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Original paper

Evaluation of G6PD deficiency in malaria patients in the south-east of Iran

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ABSTRACT. Glucose phosphate dehydrogenasis (G6PD), the most prevalent enzymatic disease in humans, exists in south-eastern Iran. The geographic correlation of its distribution with the historic malaria endemic suggests that G6PD has increased in frequency as a result of natural selection by malaria. Based on studies, there is a controversy in terms of different analytical methods in terms of resistance to malaria. Fifty malaria patients and 50 healthy individuals from several cities south-east of Iran were included in the study and after obtaining consent, blood samples were taken from them. G6PD enzyme deficiency was investigated using a fluorescent stain test. The age, gender, and nationality of malaria patients were also assessed. The results were analyzed using SPSS software and appropriate statistical tests, and the value of $P<0.05$ was considered significant. The results showed that in malaria patients only one person had G6PD deficiency, while this number was six in the control group, which is significantly higher than the malaria group ($P<0.05$). Age group 27–42 years, men and people with Iranian citizenship also showed the highest incidence of malaria. Based on the results, it can be concluded that G6PD enzyme deficiency causes resistance to malaria and the frequency of this enzyme deficiency in malaria patients is significantly lower than in other people.

Keywords: malaria, enzyme deficiency, glucose 6-phosphate dehydrogenase, *Plasmodium*

Introduction

Lack or reduction of glucose 6-phosphate dehydrogenase (G6PD) is one of the most common enzyme deficiencies in humans. This enzyme is one of the most important enzymes in the human body that different cells, including red blood cells, have different amounts of it [1].

A lack of this enzyme can lead to problems such as mental retardation, kidney failure, neonatal jaundice, liver disease, and chronic anaemia. Children or adults with enzyme deficiency develop severe, life-threatening haemolytic attacks from certain substances, such as antimalarial drugs, oxidants [2,3]. According to available statistics, about 400 million people worldwide are affected by this complication [1,2]. G6PD deficiency varies greatly in diversity and dispersion. Approximately 7.5% of the world's population carries one or two defective G6PD genes. The prevalence in Jewish groups is about 70% [1,4]. Neonatal screening for G6PD deficiency, together with a comprehensive education programme, is advisable in those parts of the world where the severe variant of G6PD deficiency is prevalent [3]. The lowest prevalence of deficiency is reported in the Japanese and about 1% [1]. Prevalence of G6PD in Arak City showed 2.2% which is lower than that of north our country (8.7%) as well as lower than another part of the world (10–14.9%) [5]. According to the statistics of the World Health Organization, enzyme deficiency in Iran is between 10 and 14.9% [6].

Malaria is an acute and chronic infection characterized by chills, fever, and sometimes severe and fatal complications [7]. It is one of the most important infectious diseases in the tropics and subtropics, affecting approximately 515 million people and killing between one and three million people, the majority of whom are young children in sub-Saharan Africa [8]. The disease is caused by a

protozoan of the genus *Plasmodium* [9]. *Plasmodium vivax* is one of the major causes of severe malaria [10,11] and has become resistant to chloroquine and other drugs [12,13]. *Plasmodium falciparum* malaria is the most malignant type of malaria [14] and is responsible for severe malaria and mortality in humans [15]. More than 91% of malaria cases are reported from three southeastern provinces and Sistan and Baluchistan provinces have a first place [16].

There have been worldwide studies linking genetic blood diseases to malaria, one of which is G6PD deficiency. According to the results of studies, people with G6PD deficiency are more resistant to malaria and the rate of parasitic density in these people is less than people without this disorder and the fatality rate of the disease was lower in these people [17] . Previous clinical studies on G6PD and malaria have attempted to define different genotypes through phenotypic measurements of enzymatic levels, electrophoretic mobility [18–20]. Here we argue that the most common monogenetic condition in humans, namely glucose-6-phosphate dehydrogenase (G6PD) deficiency, which developed under the pressure of malaria, have survived. Selection reinforced by inbred marriages in malaria-affected parts of the world. Previous studies have shown that hemoglobinopathies and G6PD deficiency are positively selected against *Plasmodium falciparum* malaria.

People with this genetic defect (G6PD) look normal until they are exposed to the oxidative stress that causes hemolysis. The consumption of certain foods and the use of certain medications such as primaquine, sulfamides, etc. can lead to CBR lysis in G6PD deficient individuals [21].

On the other hand, difficulties in diagnosing a glucose-6-phosphate dehydrogenase (G6PDd) deficiency greatly hamper the widespread use of primaquine, because this common genetic disorder leaves patients vulnerable to potentially severe and fatal primaquine-induced hemolysis. The risk of this outcome varies widely between variants of the G6PD gene [22].

In the study of Ebrahimipour et al. [23] conducted in Bandar Abbas to investigate the relationship between G6PD and malaria deficiency, the prevalence of G6PD deficiency was estimated to be 8.3% in malaria patients and 24.2% in healthy individuals, which can be deduced. Due to the resistance of people with G6PD enzyme deficiency to malaria and their lower incidence of malaria, the prevalence of this defect is higher in healthy individuals.

Other studies have shown that people with this defect are less likely to develop vivax malaria [23]. Also, people with homozygous G6PD deficiency are more resistant to heterozygous malaria and less likely to develop fatal malaria [24]. A study conducted by Guindo et al. [24] in Africa on children under the age of five found that children with G6PD were less likely to develop severe malaria and that boys with homozygous children were less likely to develop malaria. Malaria parasitic density was much lower than female children and children with heterozygous disease, which was significant. One hundred and sixty G6PD gene mutations, which lead to amino acid substitutions, have been described worldwide [25].

In some studies, no significant differences were observed between the patients' age, nationality or G6PD status in patients with a G6PD deficiency and patients without a G6PD deficiency. A significant association was observed between gender and G6PD status in patients with malaria with the incidence of G6PD deficiency being higher in males than in females [26]

Recent findings from important case-control studies in East and West Africa demonstrate that the G6PD deficiency variant is associated with a significantly reduced risk of severe malaria [19]. In the natural selection of malaria in people lacking the G6PD enzyme, it causes resistance and, therefore, this genetic defect (gene pool) increases in societies [19].

The present study aims to determine the incidence of G6PD deficiency in patients referred to the health centers in the Sistan and Baluchistan provinces who are infected with malaria.

Materials and Methods

This cross-sectional descriptive and case-control study was performed on 50 patients referred to medical centers in Zabol, Khash, Zahedan, and Sravan in Sistan and Baluchistan province (south-east of Iran) between 2020 and 2021 in which malaria was diagnosed (Fig. 1). Also, 50 healthy people who did not have malaria were used as controls. It should be noted that the number of malaria cases may have been higher, but due to the coronavirus epidemic, fewer visits to medical centers have been made. Healthy individuals were selected and surveyed in the same number of areas.

Figure 1. Sites of samples collection in Sistan and Baluchestan provinces

Sampling

Two (2) ml of blood was collected from definite patients with malaria as well as healthy controls and transferred to the laboratory in a vial containing EDTA and kept at 20°C until the tests.

Evaluation of glucose 6-phosphate dehydrogenase activity

The fluorescent G6PD enzyme staining test method in a suitable buffer environment reduces NADP and converts it to NADPH. NADPH produced under ultraviolet (UV) light with a wavelength of 365 nm generates fluorescence. The intensity of fluorescence is high in the blood of healthy people and low or negative in the blood of people with G6PD deficiency. Fluorescent light reflectance and enzymatic activity of G6PD can be divided into three parts based on this; (1) strong fluorescence means sufficient enzyme activity, (2) weak fluorescence means relative lack of enzyme activity (partially deficient), and (3) negative fluorescence means very weak enzyme activity (severely deficient). Normal blood samples with more than 80% of healthy red blood cells have strong fluorescence and samples with less than 40% of healthy red blood cells do not have fluorescence. In the intermediate range, the fluorescence intensity will be different. In terms of enzymatic activity, samples with activity equal to 9 units per gram of haemoglobin or more have a strong fluorescence, and samples with less than 3 units per gram of haemoglobin will have no fluorescence. To evaluate the activity of the G6PD enzyme in the present experiment, one ml of blood samples was mixed with 100 microlitres of G6PD reagent, and after 10 minutes of incubation at room temperature, 20 microlitres of the sample were prepared on Whatman No. 1 paper which was in the kit, was transferred. After drying the sample, it was examined under UV light, and based on its fluorescence, the activity status of the G6PD enzyme was determined (Fig. 2).

Determination of Plasmodium type in G6PD positive individuals

Plasmodium parasite was identified in malaria patients who tested positive for G6PD. The easiest way to diagnose the parasite is to look at a blood sample directly under a microscope to see the

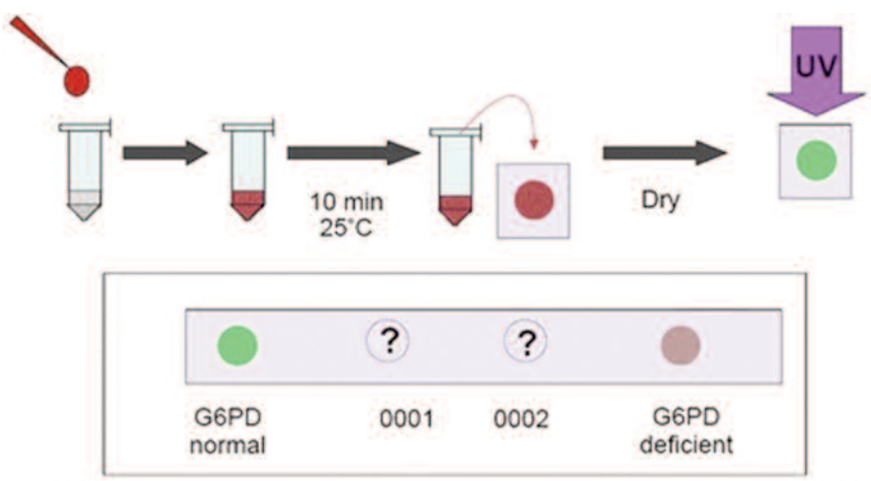

Figure 2. Schematic of the test procedure to evaluate the activity of G6PD enzyme

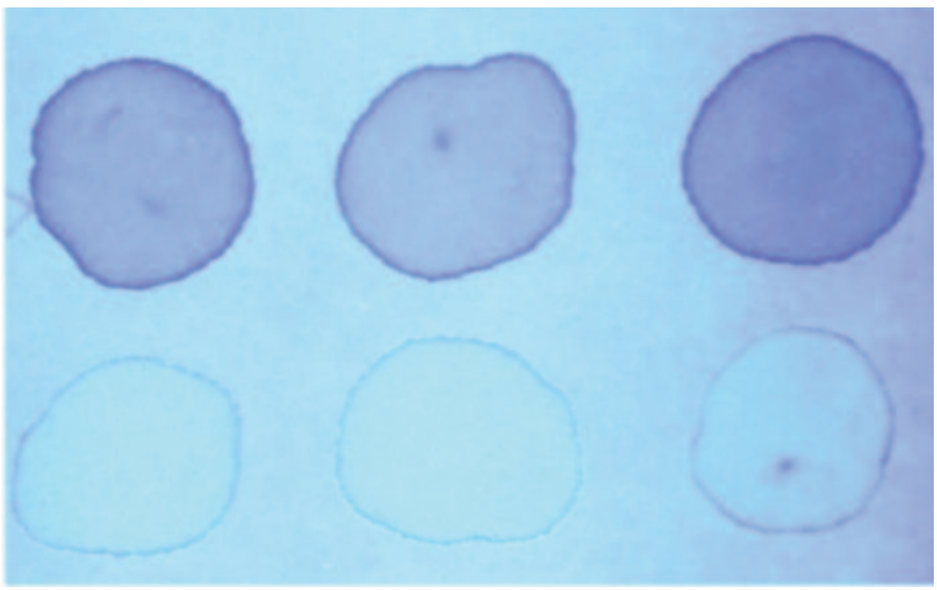

Figure 3. Examination of samples under UV light. The top row indicates cases with a G6PD enzyme defect and the bottom row indicates cases without a G6PD defect

malaria parasite, which is still the gold standard for diagnosing malaria. Microscopic examination based on thick and thin smear for diagnosis of malaria achieved. The blood was organized in ways, such as a thick blood smear, and a thin blood smear. For a thick blood smear, a drop (about 6–10 ml) was spread on a clean, dry microscope slide. After drying, the thick blood smear was stained with a Giemsa stain. To do this, a drop of blood was stirred in a circular motion with the corners of the slide. Care should be taken not to allow the sample to become too thick during preparation, and it should be allowed to dry without the addition of fixatives. Because the red blood cells are not fixed, they are lysed using water droplets.

In a thin blood smear, the smears were air-dried and fixed with methanol and examined under a microscope. In a thin blood smear, we used a smaller volume (approximately 2 ml) of blood spread into a monolayer in the preparation of a traditional blood smear and briefly immersed in methanol the erythrocytes were fixed and were not subsequently lyse during staining [27,28]. Microscopic diagnosis of malaria by staining the spread of blood on a glass slide leads to the appearance of a malaria parasite.

The statistical analysis

The data obtained from the present study were analysed using SPSS software version 26. The normality of the data was assessed by Shapiro ilk and Kolmogorov-Smirnov tests. Then they were evaluated by chi-square test. A *P*-value less than 0.05 was considered as a significant level.

Results

Demographic characteristics of the participants in the experiment, demographic characteristics of the participants in the experiment, including their gender, age, and eligibility, are shown in table 1. Attempts were made to make the control samples

Table 1. Demographic characteristics of the participants in the experiment

Selected matching	Participants number	Gender and age		Citizenship	
		Man	Women	Iranian	Foreigner
Patient (case)	50	38	12	39	11
Healthy (control)	50	41	9	41	9
P-value		0/93	0/59	0/64	0/77

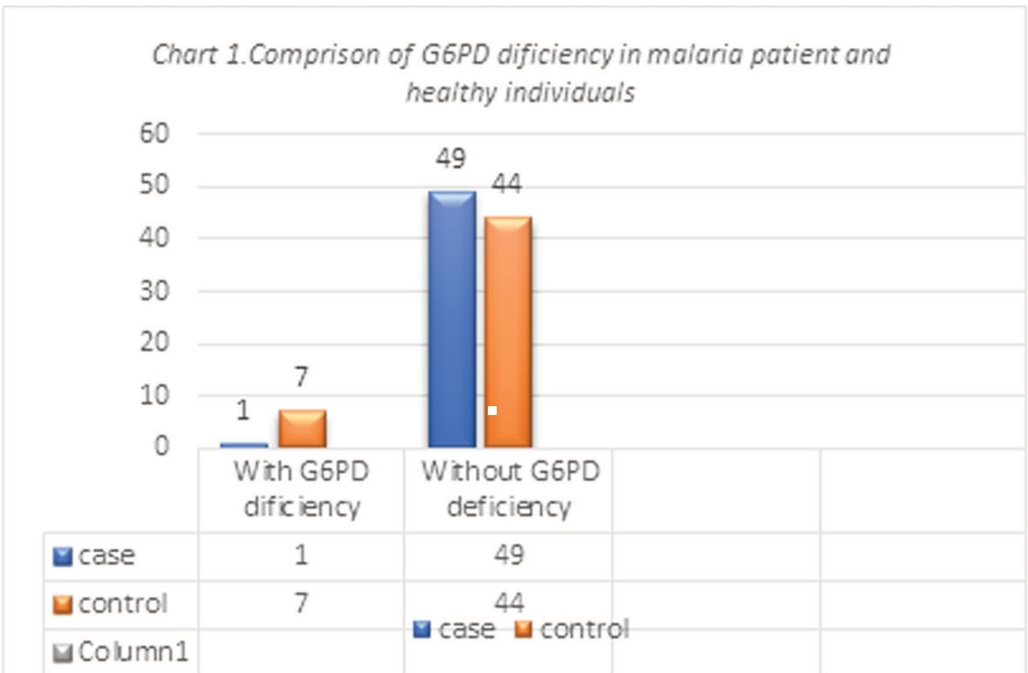

Figure 4. Comparison of G6PD deficiency in malaria patients and healthy individuals Chi-square:73.960;df:1, Asymp.Sig.:.001

similar to the patient samples in terms of demographic characteristics and, as can be seen in the table, the *P*-value for all demographic factors was more than 0.05, which indicates that there is no significant difference between the patient and control groups in terms of demographic characteristics and homogeneity of the study population.

Table 2. Results of statistical analysis with ANOVA and Tukey test to compare the prevalence of malaria in different age groups

Descriptives

Age

	N	Mean	SD	SE	95% CI for mean		Min	Max
					Lower bound	Upper bound		
10–26	17	19.29	3.981	.965	17.25	21.34	11	26
27–42	21	35.62	4.801	1.048	33.43	37.80	27	42
43–60	12	52.33	5.710	1.648	48.71	55.96	43	60
Total	50	34.08	13.430	1.899	30.26	37.90	11	60

ANOVA

Age

	Sum of squares	df	Mean square	F	Sig.
Between groups	7764.532	2	3882.266	170.029	.000
Within groups	1073.148	47	22.833		
Total	8837.680	49			

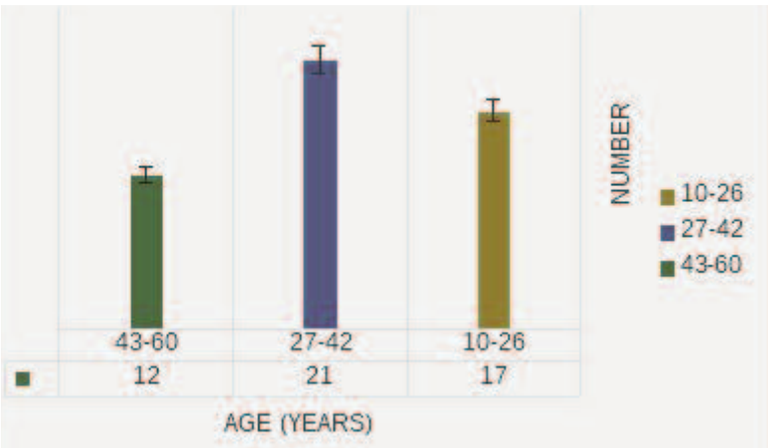

Figure 5. Comparison of the prevalence of malaria in the three age groups studied

The activity of the G6PD enzyme in the subjects was determined by examining Whatman paper under UV light. The activity of enzyme glucose 6 phosphate dehydrogenase was determined for each individual. Figure 3 shows an example of a positive case for a G6PD defect (top row) and a negative case for a G6PD defect (bottom row).The frequency of G6PD enzyme deficiency in malaria patients was compared with controls in patients without malaria (Fig. 4). In malaria patients, only one in 2% of patients had a G6PD deficiency, while in healthy individuals, 7 (14%) had a G6PD deficiency. Statistical analysis of these data using the chi-square test showed that there was a significant difference between healthy individuals and malaria patients in terms of the frequency of defects in G6PD (P=0.001).

Frequency comparison of age groups in malaria patients

Malaria patients were divided into three age groups including 10–26, 27–42 and 43–60 years, and were compared. ANOVA test and Tukey post hoc test showed that there was a significant difference between the three age groups in terms of frequency of malaria (P=0.000) and the highest frequency was related to group 27–42 years (Fig. 5 and Tab. 2).

Comparison of sex frequency in malaria patients

To compare the frequency of different sexes in malaria patients, a Chi-square test was used and the results showed that the frequency of this disease in men is significantly higher than in women (P=0.000) (Fig. 6).

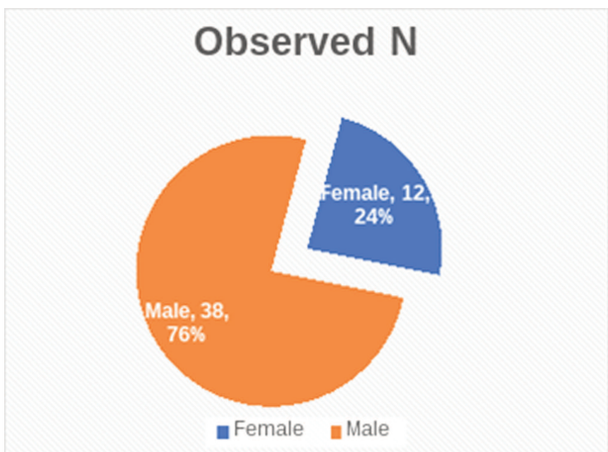

Figure 6. Comparison of the prevalence of malaria in different sexes

Comparison of the frequency of citizenship in patients with malaria Chi-square test results showed that Iranian patients were significantly more than patients with non-Iranian citizenship (P=0.001) (Fig. 7), which of course can be due to the predominance of the general population. Be more Iranian than non-Iranian.

A blood smear examination under a microscope showed that the patients were infected with *Plasmodium vivax* (46 cases) and *P. falciparum* (4 cases).

Discussion

G6PD is one of the most important enzymes in the human body that functions in redox reactions and detoxification of oxidizing agents. G6PD deficiency is one of the most common enzyme deficiencies worldwide, affecting approximately

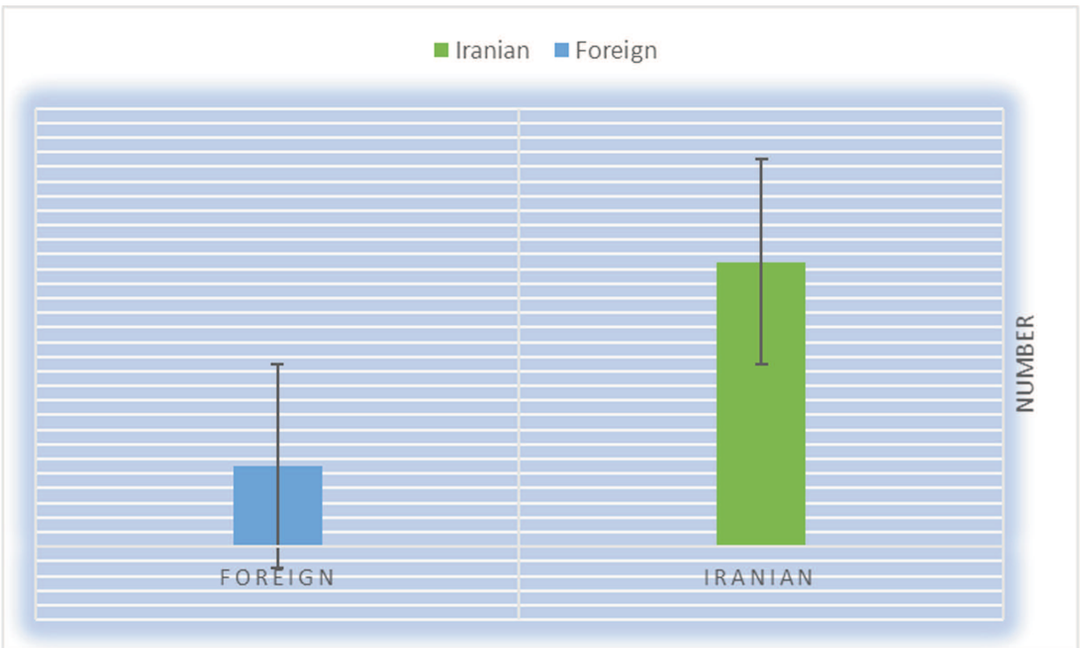

Figure 7. Comparison of the prevalence of malaria in different nationalities

400 million people. G6PD deficiency can cause a variety of clinical complications, including erythrocyte hemolysis, elevated bilirubin, and haemolytic anaemia.

The international occurrence of G6PD deficiency is geographically correlated with regions inhabited with the aid of using populations traditionally exposed to endemic malaria, which includes Africa, Mediterranean Europe, South-East Asia, and Latin America. In the United States, for example, the enzyme deficiency primarily affects populations of African and Mediterranean descent [29]. Some studies have reported incidences in other parts of Iran, and prevalence varies significantly from state to state. These surveys found high rates of 8.65% to 16.4% in the north (Mazandaran and Gilan), 12% in the south (Shiraz), and 19.3% in southeastern Iran. The first and most common variant of the Kurdish population in northern Iran's Caspian coast and western Iran is the G6PD Mediterranean [30]. One of the regions with a high prevalence of G6PD deficiency in Iran is Sistan and Baluchistan province. Studies on the association of genetic blood diseases with malaria show that G6PD deficiency is also associated with malaria, and people with G6PD deficiency are more resistant to malaria. Accordingly, this study aimed to evaluate G6PD enzyme deficiency in malaria. patients in Sistan and Baluchistan province and compare it with this enzyme deficiency in healthy individuals. The results showed that in malaria patients only one person had G6PD enzyme deficiency while in the control group 6 cases had G6PD deficiency. Predominantly, our study indicates that people with G6PD deficiency are more resistant to malaria patients.

For further investigation, G6PD deficiency was also assessed in healthy individuals for malaria. The

Table 3. Results of statistical analysis with Chi-square test to compare the prevalence of malaria in people with Iranian and non-Iranian citizenship

	Observed N	Expected N	Residual	Test statistics	
Iranian	39	25.0	15.0		Nationality
Foreign	11	25.0	–15.0	Chi-square	18.000
Total	50			df	1
				Asymp. Sig.	.000

results showed that 14% of the control group who did not have malaria had G6PD deficiency. Therefore, the frequency of G6PD deficiency in the control group was similar to the rate reported for the country, especially in Sistan and Baluchistan provinces, while in malaria patients only 2% of individuals had G6PD enzyme deficiency.

In a study by Ebrahimpour et al. [23] conducted in Bandar Abbas to investigate the relationship between G6PD and malaria deficiency, the prevalence of G6PD deficiency was estimated to be 8.3% in malaria patients and 24.2% in healthy individuals. It can be inferred that due to the resistance of people with G6PD enzyme deficiency to malaria and their lower incidence of malaria, the prevalence of this defect is higher in healthy individuals.

Daliri et al. [31], investigated the incidence of G6PD deficiency in malaria-prone areas of Fars province. The incidence of G6PD deficiency in all neonates was estimated to be 58.15%. The incidence of this defect in malaria-prone areas was much higher than in other areas. The higher the severity of malaria in the region, the more common the incidence of defects, which was significant. The findings of this experiment also indicated the role of natural selection, which made people with G6PD deficiency in malaria-prone areas more resilient [24].

These findings are consistent with the results of the present experiment and our results showed a high frequency of G6PD deficiency in healthy people in the community and also a significant reduction in this frequency in people with malaria.

In terms of the mechanism involved in the association between G6PD deficiency and malaria resistance, it is stated that in this deficiency, the malaria parasite is unable to survive in the host red blood cells. To better understand the mechanism of action of anti-malarial drugs. Dehydroepiandrosterone (DHEA) as a non-competitive mammalian G6PD inhibitor can inhibit G6PD activity in healthy red blood cells [32]. In terms of the mechanism involved in the association between G6PD deficiency and malaria resistance, it is stated that in this deficiency, the malaria parasite is unable to survive in the host red blood cells.

This does not directly affect the activity of NADPH oxidase but can inhibit the activity of G6PD by binding to the triple complexes of the coenzyme substrate [33]. As expected, G6PD activity is reduced by DHEA, and the spread of the *Plasmodium* parasite is inhibited. In the erythrocyte stage, the malaria parasite is synthesized nucleotides and is exposed to endogenously produced oxidative radicals that must be detoxified. Like other cells, the parasite needs not only energy (ATP) but also reduced NADPH to perform its anabolism. NADPH can be produced during the oxidation of glucose 6-phosphate via the pentose phosphate pathway. This pathway also produces ribose 5-phosphate, the sugar component of nucleic acids [34].

Therefore, when we use DHEA to inhibit G6PD activity in red blood cells, *Plasmodium* cannot obtain sufficient ATP and NADPH to complete the erythrocyte stage. As a result, the parasite's cell nucleus shrinks, and cellular organelles and metabolites gradually decrease with increasing DHEA concentration. In people with G6PD deficiency without the need for drugs such as DHEA, congenital defects of this enzyme have a mechanism similar to that described, thereby counteracting the spread of the malaria parasite [35]. In terms of the prevalence of malaria in age groups, it was shown that the highest frequency is related to the 27–42 age group. In this regard, Zia Sheikholeslami and Rezaian [36] studied the epidemiology of malaria in one of the cities of Kerman province and stated that the most affected age group was 20 to 29 years.

In Mazandaran, Najafi et al. [37] by examining the epidemiology of malaria during 2003, showed that the frequency of this disease in the age group of 20–30 years is higher than others. These findings are consistent with the results of the present experiment. However, because malaria in the region is unstable and caused by *Plasmodium vivax*, it seems that all age groups are at risk.

The study of malaria in different sexes showed that the frequency of this disease in men is significantly higher than in women so 76% of the patients were men. Zia Sheikholeslami and Rezaian [36] reported that the number of men with malaria was 92.3%. Najafi et al. [37] also reported this amount as 88.4%. Despite the different percentages in different studies, they all had in common that malaria was more common in men than women. Of course, gender does not directly play a role in the development of malaria, and the difference observed may be related to the type of clothing and the greater presence of men outside the home.

Finally, the nationality of the patients was examined and the results showed that 78% of the patients are Iranian and the rest are non-Iranian. Purrastgu-Haghi et al. [38] stated that 90.4% of the

patients studied were Iranian. On the other hand, Zia Sheikholeslami and Rezaian [36], 98.9% of malaria patients were Afghan. Najafi et al. [37] also considered that 80.3% of the patients belonged to non-Iranian (Afghan) people. The observed differences can be related to the study area and the conditions of non-Iranian citizenship in these areas. Also, with the strictness and monitoring of the entry of immigrants, the number of these people in the southern regions of the country have decreased significantly compared to previous years, which justifies the observed differences that can be changed.

In the present study, it was shown that G6PD deficiency can lead to resistance to malaria, and in malaria patients, it was observed that only one person had G6PD deficiency compared to the control group, which represents the general population, was significantly less. On the other hand, the incidence of malaria in the age group of 27–42 years, men and people with Iranian citizenship showed the highest frequency. Continuous monitoring of malaria epidemiology can help manage and control the disease. It also seems that natural selection can overcome malaria, in the long run, to increase the survival and prevalence of people with G6PD deficiency, especially in malaria-prone areas, which can be problematic in itself. Therefore, malaria control can also prevent the increase in the frequency of G6PD defects.

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