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ARAŞTIRMA / RESEARCH

Evaluation of the effect of glyphosate on glucose-6-phosphate dehydrogenase enzyme activity in vitro conditions

Glifosatın glukoz-6-fosfat dehidrogenaz enzim aktivitesi üzerindeki etkisinin in vitro koşullarda değerlendirilmesi

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Abstract

Purpose: The aim of this study was to investigate *in vitro* effect of glyphosate on Glucose 6-phosphate dehydrogenase (G6PD) enzyme activity.

Materials and Methods: In terms of G6PD enzyme deficiency, samples taken from healthy and enzyme deficient male individuals were studied. After the hemolysates were prepared from blood sample, G6PD enyzme activities were determined by the modified Beutler method. Then, the effects of different concentrations ($5.3x10^{-3}$, $5.3x10^{-4}$, $5.3x10^{-5}$, $5.3x10^{-6}$ mmol/mL) of glyphosate on G6PD activity were evaluated in normal and mutant enzymes. In addition, the *in vitro* effect of the antioxidant N-acetylcysteine (NAC) on the enzyme was investigated in the presence of glyphosate and without glyphosate.

Results: While the result of normal erythrocyte G6PD activity was 12U/g for the individual, the result for the individual with enzyme deficiency was 2.5U/g Hb. The glyphosate's maximum activity loss in the G6PD enzyme was observed in the 60th minute incubation. The highest inhibition was observed at $5.3x10^{-3}$ mmol/mL glyphosate. $4.7x10^{-7}$ mmol/mL N-Acetylcysteine partially increased the inhibition of glyphosate in the G6PD enzyme in healthy individuals, but had no effect on mutant G6PD.

Conclusion: In humans, it is predicted that glyphosate affects G6PD enzyme activity *in vitro* and is an interference agent in the experimental process. In case of contamination, studies on limits of glyphosate that will not cause harmful effects in humans should be continued.

Keywords: Glyphosate, G6PD enzyme, inhibition, N-acetylcysteine.

Öz

Amaç: Bu çalışmada, substrat Glukoz-6-fosfat (G6P) ile moleküler yapı benzerliği ve enzimin kofaktör olarak Mg^{+2} kullanması nedeniyle, eritrosit G6PD enzimi üzerindeki *in vitro* etkisi incelendi.

Gereç ve Yöntem: Çalışmada sağlıklı ve G6PD enzim eksikliği olan erkek bireylerin kan örneklerinden yararlanıldı. Hazırlanan hemolizatların enzim aktiviteleri modifiye Beutler yöntemi ile ölçüldükten sonra deney ortamına farklı derişimlerde ($5.3x10^{-3}$, $5.3x10^{-4}$, $5.3x10^{-5}$, $5.3x10^{-6}$ mmol/mL) glifosat eklenerek aktivite üzerindeki etkisi değerlendirildi. Daha sonra antioksidan N-asetilsisteinin normal ve mutant enzimler üzerindeki etkisi *in vitro* deney koşullarında glifosatlı ve glifosatsız ortamda karşılaştırıldı.

Bulgular: Sağlıklı örnekte eritrosit G6PD aktivitesi 12, enzim eksikliği bulunan olguda ise 2.5 U/gr Hb olarak saptandı. Deney ortamına glifosat eklenmesinden sonra en yüksek aktivite kaybı 60. dakikada saptandı. En yüksek inhibisyon oranı ise $5.3x10^{-3}$ mmol/mL glifosatın etkilediği çalışma setinde gözlendi. $4.7x10^{-7}$ mmol/mL N-Asetilsistein, normal aktivitedeki G6PD enziminde glifosatın oluşturduğu inhibisyonu azalttı ancak mutant G6PD enzim aktivitesi üzerinde herhangi bir değişikliğe neden olmadı.

Sonuç: Bu çalışmayla glifosatın insanlarda *in vitro* olarak eritrosit G6PD enzimini inhibe ettiği gösterildi. İlaveten laboratuvar ortamında G6PD enzim aktivitesinin ölçüldüğü ve/veya kullanıldığı deneysel süreçte, negatif interferans ajanı olarak davrandığı gösterildi. Ayrıca bulaşı durumlarında glifosatın insanlarda zararlı etkiye neden olmayacak limit sınırları ile ilgili çalışmalar yapılmalıdır.

Anahtar kelimeler: Glifosat, G6PD enzimi, inhibisyon, N-asetilsistein

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INTRODUCTION

Glyphosate (N-(phosphonomethyl) glycine, trade name Roundup™) is a broad-spectrum organo-phosphate derivative herbicide used in agricultural industries around the world[1]. It has phosphate and glycine moieties in its molecular structure (Fig.1). Its herbicide feature was first demonstrated by Monsanto chemist John E. Franz in 1970[2]. The main purpose of glyphosate used in agriculture is to control cover crops and weeds. A reduced tillage system is also preferred to shorten the cost and product formation process by using it in seedbed preparation, stubble management, pre-harvest treatment for drying. It is also applied in pastures, horticulture, parks, and homes for perennial tree crops[3,4].

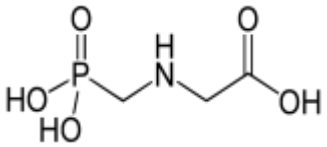

Figure 1. Chemical structure of glyphosate[5]

As weed control during the growing season becomes possible with glyphosate and genetically modified glyphosate-tolerant and resistant crops have begun to spread around the world, glyphosate consumption is increased tremendously[6]. As of 2014, over 800,000 tonnes of glyphosate per year were used in agriculture globally. Global consumption of glyphosate is expected to increase further, possibly reaching 1 million tonnes by 2023[7]. In the European Union, glyphosate has been approved until 2022, and re-evaluation procedures are ongoing[8]. While glyphosate was 300 tons in Turkey in 2001, it is estimated to reach 8,000 tons in 2019[9-12].

Glyphosate inhibits 5-enolpyruvylshikimate-3-phosphate synthase, a key enzyme of the shikimate pathway in plants (Fig.2)[13]. This enzyme is very important for the synthesis of aromatic amino acids, and its inhibition impairs protein biosynthesis, stops plant growth, and ultimately kills the plant[14]. Glyphosate has been reported to act by inhibiting enzymes, especially Manganese chelators[15]. The use of glyphosate for harvest aid causes high levels of glyphosate residues in crops, whereas pre-crop or post-harvest application of glyphosate less frequently results in detectable glyphosate levels in crops[16]. Due to its intensive use, glyphosate residues and its main product, aminomethylphosphonic acid (AMPA), have been observed in plants and soil, surface waters, and groundwater. Exposure of the general population to glyphosate primarily occurs through dietary intake of plant-based products, meat from livestock exposed to glyphosate, and contaminated drinking water[17].

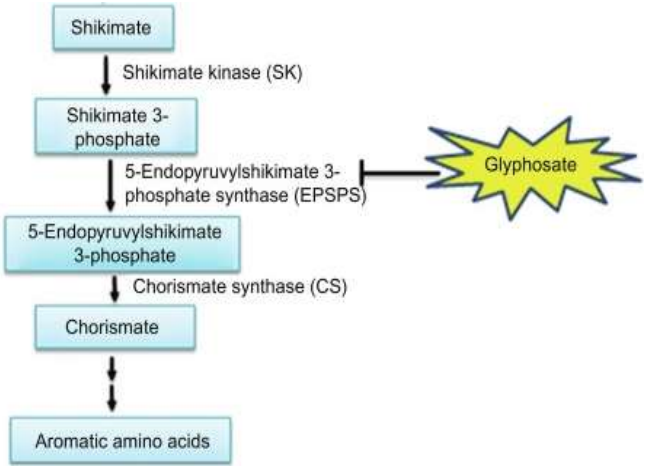

Figure 2. Shikimate pathway and glyphosate inhibition in plants[18]

Glucose-6-phosphate dehydrogenase (D-Glucose-6-phosphate: $NADP^+$ oxidoreductase, EC 1.1.1.49, G6PD) is a "housekeeping" enzyme that catalyzes the conversion of glucose-6-phosphate to 6-phosphoglucone-1,5-lactone by reduction of $NADP^+$[19]. This reaction is the first and rate-limiting

step of the Pentose-Phosphate pathway. The most important tasks of this pathway are to produce NADPH and pentose phosphate[20]. G6PD enzyme found in all tissues uses Mg^{+2} as a cofactor, and it can be found in dimeric, tetrameric, and hexameric structures. When the enzyme is dimeric, it is active, and each subunit is bound to 2 molecules of $NADP^+$ (Fig.3,4).

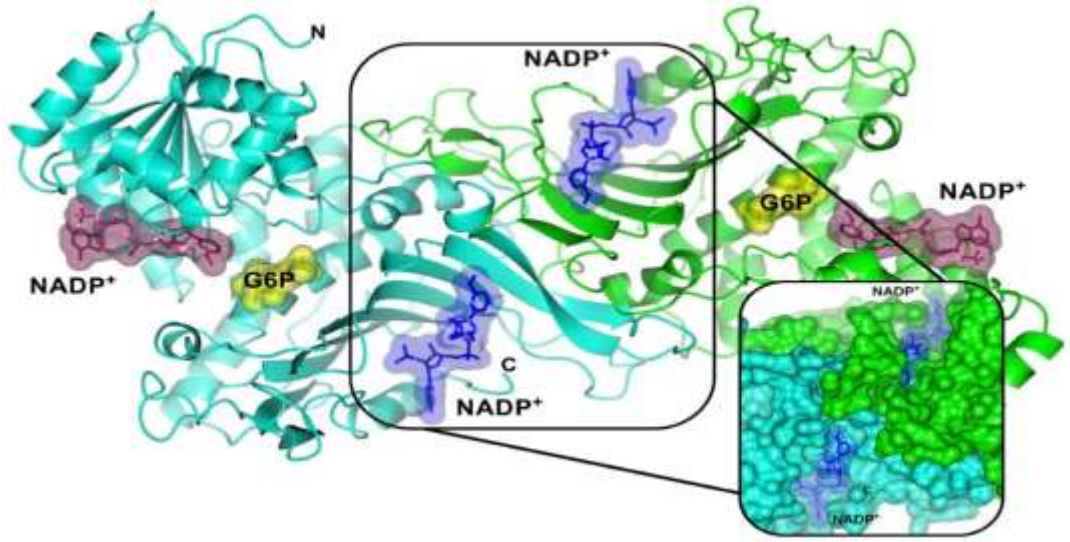

Figure 3. Molecular structure of the G6PD enzyme[21]

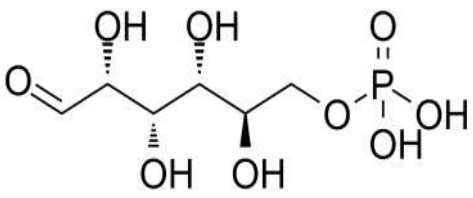

Figure 4. Chemical structure of glucose-6-phosphate[28].

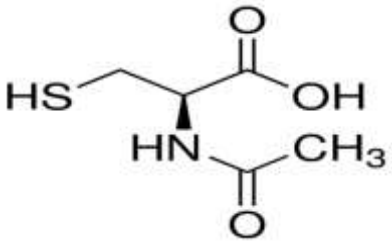

Figure 5. Chemical structure of N-acetylcysteine[29]

The most common enzyme deficiency globally is Glucose-6-phosphate dehydrogenase and affects more than 400 million people[22]. The incidence of G6PD deficiency is high in Africa, China, and the Mediterranean countries. While it is 0.5% in Turkey, it is 8.2% in the Cukurova region, and enzyme deficiency of up to 20% has been reported in some regions[23,24]. Enzyme deficiency shows its effects primarily in erythrocytes. Insufficient production of NADPH due to G6PD deficiency causes an increase in oxidized glutathione (GSSG) on exposure to oxidizing agents. It disrupts the membrane structure, causing premature destruction of erythrocytes and hemolysis in the spleen and liver[25].

Since G6PD deficiency is X-linked inherited, male individuals are more affected. The disease may present itself with different clinical findings such as acute hemolytic anemia, neonatal jaundice, favism, and hereditary non-spherocytic hemolytic anemia. Clinical findings are generally seen as a result of *Vicia faba* consumption, oxidant drugs, or infection[26]. The main function of N-acetylcysteine (NAC), which is

the acetylated derivative of cysteine amino acid, is to contain cysteine for reduced glutathione (GSH) synthesis and storage (Fig.5). In previous studies, the effect of the presence of NAC on the erythrocyte G6PD enzyme activity was investigated, and it was found that NAC had partial increase activity in healthy individuals ($G6PD^{B+}$) and 50% inhibitory effect in G6PD Mediterranean mutants[27].

There are very few studies on the effects of glyphosate, which is an important herbicide, and which the use of it increases by tons every year, on the activities of enzymes in metabolic pathways[30-32]. In this study, it is aimed to investigate the *in vitro* effect of glyphosate, on the G6PD enzyme due to its structural similarity with G6P substrate phosphate group and because the enzyme uses Mg^{2+} as a cofactor. In addition the effect of NAC on the inhibition of glyphosate was investigated because it is an important antioxidant and contains a free -SH group. In this study, we contributed to the literature regarding the concentration-dependent inhibition of glyphosate on erythrocyte G6PD enzyme activity and the possible effect of NAC, which is used as an antioxidant, on normal and mutant G6PD.

MATERIALS AND METHODS

Materials

D-Glucose-6-phosphate, glyphosate ($C_3H_8NO_5P$), trihydroxymethyl-aminomethane (Tris), magnesium chloride ($MgCl_2$), $NADP^+$, N-acetylcysteine, digitonin used in our experimental studies were obtained from Merck company and quartz cuvettes were obtained from Hellma company. The de-ionized water used was obtained using MilliPore with a resistance of ≥18.2 MΩcm.

Experimental flow

This study was carried out in Cukurova university faculty of medicine, department of medical biochemistry. There is no requirements of Ethics Committee Approval for this study. The experimental procedure was carried out in 3 stages. Samples were obtained from male individuals. First, activity was determined in a blood sample with a reference value of erythrocyte G6PD enzyme. The effect of glyphosate solutions at different concentrations ($5.3x10^{-3}$, $5.3x10^{-4}$, $5.3x10^{-5}$, $5.3x10^{-6}$ mmol/mL) added to the experimental medium in this sample on the enzymatic activity was investigated[33]. Then, the effect change was investigated by adding NAC to the medium with the highest inhibition rate. Finally, following the measurement of G6DP activity in a case with low erythrocyte G6PD activity, the change in activity was compared with the addition of $5.3x10^{-3}$ mmol/mL glyphosate and $4.7x10^{-7}$ mmol/mL N-acetylcysteine[34].

Preparation of Hemolysate

2 mL of blood in EDTA tubes was centrifuged at 3000 rpm for 10 minutes, and its plasma was discarded. 1:3 of saline was added onto the erythrocyte pellet, gently inverted and centrifuged again, and the supernatant was discarded. After this process is repeated three times, hemolysate was prepared using 50 µl of erythrocyte pellet, 100 µl of sodium phosphate buffer (5 mM, pH 7.0), and 650 µl of 0.02% digitonin. For activity calculations, the amount of hemoglobin in the hemolysate was measured in the KX-21N blood counter.

Principle of quantitative measurement

The modified Beutler method was used for erythrocyte G6PD activity. It is based on the measurement of the absorbance increase at 340 nm wavelength of Shimadzu UV-260 within 5 minutes of reduced $NADP^+$ during the conversion of glucose-6-phosphate to 6-phosphoglucolactone in the presence of the enzyme[35]. This reaction was carried out at 37°C in a quartz cuvette with a 1 cm light path. It was calculated by the optical density values in the time interval in which the increase in absorbance was linear for 5 min. Each set of experiments was repeated three times.

Application of the Beutler method

Activity was calculated from the formula using the OD difference caused by NADPH, which was created by applying according to the table 1. After basal values were taken, the protocol was repeated by adding glyphosate solution to the experimental media with final concentrations of 5.3x10-3, 5.3x10-4, 5.3x10-5, and 5.3x10-6 mmol/L, respectively. G6PD activity was measured after incubation times of 0/10/30/60 minutes in a medium with glyphosate. Inhibition experiments were repeated in normal and G6PD deficient samples. Activity changes were compared by adding 4.7x10-7 mmol/mL N-acetylcysteine to the medium with and without glyphosate.

Table 1 Determination of G6PD activity by Modified Beutler Method

Reagents	Sample (mL)	Blank (mL)	Blank Sample (mL)
Distilled water	1.75	3	2.05
0.1M $MgCl_2$	0.3	-	0.3
1 M Tris Buffer (pH 8.0)	0.3	-	0.3
Hemolysate	0.05	-	0.05
2 mM β-NADP	0.3	-	0.3
6mM D-G6P	0.3	-	-
Total Volume	3	3	3

$MgCl_2$: Magnesium chloride; β-NADP: β-Nicotinamide Adenine Dinucleotide Phosphate; D-G6P: D-glucose-6-phosphate.

$$\boldsymbol{Enzyme\ Activity}\left(\frac{\boldsymbol{U}}{\boldsymbol{mL}}\right)=\frac{\boldsymbol{\Delta\ OD}}{\boldsymbol{Time\ (min.)}}\ \boldsymbol{X}\ \frac{\boldsymbol{Total\ Volume}}{\boldsymbol{Hemolysate\ Volume}}\ \boldsymbol{X}\frac{\boldsymbol{1}}{\boldsymbol{6.22}}$$

Statistical analysis

All of the data in the study are enzymatic kinetic analyzes. After the results were repeated 3 times, their arithmetic averages were taken. The effect of glyphosate on the erythrocyte G6PD enzyme was evaluated by monitoring changes in enzyme activity at all glyphosate concentrations.

RESULTS

It was observed 48% that the enzyme activity was decreased by making the maximum inhibition of glyphosate at the 60th minute. While normal erythrocyte G6PD activity was 12 U/g Hb in the experiments, it was 2.5 U/g Hb in case of enzyme deficiency. Percentages of G6PD inhibition of the *in vitro* study in which glyphosate was added at final concentrations of $5.3x10^{-3}$ mmol/mL, $5.3x10^{-4}$ mmol/mL, $5.3x10^{-5}$ mmol/mL, and $5.3x10^{-6}$ mmol/mL illustrated graphically. The loss of activity observed at 60 minutes is when glyphosate inhibits the G6PD enzyme the most. The highest inhibition was observed in the study set, which was affected by $5.3x10^{-3}$ mmol/mL glyphosate.

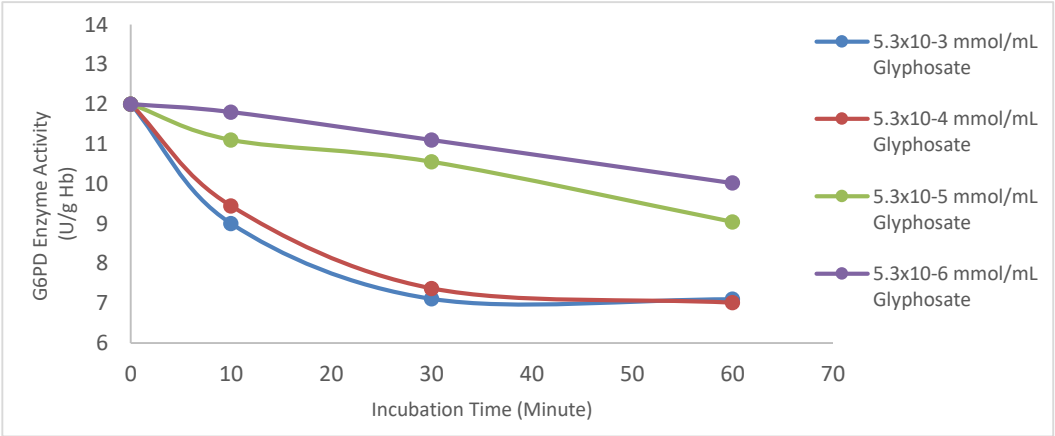

Figure 6. The effect of different glyphosate concentrations in healthy males on the erythrocyte G6PD enzyme in 0/10/30/60 minute incubations.

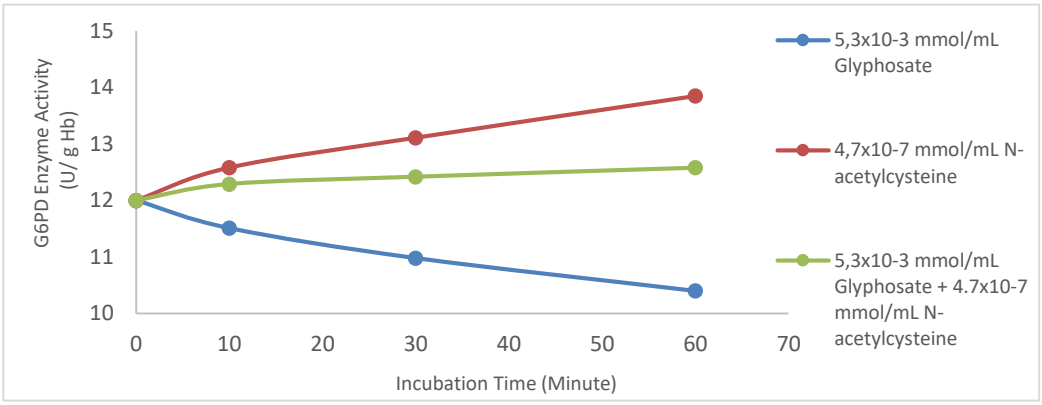

Figure 7. The effect of N-acetylcysteine and glyphosate combination on enzyme activity in an individual whose erythrocyte G6PD activity is within the reference range.

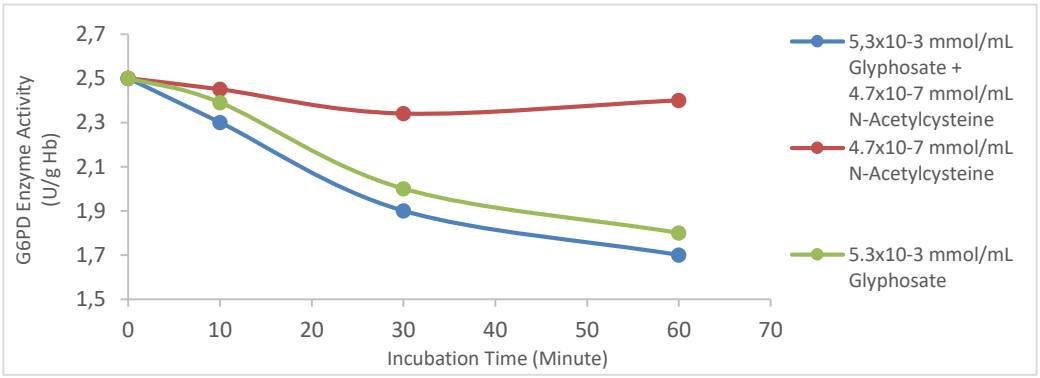

Figure 8. The effect of N-acetylcysteine and glyphosate combination on enzyme activity in an individual with erythrocyte G6PD deficiency

In Figure 7 and Figure 8, $4.7x10^{-7}$ mmol/mL N-Acetylcysteine increased the activity by decreasing the inhibition of glyphosate in the G6PD enzyme in an individual with normal activity (13.3%) but did not cause any positive effect in an individual with mutant G6PD enzyme.

DISCUSSION

36% of Turkey's surface area is cultivated as agricultural, and 34.5% of the population earns their living from this sector[36]. As in the whole world, applications for pesticides in the agricultural sector have led to significant increases in production. However, while these developments increased the efficiency of the products, they also caused serious adverse effects in terms of human and animal health[37].

Glyphosate, which has been used for many years, claiming that it is cost-effective and non-toxic, is frequently preferred, especially in products with genetically modified organisms[16]. Therefore, its use is increasing exponentially every year. While the half-life of glyphosate in aerobic soil conditions is 1.8-7 days, the plasma half-life is 14.38 hours after oral ingestion. In plants, the hydrolysis half-life of glyphosate has been reported to be >35 days. Glyphosate inhibits the absorption of calcium, magnesium, iron, manganese, and zinc in plants. One glyphosate molecule can chelate one-to-one with +2 and +3 metal ions or one metal ion against two glyphosate[38]. While it was thought that its major effect on plants did not cause any toxicity in humans

in the 1970s, it has been reported as a carcinogenic agent in addition to having negative effects on the cardiovascular system, liver, kidney, reproduction, and nervous system in recent studies[39-43].

G6PD is considered an antioxidant enzyme; this is because GSH is used by antioxidant defense mechanisms. Its production by glutathione reductase enzyme is mainly dependent on NADPH synthesized in the pentose phosphate metabolic pathway by G6PD activity. Some of the drugs used alter G6PD activity or gene expression, creating a response in metabolism. In addition, different chemicals used can affect many enzymes *in vivo* or *in vitro*, and this is based on the assumption that it is due to biochemical functions[44]. For this reason, the effect of glyphosate on the G6PD enzyme, which is commonly deficient in the region, was curiously investigated. The incidence of G6PD deficiency in Turkey shows significant regional differences. Published studies have revealed that it is <1% in Central and Northeast Anatolia, 2.3% in Southeastern Anatolia, and 5-20% in Southwest Anatolia[45]. In the Cukurova region, this rate is 8.2%[46]. The fact that Cukurova has the most fertile soils in terms of agriculture in Turkey, the agricultural sector, and the associated job employment increase the exposure of agricultural workers, local people, and those working in the pesticide sector to pesticides.

Although there are few studies in the literature on the effects of glyphosate on human erythrocyte G6PD, many studies are showing the effects of different herbicides on the G6PD enzyme in other species. In an *in vivo* study of pregnant rat tissues and fetuses, exposure to glyphosate was found to have a dose-dependent variable effect. In a study examining the effect of glyphosate on the G6PD of a worm species, *Eisenia fetida*, an inhibitory effect was also shown[47]. In our study to investigate the effect of glyphosate, one of the frequently used organophosphate group herbicides, on healthy and G6PD enzyme deficient erythrocytes, it was determined that glyphosate decreased enzyme activity and NAC can not show a protective effect in individuals with G6PD enzyme deficiency. We think that inhibition is eliminated by NAC competing with glyphosate in normal enzyme and may be useful in the supportive treatment of glyphosate exposure. We believe that the lack of this effect in mutant enzymes may be due to the 3-dimensional structure change in the enzyme molecular structure, and NAC may not be beneficial in people with G6PD deficiency.

G6PD uses $NADP^+$ as the coenzyme, and this is essential for structural stability. The $NADP^+$ molecule on the enzyme has been shown to interact with amino acid residues Gly41 and Asp42. It has been shown that the Adenine region in the $NADP^+$ molecule binds to the enzyme between Tyr503 and Arg487 amino acids, and the Nicotinamide region binds to the enzyme between Trp509 and Tyr401. The interaction between Gly41 in the G6PD structure and glycine in the glyphosate molecule can competes for the substrate binding[48]. This theory supports the formation of an inhibitory effect. In addition, G6PD uses Mg^{2+} as a cofactor. We think that since glyphosate is a +2 valent metal chelator, it increases the inhibitory activity by binding Mg^{+2} in the medium. In addition, its natural substrate, G6P is located in a separate region about 20 Å from the enzyme's NADP binding. Therefore, it may contribute to this inhibition by entering competition due to spatial similarity with glyphosate.

However, some limitations should be noted. It may be preferable to use sufficient sample size in the study. Determining the mutant type of the enzyme and reflecting the results to the clinic will contain more reliable results. These results should be supported by animal experiments or cell culture and studies should be continued. Additionally it has been shown that glyphosate affects the G6PD enzyme *in vitro* in humans, and it is predicted that it may be a negative interference agent in the experimental process. Studies on the dose of glyphosate use that will not cause harmful effects in humans are insufficient, and studies on this subject should be continued. In the clinic, individuals with G6PD deficiency should be informed about glyphosate exposure.

Yazar Katkıları: Çalışma konsepti/Tasarımı: KK, ND; Veri toplama: KK; Veri analizi ve yorumlama: KK, ND; Yazı taslağı: KK, ND; İçeriğin eleştirel incelenmesi: KK, ND; Son onay ve sorumluluk: KK, ND; Teknik ve malzeme desteği: KK, ND; Süpervizyon: ND; Fon sağlama (mevcut ise): yok.
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