



## IMPACT OF pH, TEMPERATURE AND GAMMA RADIATION ON G6PD ACTIVITY AND ERYTHROCYTE MORPHOLOGY IN G6PD DEFICIENCY

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### ABSTRACT

Glucose-6-Phosphate dehydrogenase (G6PD) deficiency is a genetic condition that primarily affects erythrocytes. G6PD is an enzyme predominantly found in erythrocytes, where it catalyses the oxidation of glucose-6-phosphate in glucose metabolism. Erythrocytes are blood cells produced by stem cells in the bone marrow. Previous research on G6PD deficiency has mainly focused on affected patients. Therefore, the objectives of this study are to optimise the effects of pH and temperature on G6PD enzyme activity, investigate the impact of G6PD deficiency on erythrocytes, and examine the morphology of both normal and G6PD-deficient erythrocytes after exposure to gamma radiation. For the methodology, the dilution method was employed to identify the optimal parameters for G6PD activity. Erythrocytes were obtained from both G6PD-deficient patients and healthy individuals. To study the reactions, substances such as uncoated aspirin and broad bean solutions were added to the erythrocytes. The erythrocytes' morphology was then examined after exposure to gamma radiation from Cesium-137 for a week. After one week, the samples were observed under a Nikon Eclipse LV/UDM microscope. The results from this irradiation serve as evidence of the effects of G6PD deficiency on erythrocytes and highlight substances that may contribute to the deficiency.

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### Introduction

Glucose-6-phosphate dehydrogenase (G6PD) is an enzyme that helps the body process carbohydrates and convert them into energy (Tiwari, 2017). G6PD deficiency is the most common enzyme disorder in humans, and it is inherited as an X-linked trait (Reclos *et al.*, 2000). Chromosomes are thread-like structures made of DNA that carry genes. The X and Y chromosomes determine gender, with females having two X chromosomes and males having one X and one Y. Additionally, data from the World Health Organization (WHO) confirms the high global burden of G6PD deficiency, with approximately 400 million people affected worldwide (Noori *et al.*, 2004), especially in Africa, Asia, and Mediterranean countries

(Frank, 2005). This widespread prevalence is linked to the genetic nature of the disorder, which is X-linked and passed down through generations in affected populations.

G6PD is important for maintaining the stability of red blood cells (erythrocytes) (Garcia *et al.*, 2022). It protects erythrocytes from harmful byproducts that can form when taking certain medications or fighting infections. G6PD helps produce nicotinamide adenine dinucleotide phosphate (NADPH) and glutathione (GSH). GSH reacts with hydrogen peroxide ( $H_2O_2$ ), converting it to water ( $H_2O$ ), which prevents oxidative stress in the erythrocytes (Allahverdiyev *et al.*, 2015). When G6PD is deficient, oxidative stress occurs because GSH

is not produced and hydrogen peroxide is not broken down, leading to hemolysis (Xu *et al.*, 2010; Karadsheh *et al.*, 2021). Erythrocytes are blood cells that carry oxygen throughout the body and all blood cells are made in the bone marrow from stem cells. G6PD deficiency affects erythrocytes more clearly because these cells lack a nucleus and live longer than other cells. Additionally, erythrocytes have proteases that degrade the mutant G6PD enzyme more than in other tissues (Park *et al.*, 2017). The defense mechanisms of erythrocytes depend on a steady supply of reduced NADP (Bonilla *et al.*, 2007).

Gamma radiation serves as a suitable experimental stressor to mimic oxidative stress conditions in G6PD-deficient individuals because of its ability to generate reactive oxygen species (ROS) within cells. When gamma rays interact with cellular water molecules, they induce radiolysis, leading to the formation of highly reactive radicals such as hydroxyl radicals, superoxide anions, and hydrogen peroxide. These ROS initiate oxidative damage to lipids, proteins, and nucleic acids, thereby creating an oxidative stress environment. In normal cells, oxidative insults are counteracted by glutathione in its reduced form (GSH), which depends on a continuous supply of NADPH generated through the pentose phosphate pathway via G6PD activity. However, in G6PD-deficient cells, the reduced capacity to produce NADPH impairs the regeneration of GSH, leaving the cells more vulnerable to ROS accumulation and subsequent damage. Thus, gamma radiation provides a controlled and reproducible method to simulate the heightened oxidative stress that characterises the cellular environment in G6PD deficiency, making it a relevant model for studying the condition.

Aspirin and broad bean (*Vicia faba*), extracts were selected as stressors in this study due to their well-documented roles in triggering oxidative stress in erythrocytes, particularly in G6PD-deficient individuals. Aspirin metabolism can generate reactive oxygen species and deplete cellular antioxidants, while several studies have reported its hemolytic potential in sensitive

patients. Broad beans, also known as fava beans, on the other hand, contain vicine and convicine, compounds that undergo biotransformation to produce divicine and isouramil, which are strong redox-active agents known to cause oxidative damage to erythrocytes. These agents overwhelm the glutathione-based defense system, leading to hemolysis in individuals with impaired NADPH production. Previous studies have consistently demonstrated the oxidative and hemolytic effects of both aspirin and broad beans on red blood cells, thereby validating their use as experimental stressors in assessing erythrocyte vulnerability under conditions of G6PD deficiency.

An enzyme is a large molecule that acts as a biological catalyst, speeding up chemical reactions in the body without being used up. It lowers the activation energy, defined as the initial energy required to initiate a reaction (Solomon *et al.*, 2010). All enzymes are proteins, made from amino acids that contain a carboxylic acid group and an amino group on the alpha carbon. Each amino acid also has a unique side group (R). Protein structure is divided into four levels: primary (the sequence of amino acids), secondary (coiling or folding of the polypeptide chain), tertiary (the overall 3D shape), and quaternary (when multiple polypeptide chains combine).

Enzymes have a special region called the active site, which fits specific molecules called substrates. The enzyme binds to these substrates, forming an enzyme-substrate complex. These binding stresses or weakens certain bonds in the substrate, helping them react and form a new molecule. Once the reaction occurs, the product is released, and the enzyme returns to its original shape, ready to catalyse another reaction. Enzymes can also break down one substrate into two products. By binding to substrates, enzymes lower the activation energy needed for reactions.

Glucose-6-phosphate dehydrogenase (G6PD) is an enzyme and its deficiency are one of the most common hereditary genetic disorders, affecting over 400 million people worldwide (Sulaiman *et al.*, 2013). There are

several genotypes of G6PD deficiency with the most common being G6PD A- and G6PD Mediterranean (Carter *et al.*, 2011). According to Felix *et al.* (2002), G6PD deficiency is typically caused by missense mutations in the X-linked gene that encodes G6PD. The common African variant, G6PD A-, differs from the normal G6PD B by two amino acid changes. One of these mutations, on its own, results in the non-deficient G6PD A- variant.

Mutations in the G6PD gene cause the deficiency by altering the production of the enzyme (Verrelli *et al.*, 2002). G6PD plays a key role in carbohydrate metabolism and helps protect erythrocytes from damage caused by reactive oxygen species (ROS), which are byproducts of normal cellular processes. G6PD catalyses reactions that prevent ROS from reaching toxic levels in erythrocytes. When mutations reduce the amount or alter the structure of G6PD, the enzyme can no longer perform this protective function. As a result, ROS levels rise, leading to damage and destruction of erythrocytes. Factors such as infections, certain medications, and broad beans can trigger increased ROS production, causing erythrocyte destruction that outpaces the body's ability to replace them (Beutler & Duparc, 2007). Broad beans can trigger a harmful reaction in people with G6PD deficiency due to the presence of specific compounds known as vicine and convicine. These compounds are glucosidic vicine alkaloids found in broad beans, which can cause oxidative stress in red blood cells.

G6PD is an enzyme that catalyses the first and key step of the pentose phosphate pathway. It converts D-glucose-6-phosphate (G6P) into 6-phosphoglucono-lactone, while turning  $\text{NADP}^+$  into NADPH. This helps cells produce pentoses for DNA and RNA and maintain their redox balance. G6PD is the only enzyme that makes NADPH and is activated during oxidative stress (Filosa *et al.*, 2003). In humans, G6PD switches between dimer and tetramer forms, which is influenced by pH and ionic strength (Haeussler *et al.*, 2019). The exact role of this change is not fully understood, but studies

suggest that the enzyme works through a rapid, random-order mechanism (Wang *et al.*, 2002).

Erythrocytes, or red blood cells, transport oxygen in the blood. Their shape is ideal for this function. Viewed from the top, they appear circular, but from the side, they are biconcave discs. This shape increases the surface area, improving the diffusion of oxygen and carbon dioxide. Erythrocytes also have a flexible membrane, allowing them to pass through small capillaries as narrow as 3 mm. They contain large amounts of haemoglobin which is the protein that binds oxygen (Oliveira *et al.*, 2009). To make room for more haemoglobin, erythrocytes lose their nucleus and other organelles during development in the bone marrow. Because they lack a nucleus and organelles, they cannot repair damage and have a lifespan of about 120 days. The spleen removes old or damaged erythrocytes.

G6PD is a key enzyme in the oxidative pentose phosphate pathway, which starts with G6PD catalysing the first step. In this step,  $\text{NADP}^+$  is reduced to NADPH, and ribulose-5-phosphate, a building block for DNA, RNA, and ATP is produced (Turner, 2000). NADPH is the main reducing agent in the cytoplasm of cells (Koehler & Van, 2003). The second step of the pathway involves the production of NADPH, which helps reduce oxidised glutathione (GSSG) to its active form which is reduced glutathione (GSH). GSH is the only defense against oxidative stress in red blood cells (RBCs) (Peters & Van, 2009; Möller, 2022; Spinelli *et al.*, 2025). In normal, unstressed erythrocytes, G6PD activity is only about 2% of its total capacity. The main job of the pentose phosphate pathway is to produce NADPH and GSH, which are crucial for cell survival and help erythrocytes maintain reducing capacity (Oka, 2012).

Individuals with G6PD deficiency are usually symptom-free unless triggered by factors like oxidative stress. Oxidative stress can be caused by three main factors: infections (like flu, colds, and bacterial infections), drugs (such as naphthalene or menthol), and foods (like broad beans, which should be avoided by

G6PD-deficient individuals). Previous research has focused on G6PD deficiency in diseases like malaria and jaundice, as well as the effects on enzyme activity in bacteria. G6PD plays a vital role in the body, especially in red blood cells, which have no other way to produce NADPH. A decrease in G6PD activity lowers NADPH and glutathione levels, which can lead to hemolysis (destruction of red blood cells). Hemolysis is a major health issue associated with G6PD deficiency. This research aims to reduce the health problems caused by this deficiency.

### Materials and Methods

Glucose-6-phosphate dehydrogenase (G6PD), 0.1 M hydrochloric acid (HCl), 0.1 M sodium hydroxide (NaOH), 0.1 M ethanol, 1 mL blood sample of normal people, 3 mL blood sample of G6PD-deficient patient, distilled water, broad beans, and aspirin were used in this research.

#### Erythrocytes

Two volunteers, a G6PD-deficient patient and a normal person were chosen to be part of the research. 3 mL of blood was drawn out from each volunteer and then transferred into two test tubes that were equipped with an anticoagulant. This procedure was done by the staff nurse at the Universiti Tun Hussein Onn Malaysia (UTHM) Health Centre. This study is a preliminary study to gather insights into the possible effects or differences between the two groups. The normal erythrocytes and G6PD-deficient erythrocytes were also subjected to irradiation for comparison. Both erythrocytes were kept on ice immediately after irradiation.

#### Broad Beans Powder

The cardiprin uncoated aspirin and broad beans were purchased in the pharmacy and supermarket, respectively. Using a grinder, the broad beans were ground until they became powder. The powder was kept in an airtight container. The procedure was carried out in the laboratory at room temperature.

#### Stock Solution Preparation

The HCl, NaOH, and ethanol were all in the concentrated form of liquid. A formula of

dilution was used in order to obtain the volume of each concentrated HCl, NaOH, and ethanol. They were diluted with distilled water in 500 mL of a volumetric flask separately. The specific volume of the concentrated HCl was poured into the volumetric flask, and then distilled water was added to the volumetric flask until it reached the calibration mark. The steps were repeated using the NaOH, and lastly the ethanol.

5 g of broad bean powder was weighed using a weighing scale and then added to 50 mL of distilled water. The solution was heated until the powder dissolved, and then it was stored in a container. For the aspirin, one tablet of aspirin was dissolved in 15 mL distilled water and then heated until it was completely dissolved. The solution of aspirin was also stored in a container. The relationships between the parameters and G6PD enzyme activities were studied through a plotted graph using Ultraviolet-Visible spectroscopy (UV-Vis) and a Fourier Transform Infrared (FTIR) spectrometer.

### Results and Discussions

The effect of pH level and temperature of G6PD on enzyme activities was investigated by using UV-Vis spectroscopy. For instance, at different pH levels, the enzyme may undergo conformational changes that alter its catalytic activity. The production of NADPH is directly linked to the activity of G6PD. By measuring the absorbance at 340 nm, UV-Vis spectroscopy can track the rate of NADPH formation as a function of time, providing a real-time measurement of enzyme activity. For the pH level parameter, the G6PD enzyme solution was adjusted by adding 0.1 M HCl and 0.1 M NaOH until the desired pH was obtained. The pH level was measured by using pH meter. The solution was carried out at a temperature of 37°C. From the graph in Figure 1, pH 7 showed the highest absorbance of UV light at a wavelength of 200 nm, which was 1.40, while pH 11 had the lowest absorbance of UV light at a wavelength of 200 nm, which was 0.20. The absorbance of UV light decreased gradually for pH 3, 5, 7, 9, and 11 as the wavelength increased.

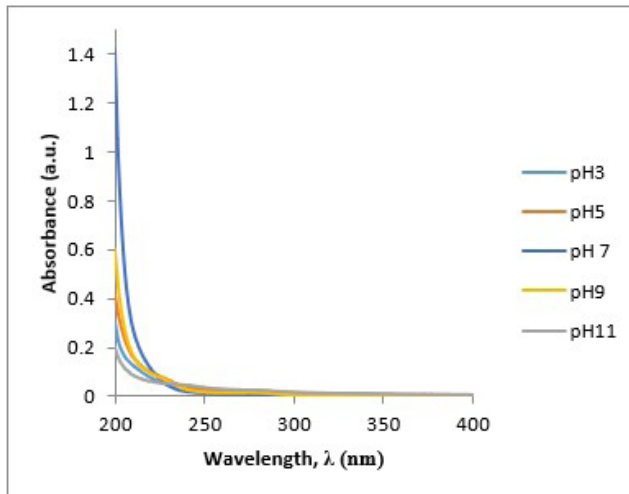


Figure 1: The effect of different pH levels on the absorbance of ultraviolet light in enzyme G6PD using UV-Vis

For the parameter of temperature, the effect can be seen in the graph shown in Figure 2. The temperature chosen were 29°C, 31°C, 33°C, 35°C, 37°C, 39°C, and 41°C. To obtain the desired temperature value, the enzyme solution in the test tube was placed in a beaker filled with water and heated on a hot plate, and the temperature was measured using a digital

thermometer. From Figure 2, the temperature of 33°C had the highest UV light absorbance at 200 nm, which was 0.40 AU, while the lowest UV light absorbance at 200 nm, was recorded at 0.17, at the temperature of 39°C. Temperature of 29°C, 31°C, 33°C, 35°C, 37°C, and 41°C exhibited a similar trend, with the graph gradually decreasing as the wavelength increased.

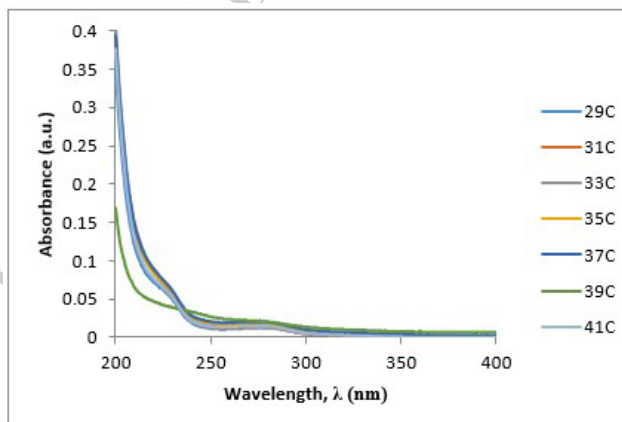


Figure 2: The effect of different temperature on the absorbance of ultraviolet light in enzyme G6PD using UV-Vis

UV-Vis spectroscopy was used to observe the G6PD enzyme activity. The function of UV-Vis is to measure the amount of ultraviolet light or visible radiation absorbed by the substance in a solution, and it is categorised as one of the enzyme assays. The conversion of substrates to products catalyzed by the enzymes exhibited different ultraviolet light absorbance. As for G6PD enzyme, the structures are made up of a coenzyme domain, a structural NADP<sup>+</sup>, an  $\alpha+\beta$  domain, a substrate site, and a coenzyme site (Kotaka *et al.*, 2005). Siddharta Singh *et al.* (2012) stated that NADP<sup>+</sup> is an important coenzyme in the G6PD enzyme. NADP<sup>+</sup> is in oxidised form and in this form, NADP<sup>+</sup> does not absorb ultraviolet light at 340 nm.

As for the pH level, the optimum pH level for G6PD enzyme in human blood is pH 7 to pH 7.5. Based on its properties, the enzyme functions most efficiently at an optimum pH near neutrality. This is because the change in pH can affect the structure of amino acids in the active site of the enzyme (Milanowski *et al.*, 2013). Figure 8 shows that pH 7 has the highest absorbance at 200 nm, which makes

pH 7 the optimum pH level. On the other hand, at 200 nm, NADP<sup>+</sup> in G6PD enzyme solution shows the highest amount compared to other pH. In this situation, NADP<sup>+</sup> is then reduced to NADPH (Gowda *et al.*, 2025). The optimum temperature for the G6PD enzyme is 37°C, which corresponds to the normal temperature of the human body. This can be seen in Figure 2, where at 200 nm, the enzyme solution of G6PD with a temperature of 37°C had the highest absorbance of ultraviolet light compared to other temperatures. This is due to the amount of NADP<sup>+</sup> in the solution.

### **Morphology Structure of Erythrocytes with G6PD Deficiency**

The structure of G6PD-deficient erythrocytes was examined using a Nikon Eclipse LV/UDM microscope. Two samples of G6PD-deficient blood were added with aspirin and broad beans solutions separately, while three samples of pure G6PD-deficient blood were used to study the structure of erythrocytes. The samples were put on glass slides using a thin film method. The results are shown in Figure 3, 4, and 5 below.

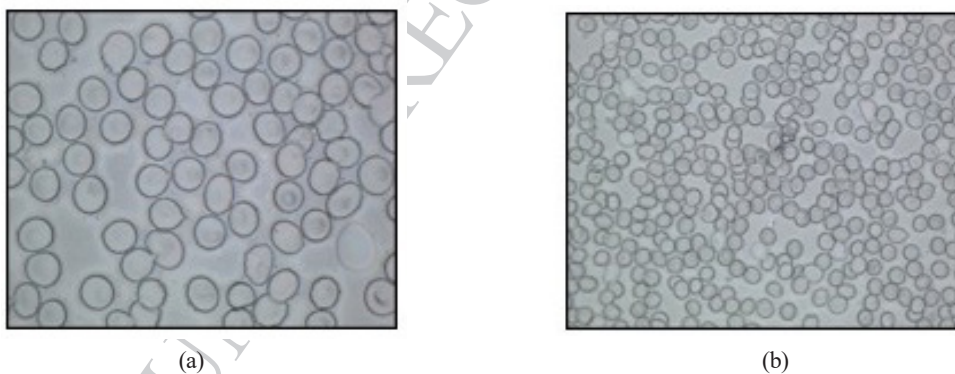


Figure 3: The erythrocytes of pure G6PD-deficient blood with (a) 50 times and (b) 100 times magnification under the Nikon Eclipse LV/UDM microscope

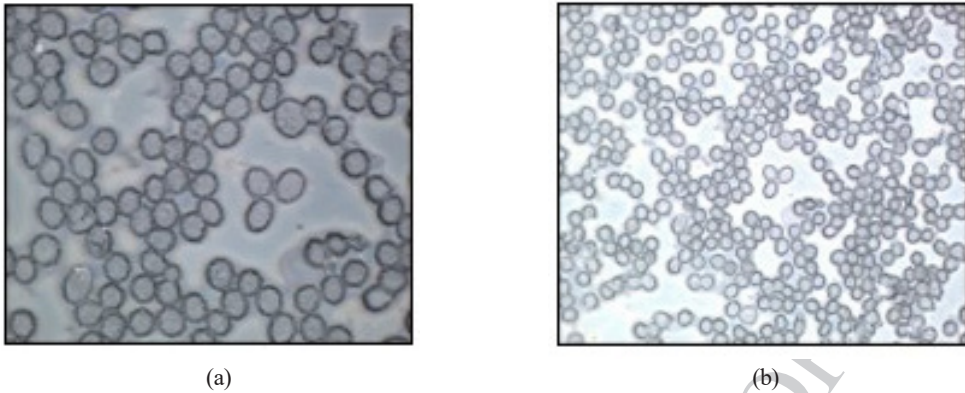


Figure 4: The erythrocytes of the aspirin added to G6PD-deficient blood with (a) 50 times and (b) 100 times magnification under the Nikon Eclipse LV/UDM microscope

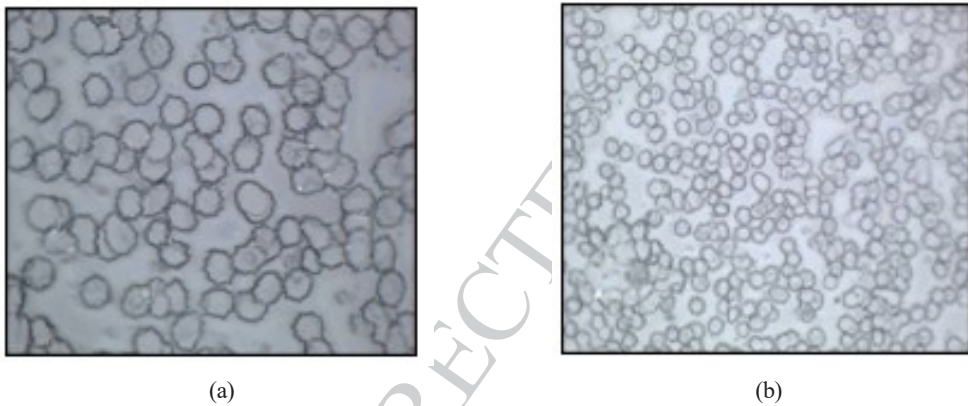


Figure 5: The erythrocytes of the broad beans added to G6PD-deficient blood with (a) 50 times and (b) 100 times magnification under the Nikon Eclipse LV/UDM microscope

Based on Figure 4, the structure of erythrocytes was observed. The image clearly shows that the shape of the erythrocytes is round and the surface of the erythrocytes is smooth. There are also some irregular shapes found in the image. In order to observe the effect of G6PD deficiency on erythrocytes, aspirin and broad bean solutions were added to the pure G6PD-deficient blood where the images are shown in Figure 4 and 5, respectively. In Figure 4, where the aspirin was added, the image shows that the shape of erythrocytes is similar to that of a sea urchin. While in Figure 5, where the broad beans solution was added, the shape of the erythrocytes looks like a “thorn” (Arielle, 2024).

The effect of G6PD deficiency on the structure of erythrocytes is the occurrence of hemolysis. Hemolysis is the rupture or destruction of red blood cells, or in other words, the normal structure of red blood cells is turned into an abnormal structure (Fernando *et al.*, 2024). There are several abnormal structures of red blood cells obtained when the aspirin and broad bean solutions were added to the G6PD-deficient blood. The result for aspirin is clearly shown in Figure 4 where the shape of erythrocytes is like “sea urchin”. The abnormal erythrocyte is known as an ‘echinocyte’ or ‘burr cell’ (Ariel *et al.*, 2025). Echinocytes often occur due to the influence of an oxidising

agent, which is acetylsalicylic acid that exists in aspirin. While, the result for the added broad beans is shown in Figure 5. The structure of erythrocytes after the broad bean solution was

added resembles a “thorn”. The structure is known as an ‘acanthocyte’ or ‘spurr cell’. The exchange in structure is due to the existence of divicine, isouramil, and hydrogen peroxide, which are the oxidising agents in broad beans.

### ***Morphology of Normal Erythrocytes and G6PD Deficiency Erythrocytes after Irradiation with a Gamma Source***

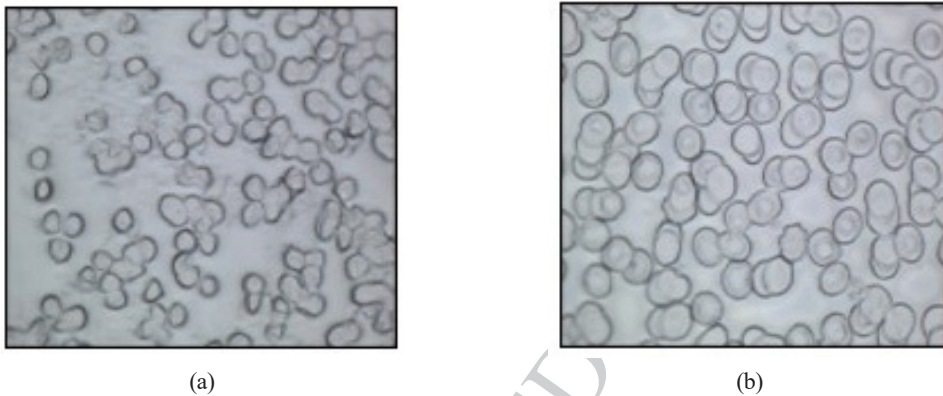


Figure 6: The erythrocytes of normal blood with 100 times magnification under the Nikon Eclipse LV/UDM microscope (a) before irradiation and (b) after being exposed to Caesium-137 for one week

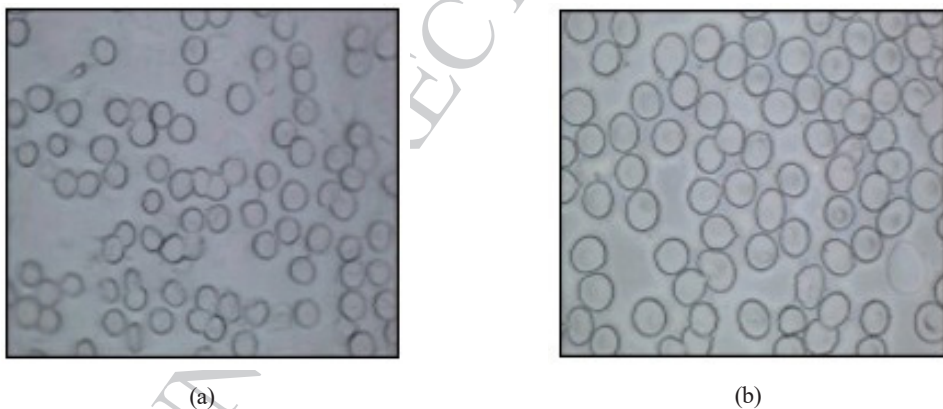


Figure 7: The erythrocytes of pure G6PD-deficient blood with (a) 50 times and (b) 100 times magnification under the Nikon Eclipse LV/UDM microscope

Figure 6 (a) is the image of the normal blood and G6PD-deficient blood before irradiation, respectively, while Figure 6 (b) is the image of the blood after irradiation with Caesium-137 for one week. Comparison of images between Figure 7 (a) and (b) shows that in G6PD-deficient blood, abnormal shapes of erythrocytes were

observed compared to the normal blood. Both Figure 7 (a) and (b) show that the erythrocytes were damaged by the decrease in size.

Theoretically, the erythrocytes will undergo process hemolysis and membrane damage. Based on Figure 6 and 7, there are differences between the structure of normal erythrocytes

and G6PD-deficient erythrocytes. Some of the structure of the G6PD deficiency erythrocytes is abnormal that resembles “teardrop” shape (Parth Thakor *et al.*, 2024). As for the normal erythrocytes in Figure 6, the structures are seen as a round shape. Both the normal and G6PD-deficient erythrocytes are exposed to Caesium-137, which has a maximum activity of 30 mCi for about a week. The results are shown in Figure 6 and 7 for normal erythrocytes and G6PD-deficient erythrocytes, respectively. The size of erythrocytes for both types of blood decreased and the round shape of erythrocytes disappeared.

### Conclusions

From the research, the parameters of the pH level and temperature of the enzyme G6PD are optimised. For pH level, the optimum pH is 7, and the optimum temperature is 37°C. Besides the parameter, the effect of the G6PD deficiency on erythrocytes is also determined. There are two ways of causing the G6PD deficiency in erythrocytes, which are by adding aspirin and broad bean solution to the G6PD-deficient blood. As a result, the shape or structures of the erythrocytes became abnormal shapes, which are echinocyte for the aspirin and achanoocyte for the broad beans. Lastly, the morphology of the normal and G6PD-deficient erythrocytes after irradiation with gamma radiation, which is Caesium-137 was studied. After a week of exposure, the result showed that the erythrocytes lost their round shapes while some of them burst.

This study has several limitations. Although morphological alterations in erythrocytes were observed, the extent of damage was not quantified, hence, future studies are recommended to apply quantitative methods for a more comprehensive assessment. Absorbance data were obtained from limited replicates without statistical analysis, underscoring the need for repeated measurements and proper statistical evaluation. In addition, the sample size was very small, including only one G6PD-deficient subject and one healthy control; thus,

the findings should be considered preliminary and exploratory, aimed at generating hypotheses rather than providing confirmatory evidence.

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### Conflict of Interest Statement

The authors declare that there is no conflict of interest regarding the publication of this article.

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