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Oxidative stress and antioxidant defense in Egyptian favism patients

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Abstract. - BACKGROUND: Favism occurs as the result of intolerance to the ingesting of fava beans or to the inhalation of pollen from the *Vicia faba* plant. Patients with favism are always Glucose-6-phosphate dehydrogenase (G6PD)-deficient, but not all G6PD-deficient individuals develop hemolysis as a result of fava beans consumption.

PATIENTS AND METHODS: Blood samples were collected from children with favism (n = 55) between age (2-12 years) on EDTA tubes divided into 3 groups: group 1 control group (n = 15), group 2 before blood transfusion (during hemolytic action) (n = 20) and group 3 after blood transfusion (treated) (n = 20).

RESULTS: It was found that in group 2 GSH level was significantly low; (1.11 ± 0.39, $p < 0.001$) compared to controls (26.31 ± 5.26, $p < 0.001$). In group 3 after blood transfusion Level of GSH rose but remained lower than normal level (5.88 ± 2.33, $p < 0.001$) compared to controls. As for oxidative stress parameters, both levels of H_2O_2 and MDA were highly significant in group 2; (213.49 ± 57.56, $p < 0.001$), (98.05 ± 22.34, $p < 0.001$) compared to controls (3.75 ± 1.164, $p < 0.001$), (7.38 ± 2.07, $p < 0.001$), respectively.

Moreover, in group 3 after blood transfusion, levels of H_2O_2 and MDA were decreased but remained high compared to controls (66.55 ± 22.49, $p < 0.001$), (47.18 ± 9.62, $p < 0.001$) sequentially. Also, there was a negative correlation between GSH that acts as antioxidant defense enzyme and each one of oxidative stress parameters MDA & H_2O_2. However, there was a positive correlation between H_2O_2 and MDA.

CONCLUSIONS: From this study, it could be concluded that the favic patients have high oxidative stress (H_2O_2 and MDA) more than normal individuals and less antioxidant defense (GSH). With the passage of time these individuals, cells would be more vulnerable for H_2O_2-induced senescence.

Key words:
Favisim, Oxidative stress, Antioxidant defense.

Introduction

Exposure to fava beans (*Vicia faba*; broad bean) is known to be toxic and potentially fatal for some individuals since the era of the old Greeks. Favism is the acute hemolysis that follows the ingestion of fava beans. This syndrome appears to be limited to those who have the Mediterranean variant with more prevalence among males than females. There is an increased frequency between children who are between the ages of 2 and 6 years. Also, the breast-fed infant whose mother had ingested fava beans can develop favism[1].

Favic patients are always G6PD deficient, but not all G6PD-deficient individuals show symptoms of hemolysis when they ingest fava beans. Therefore, G6PD deficiency is essential but not enough to cause favism. Favism is the most common in patients who develop G6PD class II variants, but it is uncommon in patients with the G6PD class V A-variant[2]. Fava beans are assumed to be the cause of oxidative damage produced by an unknown component, probably vicine, convicine, or isouramil[3,4].

G6PD deficiency is the most common existing enzymatic disorder of red blood cells in human beings. About 400 million people are considered to be affected by this deficiency[5]. The G6PD enzyme is involved in catalyzing the first step in the pentose phosphate pathway (PPP), which leads to the production of antioxidants that protect cells against oxidative damage[6]. This pathway shows the production of NADPH, which maintains the reduced glutathione within the cell. Reduced glutathione acts as an antioxidant and protects cells against oxidative damage[6]. A G6PD-deficient patient, consequently, is not capable of protecting red blood cells against oxidative stresses from certain drugs, metabolic conditions, infections, and consumption of fava beans[7].

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G6PD is reported to be more common in Africa, southern Europe, the Middle East, South-East Asia, and the central and Southern Pacific islands. However, deficient alleles are quite widespread in North and South America as well as in some areas of northern Europe due to migration[8]. G6PD deficiency is an X-linked hereditary genetic defect produced by mutations in the G6PD gene. The inheritance of G6PD deficiency, a typical X-linked, exhibits pattern with higher frequency in males than in females[7].

In most cells, the generation of the necessary intracellular NADPH has been backed up by other metabolic pathways. In contrast, red blood cells do not have other options to produce NADPH. Thus, G6PD deficiency becomes fatal in red blood cells, where hemolytic anemia will be the outcome of any oxidative stress. Numerous factors may be the cause of oxidative stress including the ingesting of fava beans, certain drugs, infections, and certain metabolic conditions such as diabetic ketoacidosis[6].

NADPH has a key function in the reduction of oxidized glutathione (GSSG) to a tripeptide known as reduced glutathione (GSH). This tripeptide acts as a reducing agent together with the enzyme glutathione peroxidase, which plays an important role in the detoxification of hydrogen peroxide. During this process, GSH is converted into GSSG which in turn decreases the GSH levels. Glutathione reductase catalyzes the reduction of GSSG to GSH in the presence of NADPH leading to the regeneration of GSH[9]. Red blood cells have no other sources of NADPH, *i.e.* G6PD is required for the protection of hemoglobin sulfhydryl groups and preventing red blood cell membrane oxidation[10]. Lipid peroxidation in red blood cells results in disorganization of the lipid moiety of cell membranes causing in lethal damage to the cell[11].

The aim of our study is to find out the correlation between oxidative stress (H_2O_2 and MDA) and antioxidant defense (GSH) in favism patients.

Patients and Methods

Patients

This study was conducted on 55 children dividing to 3 groups:

Group 1, [control group, (n = 15)], group 2, (before blood transfusion (during hemolytic action) (n = 20) and group 3, after blood transfusion (treated) (n = 20). They were enrolled from those presented and followed up at Pediatric Hospitals in both Mansoura and Zagazig University Children's Hospital and after taking a written formal consent from the patient's parents.

Methods

G6PD assay

All patients were screened for G6PD deficiency using qualitative visual method from the red cell hemolysate (Span Diagnostics Ltd, Surat, India)[12,13].

Estimation of glutathione reduced (GSH)

The concentration of GSH in RBC_S was assessed by the method based on the reduction of 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) with glutathione (GSH) to produce a yellow compound. The reduced chromogen directly proportional to GSH concentration and its absorbance can be measured at 405 nm[14].

Estimation of hydrogen peroxide (H_2O_2)

The concentration of H_2O_2 in plasma was assessed by the method based on the reaction of H_2O_2 with 3, 5'dichloro-2-hydroxy benzene sulfonic acid (DHBS) and 4- aminophenazone (AAP) in the presence of peroxidase (HRP) to form achromophore[15,16].

Estimation of Malondialdehyde (MDA)

The lipid peroxidation products were estimated by the formation of thiobarbituric acid (TBA) and quantified in term of MDA, where TBA reacts with MDA in acidic medium at temperature of 95°C for 30 min. to form thiobarbituric acid reactive product, the absorbance of the resultant pink product can be measured at 534 nm[17,18].

Statistical analyses

Statistical differences between groups were evaluated using Student's *t*-test. Pearson-product correlation was used to test for linear relationship between different variables. The software SPSS 11.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. Results were reported as means ± SD, and a p value < 0.05 was considered statistically significant.

Results

It was found that in group 2 GSH level was significantly low; (1.11 ± 0.39, $p < 0.001$) com-

Table I. Minimum, Maximum, and Standard Deviation values of GSH, H_2O_2 and MDA obtained from groups 1, 2 and 3.

Group	Variable	No.	Min.	Max.	Mean	St. deviation
Control	GSH	15	19.89	35.00	26.3086*	5.25638
	H_2O_2	15	2.39	5.75	3.7497*	1.16428
	MDA	15	3.66	11.19	7.3831*	2.74692
Hemolytic action	GSH	20	0.42	1.98	1.1065*	0.38544
	H_2O_2	20	104.50	300.00	213.4863*	57.55669
	MDA	20	65.60	153.80	98.0538*	22.33509
Treated	GSH	20	2.60	10.13	5.8806*	2.33060
	H_2O_2	20	38.36	100.20	66.5498*	22.49079
	MDA	20	32.35	60.50	47.1839*	9.62414

The mean difference is significant at 0.001 levels ($p < 0.001$).

pared to controls (26.31 ± 5.26, $p < 0.001$). In group 3 after blood transfusion Level of GSH rose but remained lower than normal level (5.88 ± 2.33, $p < 0.001$) compared to controls. As for oxidative stress parameters, both levels of H_2O_2 and MDA were highly significant in group 2; (213.49 ± 57.56, $p < 0.001$), (98.05 ± 22.34, $p < 0.001$) compared to controls (3.75 ± 1.164, $p < 0.001$), (7.38 ± 2.07, $p < 0.001$), respectively.

Moreover, in group 3 after blood transfusion, levels of H_2O_2 and MDA were decreased but remained high compared to controls (66.55 ± 22.49, $p < 0.001$), (47.18 ± 9.62, $p < 0.001$) sequentially (Table I).

This high significant difference between studied groups was shown in (Figure 1).

Also, there was a negative correlation between GSH that acts as antioxidant defense enzyme and each one of the oxidative stress parameters MDA & H_2O_2 (Figures 2 and 3). However, there was a positive correlation between H_2O_2 and MDA (Figure 4). These correlations were significant at the (0.01 and 0.05 level) as shown in (Table II).

Discussion

Oxidative stress results from a disturbance in the balance between the production of Reactive Oxygen Species (ROS) and the antioxidant defense efficiency. Furthermore, oxidative stress occurs if excessive production of ROS overcomes the antioxidant defense system or when there is a significant decrease of antioxidant defense[19]. Free radicals attack potential biological targets such as lipids, proteins and nucleic acids[20]. The epoxides are formed as the result of an increase in oxidative stress. These epoxides

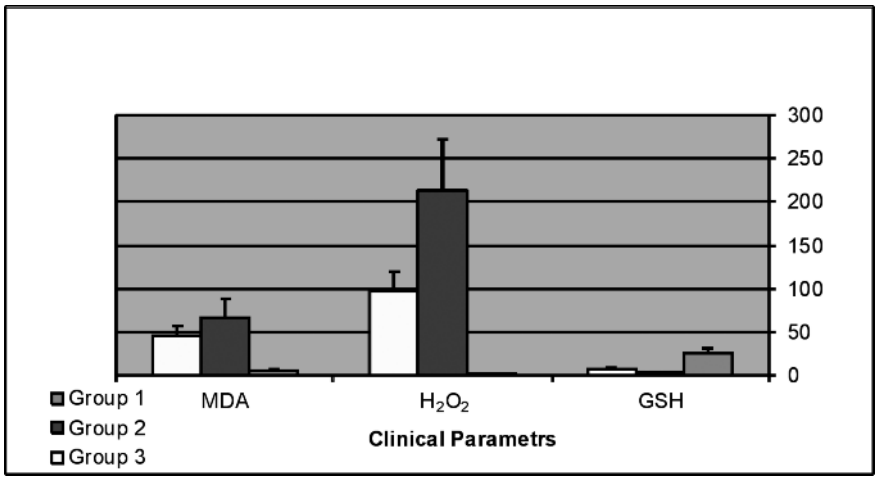

Figure 1. MDA, H_2O_2 and GSH levels in studied groups.

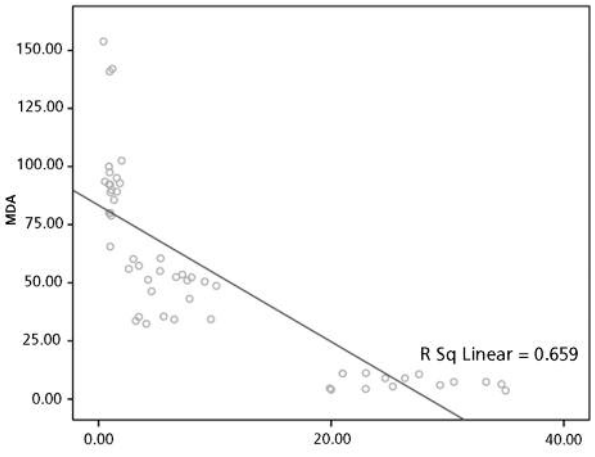

Figure 2. The Pearson negative correlation between GSH and MDA.

may spontaneously react with the nucleophilic centers present in the cell and, thereby, covalently bind to DNA, RNA and protein. These reactions leading to carcinogenicity and cytotoxicity are dependent on the properties of the epoxides[21].

This present study showed that the patients with favism had significantly low level of Reduced Glutathione (GSH), (26.31 ± 5.26, $p < 0.001$) in comparison to those of healthy controls (26.31 ± 5.26, $p < 0.001$), (Figure 1). These findings are consistent with the results obtained by some investigators all over the world[22,23], where it has been established that, the glycosides vicine and convicine play a role in generating the damage and ultimate lysis of the affected red cells. Actually, the latter substances represent about the 0.5% (w/w) of the bean seeds and can produce both *in vivo* and *in vitro,* aglycones (divicine and isouramil, respectively) which have been reported to produce in G6PD-deficient red blood cells a rapid decline of reduced glutathione (GSH)[24].

As G6PD is crucial in maintaining redox balance and detoxification of ROS, it is possible that G6PD deficiency retards the antioxidant defense, leading to oxidative damage and, therefore, cellular senescence[24]. Moreover, G6PD-deficient cells display increased tendency for H_2O_2-induced senescence[25]. Consequently, favism patients in the present study had high significant levels of H_2O_2 (213.49 ± 57.56, $p < 0.001$), (Table I), even after blood transfusion (66.55 ± 22.49, $p < 0.001$) when compared to controls (3.75 ± 1.164, $p < 0.001$), (Figure 1), which may lead to conclude that these favism patients are more vulnerable for H_2O_2-induced senescence. In other *in vitro* studies it was reported that high levels of H_2O_2 cause damage to lipids, proteins, mitochondrial DNA and genomic DNA in a relatively undiscriminating manner. The buildup of this damage cripples the ability of cells to grow, provoking senescence[26].

Furthermore, severe oxidative stress is not only known to be the reason for DNA damage and mutations of tumor suppressor genes, which are known to be the initial events in carcinogenesis [19], but can also play a key role in the promotion of multistep carcinogenesis[27]. Lipids, especially polyunsaturated fatty acids (PUFA), are very vulnerable to free radical attack, which can result in lipid peroxidation[28]. Lipid peroxidation plays a vital role in the control of cell division[29]. Malon-

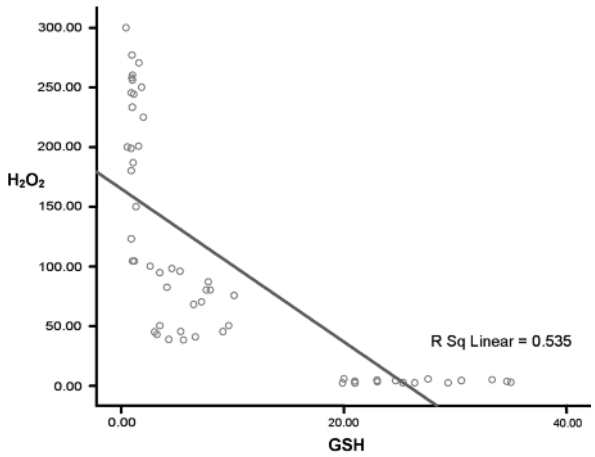

Figure 3. The Pearson negative correlation between GSH and H_2O_2.

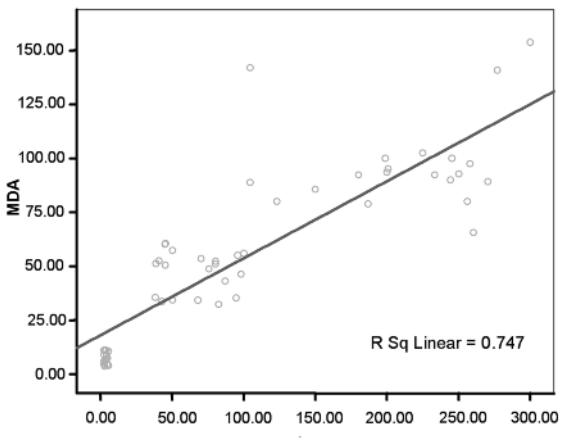

Figure 4. The Pearson positive correlation between MDA and H_2O_2.

Table II. Correlations between studied groups

		group	GSH	H_2O_2	MDA
group	Pearson Correlation	1	-.693(**)	.185	.329(*)
	Sig. (2-tailed)		.000	.177	.014
	N	55	55	55	55
GSH	Pearson Correlation	-.693(**)	1	-.731(**)	-.812(**)
	Sig. (2-tailed)	.000		.000	.000
	N	55	55	55	55
H_2O_2	Pearson Correlation	.185	-.731(**)	1	.865(**)
	Sig. (2-tailed)	.177	.000		.000
	N	55	55	55	55
MDA	Pearson Correlation	.329(*)	-.812(**)	.865(**)	1
	Sig. (2-tailed)	.014	.000	.000	
	N	55	55	55	55

** Pearson Correlation is significant at the 0.01 level (2-tailed). *Pearson Correlation is significant at the 0.05 level (2-tailed).

dialdehyde (MDA); the end product of lipid peroxidation, is suggested to act as a tumor promoter and a cocarcinogenic agent due to its high cytotoxicity and inhibitory action on protective enzymes[30]. In our study, it was found that the favic patients had significant high levels of MDA (98.05 ± 22.34, $p < 0.001$), even after blood transfusion (47.18 ± 9.62, $p < 0.001$), when compared to controls (7.38 ± 2.07, $p < 0.001$) (Figure 1), thus the cell growth of these patients will be affected with passage of time. These results are in accordance with the reports which have referred to fatty acid composition of red cells deficient in G6PD and their susceptibility to lipid peroxidation[30]. In addition, these reports have demonstrated that the red cell sensitivity to lipid per oxidation was found to be higher in patients with glucose-6- phosphate dehydrogenase deficiency than in normal subjects[31]. This may indicate the presence of increased oxidative stress. Increase in MDA levels could be caused by a rise in the generation of reactive oxygen species (ROS) due to the excessive oxidative damage generated in these patients produced from fava beans components divicine and isouramil.

In our study, it was also found that there was a negative correlation between GSH and MDA (Figure 2), in other reports it was stated that GSH is necessary for the activity of glutathione peroxidase (GPx) which efficiently protects the cell membrane from lipid peroxidation and catalyzes the reaction of hydro peroxides with GSH to form GSSG[32].

Moreover, it was found that there was another negative correlation between GSH and H_2O_2 (Figure 3), this finding is consistent with other *in vitro* reports which described that the decrease of GSH in RBCs of aged rats could be the reason for the inhibited activities of GPx and glutathione S transferase (GST), since these enzymes act only in the expense of GSH[32], as glutathione is a reducing agent because of its sulphydryl groups against oxidative stress, depletion of glutathione causes a proportional decrease of GPx activity. Reduced glutathione (GSH) is the main non-enzymatic antioxidant defense within the cell, reducing different peroxides, hydroperoxides and radicals (alkyl, alkoxyl, peroxyl, etc.)[33]. Thus, it is usually assumed that GSH depletion reflects intracellular oxidation.

It has been reported that divicine and isouramil react with oxygen to produce H_2O_2, and this reaction can be maintained as a redox cycle in the presence of reduced glutathione, which regenerates the reduced, autoxidizable pyrimidine form[34]. This mechanism can be responsible for the observed effect on sensitive red blood cells.

In other works it was found that, an acute hemolytic anemia can occur in some dehydrogenase-deficient subjects as a result of administra-

tion of exogenous substances other than fava beans[35], particularly hemolytic drugs[36]. These compounds are likely to interact with oxygen, or, in the red cell, with oxyhemoglobin, and eventually produce H_2O_2, *via* O_2^-, as intermediate[37,38].

The present study showed a positive correlation between H_2O_2 and MDA (Figure 4), where as H_2O_2 level increased MDA would be increased consequently. Other investigators have reported, that when RBCs are incubated with high concentration of H_2O_2 in the presence of a catalase inhibitor (Sodium Azide), membrane lipids are slowly and partly oxidized[39]. The result of that lipid peroxidation usually MDA, which is one of thiobarbituric acid (TBA) reactive species[30].

Conclusions

Favic patients had increased oxidative stress parameters, (H_2O_2 and MDA) more than normal individuals and less antioxidant defense enzyme (GSH). With the passage of time, these individuals· cells would be more susceptible to H_2O_2 induced senescence. The implication of oxidative stress in the etiology of several chronic and acute degenerative disorders suggests that antioxidant therapy represents a promising avenue for the treatment. Hence, it is recommended for these patients to take dietary antioxidant factors from food and medicinal plants.

Therefore, according to the significant correlations obtained between these parameters and favism, it is recommended that these parameters may be used as a biochemical marker for this disease.

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Conflict of Interest

None declared.

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