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Original article

Level of apoptosis in Saudi patients with a defect in Glucose-6-phosphate dehydrogenase (G6PD)

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ABSTRACT

Introduction: Glucose-6-phosphate dehydrogenase (G6PD) is an enzyme responsible for the production of NADPH pathway. Mutation in G6PD gene results in abnormal functional and structural changes in red blood cells (RBCs). The aim of the current study is to compare the levels of apoptosis in Saudi people suffering from G6PD deficiency.

Materials and methods: Twenty samples from unrelated Saudi children and neonates between the ages of 1 month to 10 years were collected. Ten ml Blood samples were collected by venipuncture in EDTA tubes, and hematological parameters assessed using Coulter Counter Analyzer. MNC cells were isolated. Apoptotic leukocytes were determined and concentaration of IL-107 release was measured using the FACS machine.

Result: There was a significant increase in white blood cells (WBCs) count in G6PD deficiency group compared to healthy controls. Also, there was a sgnificant increase in the level of apoptosis observed in the freshly isolated RBCs obtained from G6PD deficiency patients.

Conclusion: The findings may indicate that apoptosis of RBCs represents an important factor in the pathogenesis of G6PD.

1. Introduction

Glucose-6-phosphate dehydrogenase (G6PD) is an enzyme which is actively involved in the metabolism of all type of cells. G6PD is an initiating effector enzyme which catalyzes a series of chemical reactions that lead to the formation of ribose: the main sugar component of DNA and RNA (Richardson and O'Malley, 2019; Luzzatto et al., 2016; Isa et al., 2017). The enzymatic activity of G6PD with respect to the production of a hydrogen carrier and a reducing agent referred to as nicotinamide adenine dinucleotide phosphate (NADPH) in the functioning of RBCs. NADPH protects RBCs from potentially harmful reactive oxygen species (ROS), which are generated during routine cellular metabolism. NADPH also prevents the accumulation of toxic ROS in human cells, particularly in RBCs that are more susceptible to damage by these ROS (Richardson and O'Malley, 2019; Isa et al., 2017).

Mutation in G6PD gene causes G6PD enzyme deficiency that results in the abnormal functional and structural changes in G6PD enzyme or reduce its production in the cells. RBCs with deficient G6PD enzyme are unable to protect themselves from the damaging effects of the ROS (Dos et al., 2016). Signs and symptoms of hemolytic anemia manifests in the body when the rate of RBCs hemolysis exceeds the ability of the body to replace them; this anemia is caused by the loss of RBCs, which is a characteristic feature of G6PD deficiency (Kaplan et al., 2018). Individual suffering from G6PD deficiency are mostly asymptomatic; however, they can suffer from severe jaundice or hyprbiliruminemia during the neonatal period and adults might experience acute hemolytic

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anemia when they consume fava beans or when they are exposed to certain infections or drugs (Luzzatto et al., 2016; Isa et al., 2017; Alabdulaali et al., 2010). The most common symptoms and laboratory findings of G6PD deficiency are hemolytic anemia and possibly RBC sequestration by the spleen. Some of these manifestations of anemia include pallor, jaundice, fatigue, splenomegaly, and dark urine.

G6PD enzyme deficiency is an X-linked genetic disorder that predominantly affects males than females, and causes mild to severe jaundice in neonates (Nkhom et al., 2009). The tropical areas of Africa and the Middle East, tropical and subtropical areas in Asia, as well as some areas in Mediterranean Countries generally have high prevalence of G6PD deficiency (Usanga and Ameen, 2000). Some studies also revealed association between prevalence of G6PD deficiency and prevalence of malaria endemic (Doss et al., 2016; Kaplan et al., 2018; Usanga and Ameen, 2000).

In Arab countries, G6PD deficiency is one of the most prevalent genetic diseases. Saudi Arabia has the highest prevalence (39.8%), followed by Syria (30%), and Oman (29%) (Alabdulaali et al., 2010; Nkhoma et al., 2009; AlRiyami and Ebrahim, 2003; Gandapur et al., 2002). In 2001, El-Hazmi and others revealed that G6PD deficiency is a common genetic diseasein all provinces of Saudi Arabia and thatthe Eastern Province has the highest frequency of G6PD deficiency recorded in both males and females (Alabdulaali et al., 2010; Nasserullah et al., 1998, 2003; Muzaffer, 2005; Hayakawa et al., 2007). Isa et al also showed that G6PD deficiency is an important risk factor for severe Neonatal Indirect Hyperblirubinema (NIH), and, therefore, special attention should be paied to avoid irreversible neurological complications (Isa et al., 2017). Neonates with G6PD deficiency are two times more likely to develop hyperbilirubinemia than the normal population, and approximately 20% of kernicterus cases are associated with G6PD deficiency (Isa et al., 2017).

CD107 or LAMP-1 is a marker for T cells cells degranulation as part of the perforin-dependent cytotoxic pathway; CD107a can work as an activation marker since on stimulation CD107a is transported to the surface of the cells. Moreoever, IL-33 is released from necrotic cells and is inactivated by caspase-1 during apoptosis (Schmitz et al., 2005; Reading et al., 2016). The aim of the current study is to investigate the levels of apoptosis in Saudi people suffering from G6PD deficiency.

2. Materials and methods

The patients' sample include unrelated Saudi children and neonates between the ages of 1 month to 10 years. After obtaining a consent from their parents or guardians, the selected children and neonates were screened for G6PD deficiency. Twinty samples from age-matched healthy controls were run in parallel. This study was conducted between June2018 and Jan2020 at the Immunology Research Lab-PSAU. The Samples were obtained from King Fahad Medical City (KFMC-Riyadh) and King Khalid Hospital (KKH-Alkharj), Saudi Arabia.

Ten ml Blood samples were collected by venipuncture in EDTA tubes, and hematological parameters assessed using Coulter Counter Analyzer. Quantitative Sigma G6PD Kits were used to measure the activity of the G6PD enzyme in the RBCs (Sigma, USA), in which samples with < 5U/g hemoglobin (Hb) were considered G6PD deficient. Hyperbilirubinemia was diagnosed using the following laboratory tests: complete blood count, blood smear for red cell morphology, blood groups of mother and infant, Coombs' test, total serum bilirubin and G6PD screening test. The characteristics of selected patients are shown in Table 1. Written informed consent from all patients were obtained.

Table 1
Patients did not receive any treatment at the time of investigation.

	Number of samples	Age	Sex
Healthly Controls	20	13 ± 1.5	All males
G6PD Deficiency Patients	20	8 ± 2.1	Male:Female 8:12

2.1. Isolation of mononuclear and polymorphonuclear leukocytes from human peripheral blood

Mononuclear cells (MNCs) were obtained by density gradient centrifugation over Ficoll-Hypaque (1.077 g/ml) (Nyegaard). After washing with hank's buffered salt solution (HBSS, GibcoBRL), cells were resuspended in HBSS. Granulocytes were prepared from the fraction of cells settled at the bottom of the test tube, through Ficoll centrifugation. Granulocytes were filtered from erythrocytes by the standard osmotic shock method in an iced water bath. To calculate the concentration of nucleated cells, the sample was diluted with 3% acetic acid to lyse RBCs and an aliquot of cell suspension was placed in a hemocytometer counting chamber.

2.2. Determination of quantity of apoptotic leukocytes

The quantity of apoptotic cells was determined from both freshly isolated peripheral blood leukocytes and granulocytes after incubation in RPMI-1640 culture medium supplemented with 50 IU/ml penicillin , 50 mg/ml of streptomycin, 10% PBS (pH 7.4) and 2 mM glutamine in a CO_2 incubator at 5% CO2 for 18hr and 36hr. The degree of apoptosis was estimated using the TUNEL kit (FragEL™ DNA Fragmentation Detection kit; Calbiochem, Nottingham, UK) as recommendeed by the company. Viable cells stained blue whilst apoptotic cells appeared as small fragmented bodies which were stained bright green**.**

2.3. Measurement ofT cell degranulation by using IL-107 release

To assess levels of CD107a surface expression in T-cells DC and healthy subjects, PBMCs from both normal controls and G6PD deficiency patients were incubated with CMV lysate and/or SEB as a positive control overnight. Peripheral Blood Mononuclear cells (PBMCs) were stimulated with Cytomegalovirus (CMV) lysate in culture medium in a humidified 5% CO_2 atmosphere at 37 °C; afterwards, the cells were labelled with anti-CD107a. Isotype and unstimulated PBMCs were applied to remove any formed background. Mononuclear cells were aliquoted into FACs tubes for staining them with different fluorochrome-conjugated monoclonal antibodies (Mab). These Mab were optimized according to the Mab manufacturer's recommendations. The staining was analyzed using the FACSCalibur and subsequently analyzed by WinMDI 2.9 software.

2.4. Statistical analysis

Statistical analysis was carried out using Microsoft Excel spreadsheet and the StatView SE + graphics software. The probability of a significant difference between groups was determined by Mann Whitney test and Wilcoxon Signed rank test. Graphs were plotted using Cricket graph graphics package. All software programs were run on a Macintosh computer.

3. Result

1. Investigating the Level of hematological parameters

Table 2
Hematological parameters between G6PD deficiency patients and healthy controls.

Item	Hemoglobin	WBCs	RBCs	Platelets
G6PD Deficiency Patients	13.1	11.8	4.7	345
Healthly Controls	14.8	7.5	4.8	240
P value	p = 0.9	p = 0.04	p = 0.3	p = 0.1

Table 2, shows the hematological levels in both G6PD deficiency patients and healthy controls. There was a significant increase in WBC count in G6PD deficiency group compared to healthy controls, while other parameters are comparable between the two groups.

2. Investigating the Level of Leukocytes:

Table 3, shows that G6PD deficiency group suffered from lymphocytosis as compared to the healthy controls. Other cells were similar to those in the control group.

The data also shows that males suffered from G6PD deficiency more than females. More interestingly, increase in serum bilirubinemia and retics in G6PD deficiency group was reported as compared to the control group. G6PD-deficient neonates had lower **hemoglobin** (Hb) level and higher serum bilirubin (p = 0.01). The hospital records did not show whether those male neonates were hospitalized for longer periods in comparison with the females.

3. Study of apoptosis in RBCs, lymphocytes of G6PD deficiency patients

A sgnificant increase in the level of apoptosis was observed in freshly isolated RBCs obtained from G6PD deficiency patients. Since the lack of significant difference could be due to the fact that apoptotic cells are quickly phagocytosed by macrophages. Table 4, also shows that G6PD deficiency group had low % of apoptotic cells in freshly isolated Lymphocytes and Granulocytes compared to those in the healthly controls.

4. Measurement of T cell degranulation by using IL-107

The level of CD107 was examined in G6PD patients. Table 3 shows the level when PBMCs were stimulated by Staphylococcal Enterotoxin B (SEB) cells, levels of CD107a expression were found to be significantly more in CTL cells (P = 0.0061) than healthy controls after stimulation with K562 cells. IL-33 expression in apoptotic T-cells was significantly different (P = 0.02) in G6PD patients (7.4% ±1.5, N = 20) than healthly controls (Table 3).

4. Discussion

G6PD enzyme deficiency Over 200 million individuals are affected with G6PD deficiency all over the world; however, higher frequencies are reported in Mediterranean Areas and in the Middle East (Cappellini and Fiorelli, 2008; Weng and Chiu, 2010; Ainoon et al., 1999; Moiz et al., 2012). For most cases, individuals with the G6PD enzyme deficiency are asymptomatic and, sometimes, healthy. Ingestions of certain drugs, food, exposure to certain chemicals, infections or hypoxia lead to acute hemolytic anemia in those affected individuals (Weng and Chiu, 2010; Ainoon et al., 1999).

Table 4
Level of apoptosis between G6PD def - subjects and healthy controls.

Group investigated	% apoptotic Lymphocyte (M ± SD)	% apoptotic Granulocytes (M ± SD)	% apoptotic RBCs (M ± SD)
Healthy Controls (n = 20)	2.0 ± 1	3.5 ± 0.8	2.4 ± 1
G6PD Deficiency Patients (n = 20)	1.5 ± 2	1.8 ± 9.8	7.5 ± 0.9
P value	p = 0.7	p = 0.5	p = 0.002

This study was conducted in the Riyadh, Saudi Arabia. Similar study has shown that most G6PD-deficient cases are males than females on account of X-linked inheritance of the disease (Weng and Chiu, 2010; Ainoon et al., 1999). Early detection of G6PD deficiency via newborn screening is feasible, cost effective and allows early prevention of severe hyperbilirubinemia and its damaging effects on the central nervous system (Isa et al., 2017; Muzaffer, 2005).

In our study, the G6PD-deficient Hb and hematocrit levels were lower than those in normal controls. These results are in agreement with Moiz et al. (Moiz et al., 2012; Faraji-Goodarzi et al., 2019). Also, our study showed that lymphocytes count was significantly higher in G6PD-deficiency patients as compared to normal neonates and children, indicating active inflammatory response (Faraji-Goodarzi et al., 2019). Higher levels of DNA damage occurred in RBC not in lymphocytes and granulocytes of G6PD patients as compared to those of healthy controls. This was also evident by the higher level of RETICS.

The most significant damage was seen in the RBC of G6PD patients. This implies that a genetic mechanism is likely to be involved in the pathogenesis of G6PD disease. Similarly and naturally, it was shown that Fas-FasL pathway represents an important factor in normal erythropoiesis, and therefore any damage might serve as an indicator of the degree of pathogenesis of the disease, and the positive influence treatment of these patients (Alenzi et al., 2004). We also showed a striking result to the present data, in that a defect in Fas-mediated apoptosis was linked to autoimmune haemolytic anaemia (AIHA). They are two different hematological diseases (Alenzi et al., 2004).

Altogether, the findings may indicate that apoptosis of RBCs represents an important factor in the pathogenesis of G6PD. It is not clear whether treatment of these children may increase Fas/FasL expression as a consequence of corticosteroids therapy and/

Table 3
Leukocytes count between G6PD deficiency patients and healthy controls.

Item	Neutrophils	Basophils	Eosinophils	Lymphocytes	Monocytes
G6PD Deficiency Patients	47	0.7	3.5	66	9.2
Healthly Controls	52	0.6	3	37	8
P value	p = 0.09	p = 0.6	p = 0.2	p = 0.02	p = 0.1

or cytotoxic drugs. Therefore, studying the role of sFas in the regulation of apoptosis in lymphocytes is another interesting point. It can thus be suggested that Fas expression is increased immediately after the activation of lymphocytes, with the increased level of sFas in G6PD patients.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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