Biochemical and hematological indicators of acute and chronic cases of Mediterranean G6PD deficiency patients from southern Jordan

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Abstract: A total of 34 patients were analyzed in this study. Samples from 21 healthy individual were used as a negative control. Twenty eight out of the thirty four G6PD deficiency patients (82%) in our study were found to be of the class II (Mediterranean variant) according to WHO G6PD variant classification system. This variant was recognized by an extremely low enzyme activity (9±5 mU/109 RBC), and a more thermolabile enzyme than normal G6PD enzyme. Also, those patients did not have chronic nonspherocytic hemolytic anemia. Six out of thirty four of G6PD deficiency patients (18%) had moderate enzyme deficiency (38±13 mU/109 RBC), which belong to class III of G6PD variants. The activity in G6PD deficiency patients, were first clinically diagnosed before acute hemolysis. Four months after the acute hemolysis, the G6PD activity was measured again. Biochemical and hematological indicators were studied in acute and chronic of Mediterranean G6PD deficiency; the biochemical indicators showed a particular elevated level of total bilirubin, indirect bilirubin and lactate dehydrogenase enzyme in patients with acute hemolysis, while the direct bilirubin did not alter in all patients. All of other biochemical indicators gave a normal range values in both acute and chronic Mediterranean G6PD deficiency. Patients with acute hemolysis showed the characteristic hematolological findings such as low RBC counts, decreased hemoglobin value and increased WBC counts. The blood film in these patients showed the characteristic presence of fragmented cells "bite and "blister" which had Heinz bodies. However, these biochemical and hematological indicators in chronic patients were normal. Mediterranean variant of G6PD deficiency accounted for 82% of the study sample in southern Jordan. Nine out of the 28 G6PD deficiency patients of Mediterranean G6PD deficiency showed acute hemolysis (32%), which indicates the presence of a good link between Mediterranean variant and acute hemolytic crisis.

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Keyword: Mediterranean G6PD variant; Biochemical indicators; Hematological indicators

1. Introduction:

Glucose-6-phosphatase dehydrogenase (G6PD) deficiency is the most common enzymopathy in humans **(Gleason, 1996; kurutas and Tuncer; 2000; Nemoto and Sasakuma, 2000)**. G6PD enzyme is the first enzyme in the pentose phosphate pathway, with main physiological function to produce NADPH and pentose sugars (**Sodeinde, 1992; Mehta, 1994**). During exposure to xenobiotics, NADPH play important role in maintaining reduced form of glutathione, which is responsible for the detoxification of reactive free radical and lipid hydroperoxide **(Wells and Winn 1996: Abboud and Awaida, 2010).**

G6PD deficiency was discovered in the late 1950s as a result of research to investigate the differential susceptibility to the hemolytic effect of primaquine **(Beutler, 1959; Mehta et al, 2000).** Moreover, a deficiency in G6PD activity was connected with increases accumulation of cellular reactive oxygen species (ROS) while an overexpression of G6PD activity decreases the accumulation of these species in response to oxidative stress **(Leopold et al, 2003; Fico et al, 2004)**.

According to the level of enzyme activity, World Health Organization classified variants of G6PD deficiency to three groups **(Mohanty et al, 2004);** Class 1: rare variants of G6PD deficiency have severe deficiency of the enzyme with chronic nonspherocytic hemolytic anemia, class 2: Severe deficiency of the enzyme, enzyme activity is less than 10% of normal. And class 3: Moderate deficiency of the enzyme, enzyme activity is 10-60% of normal.

The most common G6PD-deficient variant found in the Middle East and particularly those recorded in Jordan were categorized belongs to class II were (Mediterranean variant) of WHO classification system for G6PD variants. It is characterized by low enzymatic activity and B-like electrophoretic mobility **(Gelpi and King, 1977, Kurdi-Haidar et al, 1990, Karadsheh et al 2005)**. A common mutations detected in the G6PD Mediterranean variant is single C-T transition at nucleotide position 563, causing a serine-phenylalanine replacement of the amino acid position **(Kurdi-Haidar et al, 1990)**.

As an antioxidant enzyme, G6PD is present in all human cells but it is particularly essential to erythrocytes because these cells don not have another source for producing NADPH **(Luzzatto, 1967; Beutler, 1994)** Therefore, G6PD deficient erythrocytes were extremely sensitive to oxidative stress (**Gerli et al, 1982; Abboud and Awaida, 2010)**. The hemolysis may occur after ingestion of certain drugs or even more commonly by exposure to free radical oxidant by leukocytes in the course of infection. Some drugs are harmless to the mild G6PDA variant but cause hemolysis to those who have Mediterranean variant. Patients with G6PD deficiency may also have hemolysis after ingestion of fava beans, because these legumes generate oxidants. The favism is endemic in the Mediterranean, Middle East, and part of Africa (Mehta et al, 2000; Mohanty et al, 2004). This work aims to investigate biochemical and hematological indicators of acute and chronic cases of Mediterranean G6PD deficiency patients from southern Jordan.

2. Materials and methods

Patients

The protocol for this study was approved by the Ethics Committee of the science faculty of American University of Madaba (AUM) and all subjects gave their informed consent.

A total of 34 G6PD-defective patients were included in this study. Samples from 21 healthy individual were used as a negative control. Blood samples were collected from patients in different area in southern Jordan. The G6PD variants are classified according to the world health organization (WHO) depending on the enzyme activity and the severity of hemolysis **(Ruwende and Hill, 1998; Mohanty et al, 2004)**.

Preparation of enzyme extract and determination of enzyme activity

Fresh unhemolyzed blood sample was collected and kept no longer than 7 days at 2–8 °C before being processed for G6PD extraction. A blood sample of 200 µL was washed 3 times with 2 mL aliquots of saline solution. The washed pellet was re-suspended in solution of digitonin (0.02%) and sodium azide (0.1% w\v) then allowed standing for 15 min at 2–8 °C. After centrifugation the supernatant was used as an enzyme preparation (enzyme extract) within 2 h of the extraction procedure. Activity of the enzyme G6PD was measured kinetically. An extracted fresh enzyme of 15 µL was added to a cuvette with 900 µL of 50 mM Tris buffer at pH 7.6 containing 1mM MgCl2 and a substrate glucose 6-phosphate at 500 µm concentration. The reaction was initiated by adding $NADP^+$ (250 µm) and the rate of increase in absorbency at 340 nm due to the conversion of NADP+ to NADPH was determined for a period of 2 min **(Tian et al, 1994)**.

During acute hemolysis of G6PD deficiency patients, the older erythrocytes have been hemolyzed while young erythrocytes and reticulocytes have almost normal enzyme activity. Testing G6PD deficiency under these conditions gives false negative results. In our study, the activity in G6PD deficiency patients, were first clinically diagnosed before acute hemolysis. Four months after the acute hemolysis and the G6PD activity were measured again.

Effect of temperature on normal and Mediterranean variant of G6PD enzyme activity:

Enzyme activity for normal and Mediterranean G6PD enzyme were assayed after 10 minutes of pre-incubation in water bath at different temperature in a range from 20°C to 80°C.

Hematological indicators measurement

All hematological indicators were measured by The MEK -6318k Hematology analyzer from Nihon Kohden, Tokyo Japan.

Biochemical indicators measurement

All biochemical indicators measured have been measured by Roche company kits on a Hitachi 911 Autoanalyzer. Biochemical parameters like total bilirubin direct bilirubin, indirect bilirubin, Alanine aminotransferase(ALT), Aspartate aminotransferase (AST), Alkaline phosphatase (ALP), Lactate dehydrogenase (LDH), urea, creatinine, cholesterol, triglycerides, glucose were determine.

Blood film, staining, and examination:

Blood film, staining, and examination were performed following **Barbara, (1984)**.

Statistical analysis

Values for specific enzyme activity, biochemical and hematological indicators are expressed as mean ± standard error of the mean (SEM). Mean pairs were then compared using t-test. A p-value < 0.05 was considered significant.

3. Results

Measurement of Glucose-6-phosphate dehydrogenase activity

Twenty eight out of the thirty four of G6PD deficiency patients (82%) have very low enzyme activity (9±5 mU/109 RBC). Six out of the thirty four of G6PD deficiency patients (18%) had moderate enzyme activity (38±13 mU/109 RBC) which belongs to class III (10 to 60 percent of normal enzyme activity) of WHO classification system of G6PD variants. However, the G6PD activity in the control ~~is~~ was (127±16 mU/109 RBC). These data are in agreement with what is reported in the literature for normal levels of erythrocytes G6PD (Normal value=118-144 mU/109 RBC) **(Ruwende and Hill, 1998: Karadsheh, 1985)**. Nine out of the 28

Mediterranean G6PD deficiency patients show acute hemolysis (32%).

Effect of temperature on normal and Mediterranean variant of G6PD enzyme activity

The effect of temperature on the G6PD activity was determined after 10 minute of pre-incubation in water bath at different temperature in a range from 20°C to 80°C. Figure (1) shows that normal enzyme activity loses all of its activity at about 80°C while the Mediterranean G6PD enzyme required a temperature of 60°C to lose all of its activity.

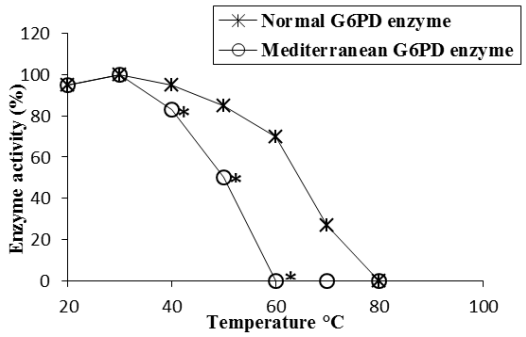

Figure (1). Effect of pre-incubation temperature on normal and Mediterranean G6PD enzyme.* Represents significant value at (P<0.05).

Biochemical indicators in acute and chronic Mediterranean G6PD patients:

Biochemical indicators were studied in acute and chronic of Mediterranean G6PD patients; the biochemical indicators show a particular elevated level of total bilirubin, indirect bilirubin and lactate dehydrogenase enzyme in patients with acute hemolysis, while the direct bilirubin did not change in all the patients(Figures 2, 3,4 and 5). All of other Biochemical indicators gave a normal range values in both acute and chronic Mediterranean G6PD deficiency (table 1).

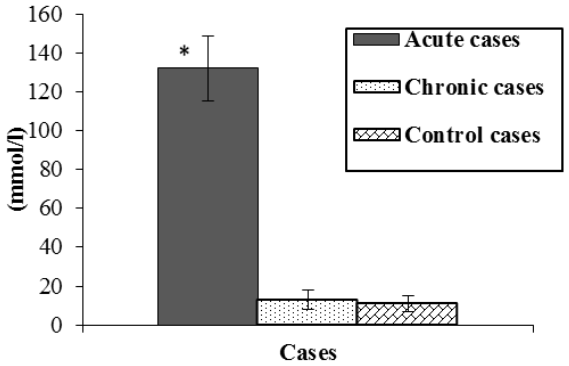

Figure (2). Total bilirubin measurement in acute and chronic Mediterranean G6PD patients.* Represents significant value at (P<0.05).

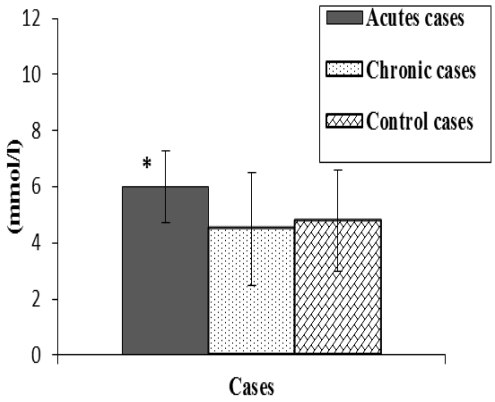

Figure (3). Direct bilirubin total measurement in acute and chronic Mediterranean G6PD patients. * Represents significant value at (P<0.05).

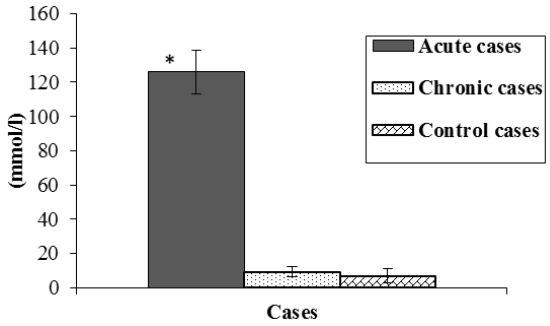

Figure (4). Indirect bilirubin total measurement in acute and chronic Mediterranean G6PD patients.* Represents significant value at (P<0.05).

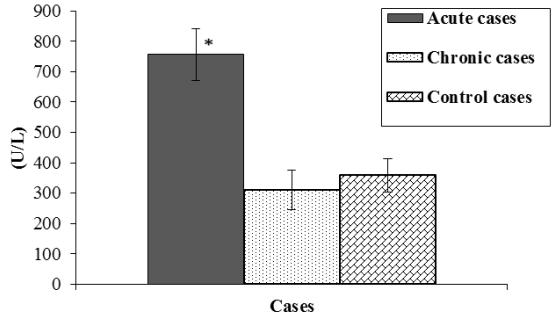

Figure (5). Lactate dehydrogenase measurement on acute and chronic Mediterranean G6PD patients.* Represents significant value at (P<0.05).

Hematology indicators in acute and chronic Mediterranean G6PD patients

The hematology indicators were studied in acute and chronic of Mediterranean G6PD patients. Patients with acute hemolysis show the characteristic hematolological findings such as low RBC counts, decreased hemoglobin value and increased WBC counts (Figures 6,7 and 8). The blood film in those

showed the characteristic presence of fragmented cells "bite" and "blister" which had Heinz bodies (Figure 9). These biochemical and hematological indicators in chronic patients were normal (Figures 6, 7 and 8).

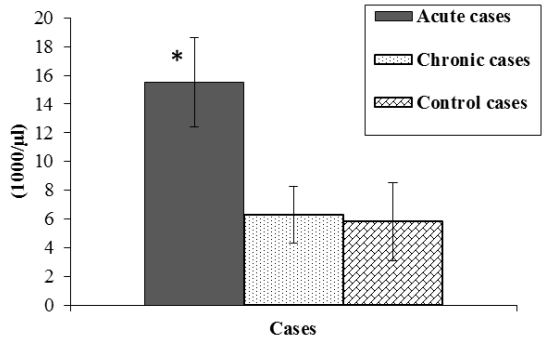

Figure (6). White blood cells count for acute and chronic Mediterranean G6PD patients.* Represents significant value at (P<0.05).

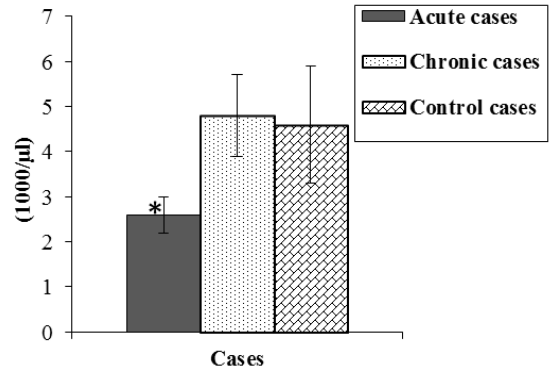

Figure (7). Red blood cells count in acute and chronic Mediterranean G6PD patients.* Represents significant value at (P<0.05).

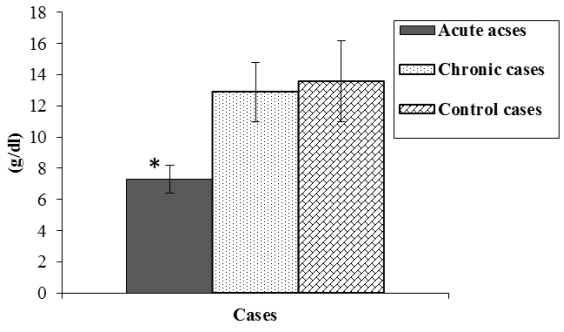

Figure (8). Hemoglobin concentration in acute and chronic Mediterranean G6PD patients.* Represents significant value at (P<0.05).

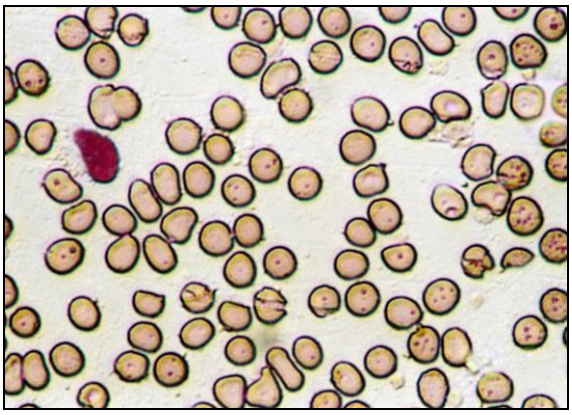

Figure (9). Blood film prepared by wright stain shows fragmented cells "bite" and "blister" cell which have Heinz bodies (magnification power 40X).

Table 1: Biochemical indicators in acute and chronic Mediterranean G6PD. patients.* Represents significant value at (P<0.05).

Biochemical indicators	control cases	Acute cases	Chronic cases
Glucose (mmol/l)	4.8±0.73	5.3±0.85	5±1
Urea (mmol/l)	4±3.5	5±2.5	4.5±3
Creatinine(um mol/l)	59±12l	64±9	66±14.5
Uric acid (ummol/l)	292±94	263±80.6	246±69.3
Cholesterol(m mol/l)	3.4±1.4	4.1±0.8	3.8±0.6
Triglyceride(m mol/l)	1.2±0.5l	1.4±0..6	1.1±0.7
Aspartate aminotransfera se (GOT)(U/L)	27±5.5	32±5.6	28±6.9
Alanine aminotransfera se (GPT)(U/L)	28±4.8	27±8.4	24±8.4
Alkaline phosphatase(U/ L)	190±60.2	159±46.5	175±58.4

4. Discussion:

A total of 34 patients were analyzed in this study. Samples from 21 healthy individual were used as a negative control. Twenty eight out of the thirty four G6PD deficiency patients (82%) in our study were found to be of the class II (Mediterranean variant) of WHO classification system for G6PD variants. This variant is recognized by an extremely low enzyme activity (9±5 mU/109 RBC), and a more thermolabile enzyme than normal G6PD enzyme. Also, those patients did not have chronic nonspherocytic hemolytic anemia. Six out of thirty four of G6PD deficiency patients (18%) had

moderate enzyme deficiency (38±13 mU/109 RBC), which belong to class III of G6PD variants. The effect of temperature indicated that the Mediterranean G6PD enzyme was more thermolabile than normal enzyme. Such enzyme instability is known to be particularly associated with the Mediterranean variant of G6PD deficiency **(WHO 1989)**. Generally, most of the discovered G6PD defective cases in the Middle East and particularly those recorded in Jordan were categorized as Mediterranean variant **(Gelpi and King, 1977, Kurdi-Haidar et al, 1990, Karadsheh et al., 2005)**.

Mediterranean variant of G6PD deficiency accounted for 82% of the study samples in southern Jordan. Nine out of the 28 G6PD deficiency patients of Mediterranean G6PD deficiency showed acute hemolysis (32%), which indicates correlation between Mediterranean variant and acute hemolytic crisis. G6PD deficient erythrocytes are sensitive to oxidative stress **(Gerli et al, 1982)**. Acute hemolysis occurs after exposure to oxidative drugs, infection and fava beans ingestion **(WHO report, 1989)**.

G6PD is not the sole NADPH producing enzyme in nucleated cell, but in red blood cell only G6PD enzyme can generate NADPH **(Luzzatto, 1967; Beutler, 1994)**. In the present study, the blood cell counting indicates low RBC counts, decreased hemoglobin value, increased WBC counts, but these indicators showed normal range in chronic patients. The NADPH plays important role in erythrocytes, by preserving the integrity of red blood cell membrane sulfhydryl groups and detoxifies harmful hydrogen peroxide and oxygen radicals. Also it is used in the regeneration of reduced glutathione which prevents haemoglobin denaturation **(Prchal and Gregg, 2005)**. Hemolysis is the destruction or removal of red blood cells from the circulation before the completion of their 120 days normal life span **(Tsun-Yee and Liu, 1997)**.

A decrease in G6PD enzyme activity leads to decrease in NADPH and reduced Glutathione. It is believed that a lack of reduced glutathione availability causes early hemolysis in the spleen **(Tian et al, 1999; Leopold and Loscalzo, 2005)**. During acute hemolysis of G6PD deficiency patients, the older erythrocytes have been hemolyzed while young erythrocytes and reticulocytes have almost normal enzyme activity. Testing G6PD deficiency under these conditions gives false negative results **(Ainoon et al, 2003)**. In our study, the activity in G6PD deficiency patients, were first clinically diagnosed before acute hemolysis. Four months after the acute hemolysis, the G6PD activity was measured again.

The blood film in acute G6PD deficient patients showed the characteristic presence of fragmented cells “bite” and “blister” which had Heinz bodies. In acute cases, Heinz bodies are formed by damage to the hemoglobin component molecules, usually through oxidant damage. Also, damaged cells are cleared by macrophages in the spleen, where the precipitate and damaged membrane are removed, leading to characteristic "bite cells" **(Bolchoz et al, 2002; Bowman et al, 2004)**. In the present study, Biochemical analysis indicated a particular elevated level of total bilirubin, indirect bilirubin and lactate dehydrogenase enzyme in acute patients, while the direct bilirubin did not change at all. These indicators in chronic G6PD deficiency patients showed normal range. All other Biochemical indicators gave normal range values in acute and chronic G6PD deficiency. Unconjugated bilirubin is fat soluble; it passes cell membranes and is potentially neurotoxic. However, toxicity is usually escaped because most un-conjugated bilirubin is bound to albumin. Hyperbilirubinaemia develops when rate of bilirubin production via the breakdown of heme by the reticuloendothelial system exceeds the rate of elimination, primarily by conjugation. Various genetic, environmental and racial factors affect the equilibrium between the methods of production and elimination **(Smitherman et al, 2006)**. Lactate dehydrogenase (LDH) is an enzyme that is present in lots of cells in the body: heart, lung, kidney, liver, muscle, and red blood cells. It’s also present in some tumor cells. Any time these cells are destroyed, LDH is released, and you can measure it in the serum **(Goldman, 1964; Heilmann et al, 2009)**.

5- Conclusion

Mediterranean variant accounted for 82% of the study sample in southern Jordan. Also 32% of Mediterranean G6PD deficiency showed acute hemolysis, which indicates relationship between Mediterranean variant and acute hemolytic. Mediterranean G6PD deficiency patients with acute hemolysis showed a particular elevated level of total bilirubin, indirect bilirubin and lactate dehydrogenase enzyme. Also, those Patients showed low RBC counts, decreased hemoglobin value and increased WBC counts. The biochemical and hematological indicators in chronic Mediterranean patients were normal.

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