification of hydrogen peroxide via reduced glutathione, and its production is crucial for the protection of cell from oxidative stress [1].

G6PD deficiency is the most common congenital enzyme deficiency in man, present in over 400 million people worldwide [2, 3]. The human G6PD gene is located on Xq28, therefore full manifestation of the defective gene is observed in the male hemizygote and the female homozygote. In the female heterozygotes, a mixed population of normal and enzyme-deficient cells can be found [4, 5].Glucose-6-phosphate dehydrogenase (G6PD) deficiency is a very common, inborn error of metabolism throughout the world. G6PD converts glucose-6-phosphate to 6-phosphogluconate and reduces nicotinamide adenine dinucleotide phosphate (NADP) to reduced NADP (NADPH), which is required by glutathione reductase. Glutathione protects hemoglobin against otherwise detrimental oxidative damage and inhibits apoptosis [6, 7]. Because of decreased levels of reduced glutathione, G6PDdeficient patients may experience severe hemolytic crises after infections or after consumption of certain drugs or foods. In contrast to hemoglobin damage in erythrocytes, damage to nucleic acids in nucleated cells from G6PD-deficient subjects is still poorly understood. Depleted G6PD enzyme activities in leukocytes of affected patients have long been recognized. Surprisingly, data on glutathione in nucleated blood cells of G6PD-deficient patients are still not available. As a consequence of a reduced capacity for detoxification by glutathione, DNA of G6PD-deficient patients may, thus, be more vulnerable to DNA damage [7, 8].

Erythrocytes and all other cells of (G6PD) deficiency, to cope with the formation of reactive oxygen species (ROS), have an antioxidant defense system that includes enzymatic and nonenzymatic antioxidants [9].In all living organisms, the levels of ROS are controlled by a complex network of antioxidant defenses, which reduce (but do not completely prevent) oxidative damage to biomolecules. In human disease, this ‘‘oxidant–antioxidant’’ balance is tilted in favor of the ROS so that oxidative damage levels increase [10, 11].

This study aims to characterize the extent and nature of DNA damage by comet assay, and the attempt to examine the status of the protective antioxidants under condition of stress due to G6PD deficiency in Baghdad Province, Iraq.

Materials and Method:

Subjects and sample collection: Thirty patients with G6PD-deficiency from pediatrics hospital and Al- Kadhumia teaching hospital in Baghdad, during March to May, 2014 were enrolled, represent different ages and gender. In addition 30 samples of healthy people (without heamoglobinopathy) were further enrolled in the study. Blood samples (2-3 ml) were taken from a peripheral vein in EDTA anticoagulant collecting tube from all patients and control groups.

Methods:

1-Neutral Comet Assay (Single Cell Gel Electrophoresis) (SCGE): Neutral Comet assay was used to investigate the possible DNA damage in peripheral blood lymphocytes from patients with G6PD-deficient in comparison to healthy controls [12].

Isolation: Lymphocytes from a 2 ml anticoagulant whole blood were isolated by lymphoprep density gradient centrifugation and washed in Phosphate Buffer Saline (PBS). Cell concentrations were adjusted to approximately 2×10^5 cell/ml in the buffer. Aliquots of 5-10 µl of the cells were suspended in 75 µl of low melting point agarose (LMA) for embedding on slides. Cells were checked for viability by trypan blue exclusion.

Preparation of slides for the SCGE/Comet assay: The microscopic slides were covered with 1% Normal Melting Aagarose (NMA) at about 45°C in PBS before the experiment. This layer was used to promote the attachment of the second layer. For the second layer, cells mixed with 80 µl of 1% LMA (pH 7.4) then and rapidly pipettd onto this slide; cells were spread using a cover slip and maintained on an ice-cold flat tray for 5 min to solidify. After removal of the cover slip, the slides were immersed in cold lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Trise- base) with 1% Triton X-100 and 10% DMSO was added just before use for a minimum of 1 h at 4°C.

Electrophoresis of slides: The slides were subjected to electrophoresis in Tris-Borate- EDTE (TBE) buffer (pH = 8.3) at 24 volts (~0.74 V/cm) for 20 min. Slides were removed from the electrophoresis chamber and fixed for 5 minutes with 70% ethanol; and then were dried at room temperature in the dark. Slides were stained with diluted Ethidium Bromide for 5 minutes at 4°C and gently tap to remove excess Ethidium Bromide and dry completely at room temperature in dark. Cells were observed at 40×magnification with inverted fluorescence microscope. Fifty randomly selected cells were count per sample to quantify the apoptotic cell percentage. Scored was calculated from the ratio of (L/W) comet to determine the Comet Index (CI). Scored range from 1.2 to 2 considered low DNA Damage (LD), from 2.1 to 3 medium DNA Damage (MD) and up to 3 high DNA damage (HD) [13, 14].

2-Measurement of oxidative stress marker

Hemoglobin concentration was determined and the G6PD activity was expressed as international units per gram hemoglobin (U/g Hb) in erythrocyte hemolysate.

The erythrocyte lipid peroxidation end product malondialdehyde (MDA) level was determined by a method depends upon the reaction with thiobarbituric acid (TBA) at 90–100 °C [15].

Glutathione Peroxidase (G-Px) and superoxide dismutase (SOD) activities were measured **a**ccording to ZeptoMetrix diagnostic kit. Measurements was performed spectrophotometrically at 340, 560 nm, respectively. [16].

Catalase activity in erythrocytes was assayed by a method described by Goth, (1991). The rate of dismutation of H_2O_2 to water and molecular oxygen is proportional to the activity of catalase. Therefore, the sample containing catalase is incubated in the presence of a known concentration of H_2O_2. After incubation for exactly one minute, the reaction is stopped with ammonium molybdate. The amount of H_2O_2 remaining in the reaction is then determined by the oxidative coupling reaction between molybdate and H_2O_2 [17].

Statistical analysis

Data were analyzed by two way analysis of variance with significant level ($P<0.05$). Data presented as means ± SD [18].

Results and Discussion

Neutral comet assay (Single Cell Gell Electrophoresis): The neutral comet assay or single cell electrophoresis was a sensitive method of detection double strand break of DNA caused by apoptosis [19]. In this study some investigators were followed to analyze the results by a manual method using ruler in photo of cell on computer monitor [12, 20] to quantify DNA damage caused by apoptosis. Based on scoring the comet into categories. Then scored was calculated from the ratio of cell diameters (L/W) comet to determined the comet index (CI), with cell exhibiting no migration having a ratio of ≈ 1 Figure-1 [21, 22].The scoring was conducted in three levels of DNA damage were assigned ranging from 1.2 to 2 considered low DNA damage (LD), from 2.1 to 3 medium DNA damage (MD), and up to 3 high DNA damage (HD) Figure-1B,C,D.

The apoptotic cells could be clearly distinguished from necrotic cells by the pattern of comet image. Neutral comet assay successfully differentiated the two types of cell death. Apoptotic nuclei

showed longer comet tails with high score, whereas necrotic nuclei yielded almost no tails, which implied that this assay could be used to differentiate apoptosis from necrosis [23, 24].

The comet image of apoptotic cells has the tail separated from the comet head [25]. The apoptotic cells could be classified into two types based on the DNA migration patterns: completely apoptotic cells and cells undergoing apoptosis. The former with apoptotic DNA fragmentation were clearly identified by the complete separation of the tail from the small comet head. Apoptosis was assessed after electrophoresis with the cells being classified according to their comet index which represent the ratio of L/W of the cells after electrophoresis, as described in procedures. When comparing percentage the values of DNA strand break of our G6PD-deficient patients and normal it was found that 7% in normal and 44.5% in G6PD-deficient patients. This revealed that there was DNA damage in G6PD-deficient patients Figure-1, Table-1.

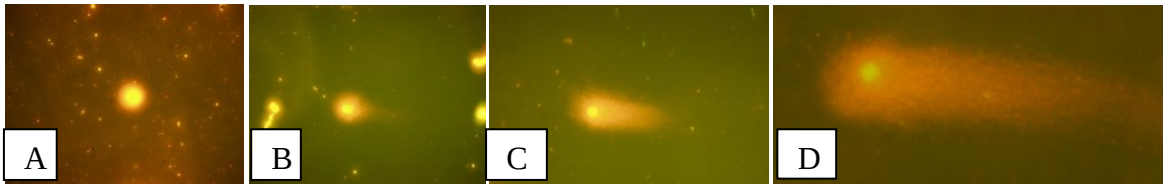

Figure 1- Three examples of scoring categories for comet assay, 1- (A) Electrophoresis under neutral conditions in low melting point agarose gel of the cells of a subject without any hemoglobinopathy and thus without DNA damage, 2- Electrophoresis under neutral conditions in low melting point agarose gel of the cells of a G6PD-deficient patient with damage classified as Comet class (B)-LD, (C)-MD, (D)-HD.

Table 1- Score range and apoptosis percentage of lymphocyte in G6PD-deficient patients by comet assay technique Score rang percentage

Groups	Score rang %				Apoptosis %
	(ND) %	(LD) %	(MD) %	(HD)%	
Control	93	7	0	0	7
G6PD-deficient patients	55.5	14.5	16	14	44.5

Oxidative stress biomarkers

Biomarkers used for oxidative stress assessment are in Table-2. A significant increase ($P<0.05$) in the means of erythrocyte antioxidant enzymes: GP-x activity (7865.2±660.4 vs. 5307.8±728.9) and SOD activity (225.94±18.29 vs. 180.90±10.82) in G6PD-deficient patients compared to controls. Whereas the activity of erythrocyte catalase was significantly decreased (70.40 ± 5. 7 vs. 93.42 ± 5.7) at ($P<0.05$) in G6PD deficiency patients compared with control. And a significant increase ($P<0.05$) in the mean of Malonyldialdehyde (MDA) (72.25 ± 10.2 nM/g Hb vs. 30.26 ± 5.64 nM/g Hb l) in patients with compared with controls. MDA values showed a significant positive correlation with G-Px levels (r = + 0.756), while it was observed negative correlated with catalase levels (r = - 0.352).

Table 2- Showing levels of antioxidants enzymes, oxidative stress profiles in controls and G6PD-deficient patients (Mean ± SD)

Parameters	Control N=30	G6PD-deficient	*P* value
GPX U/g Hb* (mean±SD)	5307.8±728.9	7865.2±660.4	0.05
SOD U/g Hb* (mean±SD)	180.90±10.82	225.94±18.29	0.05
MDA nM/g Hb	30.26 ± 5.64	72.25 ± 10.2	0.05
Catalase, kU/g Hb*	93.42 ± 5.7	70.40 ± 5. 7	0.05

*Note: All the enzyme activity units were defined previously.

Discussion

Glucose-6-phosphate dehydrogenase (G6PD) deficiency is the most common inherited disease, which causes hemolytic anemia. Considering the importance of G6PD reaction and its products NADPH and against oxidative stress, it hypothesized the failure of detoxification of H_2O_2 in G6PD-deficient white blood cells that could probably induce primary DNA damage [1]. For the evaluation of DNA damage, the analyzed mononuclear leukocytes of 30 samples using neutral single cell gel electrophoresis (SCGE) or comet assay. The level of DNA damage was compared with the level of basal DNA damage in control group represented by healthy control. Visual scoring was used for the evaluation of DNA damages. The results showed that the percentage of DNA strand breakage in mononuclear leukocytes of 30 with G6PD-deficient patients was 44.5% than those observed in the normal lymphocytes. Hydroxyl radicals ($OH^{\bullet}$) were the most reactive oxygen free radical species capable of direct oxidative damage to macromolecules including DNA, protein, and lipid membranes [26].Complication of oxidative stress in G6PD-deficient patients could be due to consumption of enzymes antioxidants and increase of oxidative stress as free radical effect of DNA [27,28]. This evidence demonstrated that free radical and iron overload might be a cause of leukocyte DNA damage [6, 29]. Mesbah-Namin et al. (2004) [27] used modified alkaline single cell gel electrophoresis (SCGE) or comet assay in G6PD in Iranian patients for detection apoptosis because it was easy, sensitive, and quantitative.

On the other hand, the study included a total of 30 samples were screened for biomarkers used for oxidative stress assessment**.** There were few studies on the incidence of G6PD deficiency and the status of oxidative stress parameters in Baghdad, Iraq.

Erythrocytes are the first react to increased activity of free radical oxidation and to exhaust their compensatory potential. Previous studies on erythrocyte antioxidant capacity and human disease relation showed that some changes in activities of the antioxidant enzymes in the cell may occur [30]. In this study, the mean ± SD of erythrocyte MDA concentrations, GPx, SOD and Catalase activity levels were determined antioxidant defense with G6PD deficiency and compared with the control group in Baghdad.

The erythrocyte lipid peroxidation product MDA levels was significantly increased ($P < 0.05$) and reached to 72.25 ± 10.2 nM/g Hb as compared with control 30.26 ± 4.55 nM/g Hb. The activity of erythrocyte catalase was significantly decreased ($P<0.05$) in G6PD deficiency patients ($P<0.05$) compared with control, whereas the activity of the other antioxidant enzyme(G-Px and SOD) are significantly increased ($P<0.05$) as compared with control group (Table 1).The data obtained from this study indicate that there are increases in free radical generation and the antioxidant defense is impaired in peroxidation which is in agreement with other report [31,32].

The lipid peroxidation product i.e. malondialdehyde (MDA) levels have been increased significantly ($P<0.05$) in erythrocytes of the G6PD deficiency compared with control group. This may indicate the presence of increased oxidative stress. Rise in MDA could be due to increased generation of reactive oxygen species (ROS) due to the excessive oxidative damage generated in these patients [33]. These oxygen species in turn can oxidize many other important biomolecules including membrane lipids. The raised MDA level in G6PD-deficient s reflects the oxidative injury due to G6PD deficiency, which is attributed to free radical formation that abstracts of hydrogen atoms from lipoproteins causing lipid peroxidation, of which MDA is the main product [34].The membrane phospholipids, specifically polyunsaturated fatty acids are converted to MDA, which can be analyzed by reactivity to thiobarbituric acid [31, 35].

In addition, the results showed that, the erythrocyte antioxidant enzyme glutathione peroxidase and superoxide dismutase were elevated in G6PD-deficient as compared with that found in control group Table-1. SOD enzyme activity showed a significant increase in G6PD deficient patients, it is clear that, SOD determination is based on the oxidation of nicotinamide adenine dinucleotide reduced form (NADH) (mediated by superoxide radical) through a free radical chain of reactions involving thiol oxidation and univalent O_2 reduction [33,36]. G-Px is an oxidative stress inducible enzyme that plays a significant role in the peroxyl scavenging mechanism and in maintaining functional integration of cell membranes [37].The rise in the activity levels of G-Px could be due to its induction to counter the effect of increased oxidative stress. G-Px provides an effective protective mechanism against cytosolic injury because it eliminates H_2O_2 and lipid peroxide products by reduction reactions utilizing GSH. Decrease in the activities of antioxidant enzyme status was reported in various studies [38, 39].

In the present study, it was also observed a significant decrease ($P<0.05$) in the activity levels of catalase in G6PD deficiency compared with control Table -1. Catalase is the enzyme which protects the cells from the accumulation of H_2O_2 by dismutating it to form water and oxygen or by using it as an anti-oxidant in which it works as a peroxidase [40, 41].

Therefore, the deficiency of G6PD leads to increased oxidant damage manifested by increased methemoglobin percentage, lipid peroxidation, crosslinking between membrane skeletal proteins, hemoglobin attachment to the membrane skeleton and altered membrane protein structure and function [35, 42]. The results showed that positively significant correlation was found between SOD levels with MDA concentrations (r = + 0.756), whereas a negative significant correlations was found between catalase activity levels and MDA concentrations (r = - 0.352). These antioxidants are classified into: primary, secondary and tertiary defense. The primary antioxidants work by preventing the formation of new free radical species. These include SOD, G-Px and metal-binding proteins (e.g. ferritin or ceruloplasmin). Secondary antioxidants trap radicals thereby preventing chain reactions. These include vitamin E, vitamin C, beta-carotene, uric acid, bilirubin and albumin. Tertiary antioxidant repair biomolecules damaged by free radicals. These include DNA repair enzymes [33, 43]. The erythrocytes are particularly prone to the free radical damage since the membrane lipids are very rich in polyunsaturated fatty acids which play an essential role in generating free radicals. Free radicals, primarily the reactive oxygen species, superoxide and hydroxyl radicals which are highly reactive having an unpaired electron in an atomic or molecular orbit are generated under physiological conditions during aerobic metabolism. As free radicals are potentially toxic, they are usually inactivated or scavenged by antioxidants before they can inflict damage to lipids, proteins or nucleic acids. [44].

In conclusion, this investigation indicates that the mononuclear leukocytes of the G6PD-deficient samples may be exposed to DNA damage due to oxidative stress. This is the first report using comet assay for evaluation of DNA damage in G6PD deficiency samples in Baghdad. The results showed that the mean level of the DNA strand breakage in mononuclear leukocytes of 30 G6PD-deficient patients was higher than those observed in the normal lymphocytes. In this study, the erythrocyte MDA concentrations, GPx, SOD and Catalase activity levels were determined antioxidant defense and oxidative stress with G6PD deficiency and compared with the control group in Baghdad. The results showed that the decreased levels catalase was observed in G6PD-deficient.And increased levels of G-Px,SOD and lipid peroxidation product. MDA were observed in G6PD-deficient which indicate an increase in ROS formation due to different causes. Negative correlations were observed between MDA and catalase, whereas the positive correlations were observed between MDA and G-Px.

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