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# Association between Glycated Hemoglobin with the Levels of Serum Proinflammatory Cytokines and Antioxidants in Patients with Type 2 Diabetes Mellitus in Universitas Sumatera Utara Hospital

Mutiara Indah Sari[1*], Zaimah Z. Tala[2], Dian Dwi Wahyuni[3]

[1]*Departement of Biochemistry, Faculty of Medicine, Universitas Sumatera Utara, Jl. Dr Mansur No.5 Medan, Indonesia;*
[2]*Departement of Clinical Nutrition, Faculty of Medicine, Universitas Sumatera Utara, Jl. Dr Mansur No.5 Medan, Indonesia;*
[3]*Departement of Microbiology, Faculty of Medicine, Universitas Sumatera Utara, Jl. Dr Mansur No.5 Medan, Indonesia*

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***Correspondence:** Mutiara Indah Sari. Departemen of Biochemistry, Faculty of Medicine, Universitas Sumatera Utara, Jl. Dr Mansur No.5 Medan, Indonesia. E-mail: mutiara@usu.ac.id

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**Abstract**

**BACKGROUND:** Hyperglycemia condition in diabetes mellitus (DM) influences proinflammatory cytokine levels and disrupts antioxidant balances. Glycated Hemoglobin is used as a biomarker of glycemic control in DM.

**AIM:** This study aimed to analyse the association between glycated Hemoglobin with the levels of serum proinflammatory cytokines (interleukin (IL)-6) and antioxidants (glutathione peroxidase (GPx) and glutathione (GSH)) in type 2 diabetes mellitus (T2DM) patients in Universitas Sumatera Utara (USU) Hospital.

**METHODS:** A total of eighty-nine T2DM patients were recruited at USU Hospital. Glycated Hemoglobin levels were measured using routine laboratory tests at USU Hospital. The IL-6, GPx, and GSH levels were measured using enzyme-linked immunosorbent (ELISA) method. The statistical significance was determined using the Kruskal Wallis test, followed by Mann-Whitney test ($p < 0.05$).

**RESULTS:** The mean of glycated hemoglobin (%), IL-6 (pg/ml), GPx (ng/ml), and GSH (ng/ml) levels in T2DM patients were 8.96 ± 2.28, 59.27 ± 16.04, 32.13 ± 12.10, and 7.42 ± 3.50, respectively. Regarding the glycated Hemoglobin levels, 28.09% of patients had controlled diabetes, 24.72% of patients had poorly controlled diabetes, and 47.19% of patients had uncontrolled diabetes. The IL-6 levels of the three study groups based on glycated Hemoglobin levels were related significantly ($p < 0.05$), but there was no statistically significant difference observed between the GPx and GSH levels ($p > 0.05$).

**CONCLUSION:** The present study suggests that the glycated Hemoglobin was associated with the levels of serum IL-6 levels but not GPx and GSH levels in T2DM patients in USU Hospital.

## Introduction

Diabetes mellitus (DM) is metabolic syndromes caused by the failure of the pancreas to produce sufficient amount of insulin, or the body cannot use insulin effectively which will increase blood glucose levels or hyperglycemia [1]. The prevalence of diabetic patients in the world in 2015 was 415 million cases, and the number of diabetic patients is estimated to increase to 642 million in 2040 [2]. According to the data of the International Diabetes Federation in 2015, Indonesia ranked as the seven highest number of diabetic patients in the world with an estimated of 10 million patients [3].

Based on the data of the Indonesian Ministry of Health in 2013, the number of diabetic patients in North Sumatra was around 205 thousand people. Hence, North Sumatra ranked 8th in terms of the number of diabetic patients in Indonesia [4].

Low levels of insulin in diabetic patients will

cause an increase in blood glucose levels (BGLs)/hyperglycemia. In hyperglycemia condition, oxidation to the glucose triggers the formation of reactive oxygen species (ROS). The accumulated ROS is the central point to an increase in oxidative stress [5], [6]. Continuous oxidative stress leads to inflammatory conditions in the cells which will ultimately increase the formation of proinflammatory cytokines. Proinflammatory cytokines, such as tumour necrosis factor (TNF)-α, interleukin (IL)-1, and interleukin (IL)-6, can induce insulin resistance by interfering insulin signalling. It will worsen the function of insulin [7]. Previous studies showed a significant relationship between increased risk of type 2 diabetes mellitus (T2DM) and increased levels of IL-6 [8], [9]. T2DM is the most common type of DM because nearly 90% of all DM cases are this type of DM [3]. IL-6 is a pleiotropic cytokine known to be involved in the amplification and protection against inflammation [10].

Also, oxidative stress in T2DM patients disrupts antioxidant balances in cells, such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and glutathione (GSH) [6]. Past research reported that T2DM patients had lower levels of total antioxidants status (TAS), GPx, and GSH than the healthy group [11], [12], [13]. GPx is an antioxidant enzyme that has several functions such as detoxifying lipid peroxide and hydrogen peroxide (H2O2), eliminating ROS, preventing the formation of new free radicals, and converting free radicals that have been formed into less reactive molecules [12]. The activity of GPx requires GSH as a direct scavenger and co-substrate for the peroxidase enzyme. GSH is the main buffer in the reduction process of intracellular oxidation. The GPx and GSH antioxidants are believed to have useful benefits for the body, such as to prevent and repair damage caused by free radicals [14], [15].

Optimum glycemic control is required to control DM and prevent worsening DM disease so that the increased proinflammatory cytokines and antioxidant imbalances due to oxidative stress can be controlled. HbA1c (glycated Hemoglobin) is used as a biomarker of glycemic control in DM condition because it describes blood glucose levels in the last 60-90 days. Glycemic control in T2DM used glycated Hemoglobin measurement which is described as follows: < 7.5% of glycated Hemoglobin describes controlled diabetes, 7-9% of glycated Hemoglobin describes poor diabetes control, and > 9% of glycated Hemoglobin describes uncontrolled diabetes [1], [16], [17]. Previous studies found that glycated Hemoglobin levels were positively correlated with cytokine levels [18] but negatively correlated with antioxidant levels [19]. Therefore, the present study aimed to analyse the association between glycated Hemoglobin with serum proinflammatory cytokine and antioxidant levels in patients with T2DM in Universitas Sumatera Utara (USU) Hospital.

## Methods

### *Research participants*

A total of eighty-nine T2DM patients were recruited at USU Hospital from March to September 2018. The diagnosis was made according to the criteria of Indonesian Endocrinology Society by endocrine specialists, namely: (1) blood glucose level was more or equal to 200 mg/dl; (2) fasting blood glucose level was more or equal to 126 mg/dl; (3) blood glucose level was more or equal to 200 mg/dl at 2 hours after administered 75 gram of glucose in the glucose tolerance test; and (4) glycated hemoglobin was > 6.5% [16].

Before participating in the research, patients were asked to sign an informed consent form explaining the research purposes, procedures, risks, and benefits of the study. The procedures have been approved by the ethics committee of the Faculty of Medicine, University of Sumatera Utara (No.227/TGL/KEPK FK USU-RSUP HAM/2018) following the Second Declaration of Helsinki. The characteristics of patients including age, medication, duration of diabetes, smoking, pregnant, and other chronic illnesses were asked using questionnaires. Patients who had one of these characteristics were excluded from the study: pregnant, took vitamin supplements, infections (e.g. HIV, tuberculosis, hepatitis, malignant diseases), anemia, hemoglobinopathy, history of blood transfusion in the last 2-3 months, other conditions that might affect the age of erythrocytes, or impaired kidney function.

The patients were divided into three groups based on the glycated Hemoglobin levels: < 7% as controlled T2DM patients, 7-9% as poorly controlled T2DM patients, and > 9% as uncontrolled T2DM patients.

### *Preparation of samples*

Blood samples (5 ml) were collected for serum separation using standard venipuncture after fasting for at least 8-12 hours. The blood samples were centrifuged at 3000 rpm for 10 minutes. Fasting blood glucose (FBG) levels were measured within 2 hours after being taken while glycated Hemoglobin was measured in the whole blood using the routine laboratory tests (Cobas 6000 analyser with hexokinase and immunoturbidimetric method, Roche Diagnostics, Switzerland) at USU Hospital. Furthermore, postprandial blood glucose (PBG) levels were measured 2 hours after the patients had meals.

Serum samples were stored at -80° C for interleukin-6 (IL-6), glutathione peroxidase (GPx), and glutathione (GSH) measurements. IL-6, GPx, and GSH were measured using enzyme-linked immunosorbent assay (ELISA) with commercial kits

from BioLegend, USA. The levels of proinflammatory cytokines and antioxidants were read with a microplate reader at a wavelength of 450 nm. All measurement process of proinflammatory cytokine and antioxidant levels were performed at the Integrated Laboratory of Faculty of Medicine, USU.

### *Statistical analysis*

Statistical Package for the Social Sciences (SPSS) version 22 was used for the statistical analysis of the data. Kolmogorov-Smirnov test was used to analyse the data normality. The mean comparison of IL-6, GPx, and GSH levels in the study groups based on glycated Hemoglobin was performed using the Kruskal Wallis test, followed with Mann-Whitney test if the test results showed a significance level. The level of significance in the study was a p-value of < 0.05.

## Results

The characteristics of T2DM patients are shown in Table 1.

**Table 1: Clinical and biochemical characteristics of T2DM patients**

| Characteristics | N = 89 |
|---|---|
| Age (years), mean ± SD | 57.77 ± 10.43 |
| Gender (male/ female), N (%) | 52 (58.4)/37 (41.6) |
| Duration of ill (years), mean ± SD | 8.60 ± 3.66 |
| Smoker N (%) | 39 (44) |
| FBG (mg/dl), mean ± SD | 220.83 ± 31.13 |
| PBG (mg/dl), mean ± SD | 301.57 ± 52.90 |
| Glycated Hemoglobin (%) | 8.96 ± 2.28 |
| Interleukin-6 (pg/ml), mean ± SD | 59.27 ± 16.04 |
| Glutathione peroxidase (ng/ml), mean ± SD | 32.13 ± 12.10 |
| Glutathione (ng/ml), mean ± SD | 7.42 ± 3.50 |

The average age of T2DM patients was 57.77 ± 10.43 years old. Based on the gender of T2DM patients in the study, there were more male patients than female patients (58.4% vs 41.6%). The average duration of diabetes in the study was 8.60 ± 3.66 years. This study also found that 44% of T2DM patients were smokers. The average FBG (mg/dl), PBG (mg/dl), glycated hemoglobin (%), IL-6 (pg/ml), GPx (ng/ml), and GSH (ng/ml) levels in T2DM patients were 220.83 ± 31.13, 301.57 ± 52.90, 8.96 ± 2.28, 59.27 ± 16.04, 32.13 ± 12.10, and 7.42 ± 3.50, respectively.

The distribution of glycated Hemoglobin of T2DM patients can be seen in Table 2.

**Table 2: Distribution of glycated Hemoglobin of T2DM patients**

| T2DM patients N = 89 | N (%) | Glycated Hemoglobin (%) Mean, SD |
|---|---|---|
| Controlled | 25 (28.09) | 6.38 ± 0.11 |
| Poorly controlled | 22 (24.72) | 8.25 ±0.09 |
| Uncontrolled | 42 (47.19) | 10.90 ±0.21 |

In Table 2, it can be seen that regarding the glycated hemoglobin levels of T2DM patients, 28.09% of the patients were categorized as controlled (mean/SD = 6.38 ± 0.11), 24.72% of the patients were categorized as poorly controlled (mean/SD = 8.25 ± 0.09), and 47.19% of the patients were categorized as uncontrolled (mean/SD = 10.90 ± 0.21).

The comparison of age, duration of illness, FBG, PBG, GPx, GSH, IL-6 levels between T2DM patients following glycated Hemoglobin groups can be seen in Table 3.

**Table 3: Comparison of biochemical levels between glycated Hemoglobin groups in T2DM patients (Kruskal-Wallis test)**

| Characteristics (Mean Rank) | T2DM patients | | | p* |
|---|---|---|---|---|
| | Controlled (N = 25) | Poorly controlled (N = 22) | Uncontrolled (N = 42) | |
| Age (years) | 58.28 ± 1.07 | 50.57 ± 1.75 | 63.18 ± 0.96 | 0.206 |
| FBG (mg/dl) | 25.00 | 39.00 | 60.00 | 0.001* |
| PBG (mg/dl) | 25.22 | 41.57 | 58.57 | 0.001* |
| Interleukin-6 (pg/ml) | 20.76 | 54.50 | 54.55 | 0.001* |
| Glutathione peroxidase (ng/ml) | 44.32 | 54.36 | 40.50 | 0.124 |
| Glutathione (ng/ml) | 44.54 | 54.09 | 40.51 | 0.135 |

p* < 0.05.

The FBG, PBG, and IL-6 levels in the uncontrolled T2DM patients were higher than the poorly controlled and controlled T2DM patients. The Kruskal-Wallis test also showed significant differences between the study groups (p < 0.05). Also, the GPx and GSH levels (ng/ml) in the controlled T2DM patients were lower than the poorly controlled T2DM patients but higher than the uncontrolled T2DM patients. Based on the Kruskal Wallis test, the association between the GPx and GSH levels with the three groups of T2DM patients based on glycated Hemoglobin levels was not significant (p > 0.05).

The comparison of biochemical levels between glycated Hemoglobin groups in T2DM patients can be seen in Table 4.

**Table 4: Comparison of biochemical levels between glycated Hemoglobin groups in T2DM patients (Mann-Whitney test)**

| Characteristics (mean rank) | T2DM patients (N = 89) | | | P* | | |
|---|---|---|---|---|---|---|
| | Controlled[a] (N = 25) | Poorly controlled[b] (N = 22) | Uncontrolled[c] (N = 42) | Ab | Ac | Bc |
| FBG (mg/dl) | 25.00 | 39.00 | 60.00 | 0.022* | 0.001* | 0.001* |
| PBG (mg/dl) | 25.22 | 41.57 | 58.57 | 0.013* | 0.001* | 0.007* |
| Interleukin-6 (pg/ml) | 20.76 | 54.50 | 54.55 | 0.001* | 0.001* | 0.899 |

p* < 0.05.

In Table 4, it can be seen that the Mann-Whitney test showed significant differences in the FBG, PBG, and IL-6 levels between the controlled and poorly controlled T2DM patients (p < 0.05). Similar results were also found between the controlled and uncontrolled T2DM patients. The FBG and PBG levels of the two study groups (poorly controlled and uncontrolled T2DM) were related significantly (p < 0.05), whereas the IL-6 levels of the poorly controlled and uncontrolled T2DM patients showed the opposite result (p > 0.05).

## Discussion

Diabetes mellitus (DM) is metabolic syndrome conditions with an increasing prevalence of patients every year in the world. Chronic DM can cause complications such as hypertension, cardiovascular disease, kidney problems, and death. The American Diabetes Association (ADA) recommends the examination of HbA1c (glycated Hemoglobin) as the long-term glycemic control in DM patients [1]. Glycated Hemoglobin describes the history of blood glucose control in the previous 60-90 days. The glycated Hemoglobin test is recognised as the gold standard in assessing the development of DM [20].

In this study, the average glycated Hemoglobin (%) of T2DM patients was 8.96 ± 2.28 whereas the average duration of DM in the study was 8.60 ± 3.66 years. A long duration of DM disease may cause a high average of glycated Hemoglobin in the study. This is because chronic hyperglycemia can increase glycated Hemoglobin levels by about 2-3 times [21].

In addition to describing a history of blood glucose control in DM, glycated Hemoglobin is also used as an indicator of prognosis in the development of DM complications [20].

The highest percentage was found in the uncontrolled T2DM patients with 47.19%, and the average glycated Hemoglobin (%) in this group was 10.90 ± 0.21. Similar results were also found in Jordanian diabetic patients [22]. Several factors might cause uncontrolled DM, such as education level, type of DM drugs consumed, duration of DM, and weight gain [22], [23]. Moreover, age, gender, and occupational factors may also affect glycated Hemoglobin levels in patients with DM [24]. Other factors that can also affect controlled diabetes regarding glycated Hemoglobin are adherence in diet, physical activity, and family history of DM [1].

Continuously increasing blood sugar levels can increase reactive oxygen compounds (ROS) through enzymatic processes, namely through oxidation, phosphorylation reactions, and ADPH-Oxidase reactions. ROS compounds formed in hyperglycemia will trigger complications in DM patients through several mechanisms. ROS tends to be oxidised which produces some free radical molecules, such as anion superoxide ($O2^-$), hydroxyl radicals (OH), and hydrogen peroxide ($H_2O_2$) [25], [26].

The increased free radicals that exceed the ability of cells in overcoming them will cause damage to various cell components. This condition leads to the damage of DNA, proteins, carbohydrates, lipids, and other macromolecules. It will also result in the fragmentation of proteins and lipids peroxidation and dysfunction of cell membranes and enzymes which will eventually cause cell death. The earliest known mechanism of cell or tissue damage due to attack by free radicals is lipid peroxidation. Malondialdehyde (MDA) is one of the results of lipid peroxidation formed by free radicals under hyperglycemia conditions [6], [27]. Previous studies have shown that there is a relationship between blood glucose and HbA1c levels with an increase in MDA [28], [29].

Another complication due to the increased ROS is the activation mechanism of Nuclear Factor-κB (NF-κB). NF-kB is transcription factors which stimulate the production of proinflammatory cytokines, such as TNF-α and IL-1. These proinflammatory cytokines have autocrine and paracrine effects which can trigger the production of other proinflammatory cytokines, such as IL-6. IL-6 is secreted by T cells and macrophages to stimulate the body's immune response. A systemic proinflammatory cascade process occurs. IL-6 is an intermediate cytokine which is known to have dual functions. First, in an inflammatory state, the production of IL-6 will increase as proinflammatory cytokines. The second function is that IL-6 can activate other macrophage and neutrophil cells to produce anti-inflammatory cytokines. Genes that encode IL-6 (IL6, MIM # 147620) are found on chromosomes (Chr) 7p21 in humans [30], [31], [32].

In the present study, IL-6 levels in the uncontrolled diabetic patients were found to be higher than those of the poorly controlled and controlled diabetic patients ($p < 0.05$). This study also found that there were significant differences in the IL-6 levels between the controlled and poorly controlled group, and between the controlled and uncontrolled group. In contrast, the IL-6 levels did not differ significantly between the poorly controlled and uncontrolled group.

A previous study has reported a significant difference in the IL-6 levels between a group of diabetic patients and a group of healthy people [8]. Furthermore, there was a significant difference found in the IL-6 levels of the controlled and uncontrolled DM patients [33].

If the formation of ROS in diabetic patients exceeds the ability of cells to overcome free radicals, it can cause oxidative stress in the cells. Continuous oxidative stress leads to a decrease in antioxidant capacity [27]. Antioxidants are compounds that can neutralise free radicals produced when ROS is oxidised. Antioxidants have a molecular structure that can give its electron compounds to free radical molecules and can break chain reactions from free radicals. The body can produce antioxidants in the form of enzymes, such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx), and glutathione (GSH) [6].

GPx levels of the controlled T2DM patients in this study were lower than the poorly controlled T2DM patients but higher than the uncontrolled T2DM patients although the difference was not statistically significant. In line with the results of this study, a past

study found that there was no relationship between the levels of GPx and glycated Hemoglobin in T2DM patients. The past study also reported that the GPx levels (U/ml) of controlled DM were lower than those of the uncontrolled DM (3696.25 ± 1199.61 vs 3914.13 ± 1219.36) [34]. Similarly, another previous study showed no relationship between blood glucose levels and glycated Hemoglobin levels with GPx levels even though adjustments have been made to age and gender [35]. In contrast, a study conducted by Wong et al. ,(2018) found that there was a relationship between GPx levels in controlled DM with chronic kidney disease (CDK) and uncontrolled DM in CDK [36]. GPx is an enzymatic antioxidant which is capable of detoxifying hydrogen peroxide and lipid hydroperoxide, preventing the formation of new free radicals, or converting free radicals that have been formed into less reactive molecules. This process occurs using other antioxidants, such as glutathione (GSH) [5], [14].

GSH plays an important role in the maintenance of cell survival, DNA replication, protein synthesis, enzyme catalysis, transport of membrane transduction, receptor action, metabolism and maturation of cells, and regulation of immune cell function. Both GSH and GPx can catalyse the process of reducing fatty hydroperoxide to alcohol and hydrogen peroxide to water. When catalysing the process, disulfide bonds from GSH will bind to form oxidised glutathione (GSSG), and the glutathione reductase (GRx) enzyme can recycle GSSG into GSH again by oxidising NADPH. When cells are exposed to oxidation stress, there will be a cumulation of GSSG, and the GSH/GSSG ratio will decrease [6], [15].

The results showed that GSH levels were higher in the poorly controlled T2DM patients than in the controlled and uncontrolled T2DM patients, but it was not related significantly ($p > 0.05$).

The increase in GSH levels in the poorly controlled T2DM patients may be related to the increase in GPx in the poorly controlled T2DM patients. GSH levels were also seen to be in line with GPx levels in the controlled and uncontrolled T2DM patients. A previous study showed an increase in the GSH levels of T2DM patients compared to the healthy controlled people (6.77 ± 0.59 vs 9.78 ± 0.58 uM /mg of protein) [36] whereas another study showed different result [37]. This might occur because of different compensations of cells to increase the expression of GSH proteins to counteract oxidative stress/ROS [38].

Furthermore, the duration of DM disease can also determine the GSH levels of patients. In addition to the duration of the disease, GSH and GPx levels are also influenced by age. GSH and GPx levels will decrease in older patients as an ageing process. It causes older people to be more vulnerable to free radicals [12]. Several previous studies revealed a decrease in antioxidant status in plasma and serum samples compared to the controls based on age. The decrease in various antioxidants is related to the formation of marker compounds for oxidative stress; for example, an increase in lipid hydroperoxide. In T2DM patients aged 50-60 years, there was an increase in lipid peroxidation since the onset of diabetes [39]. This study found that the average age of the poorly controlled T2DM patients was 50.57 ± 1.75 years. This result was lower than the average age of the controlled and uncontrolled T2DM patients (58.28 ± 1.07, 63.18 ± 0.96, respectively). This difference might have a role in the higher antioxidant levels of GPx and GSH in the poorly controlled diabetic patients than the other two groups.

This research was the first study investigating the topic at USU Hospital with cross-sectional study design. Longitudinal design research needs to be done with a larger size of samples to assess all variables in the data using complete questionnaires. Data can also be obtained regarding the history of the drug used.

In conclusion, this study suggests that glycated Hemoglobin levels were associated with the levels of serum IL-6 but not GPx and GSH in T2DM patients in Universitas Sumatera Utara (USU) Hospital. Future studies should be conducted using a longitudinal design study and a larger size of samples to assess all variables measured in complete questionnaires.

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