

Decreased peroxidase activity of glycated hemoglobin inversely correlated with levels of glycated hemoglobin in type 2 diabetes patients

Pinaki Saha¹, Pradipta Ghosh², Santanu Sen³, Usashi Biswas Bose⁴, Shreya Mandal⁵, Indira Bhaskar Biswas¹ and Utpal Kumar Biswas⁶

¹Department of Biochemistry, KPC Medical College & Hospital, Kolkata, West Bengal, India

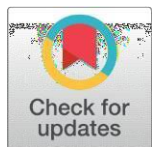
²Department of Biochemistry, R G Kar Medical College, Kolkata, West Bengal, India

³Department of Biochemistry, Calcutta National Medical College, Kolkata, West Bengal, India

⁴Research Society for Study of Diabetes in India, Jaipur National University, Rajasthan, India

⁵Drs. Lal path Lab, Kolkata, West Bengal, India

⁶Department of Biochemistry, North Bengal Medical College, West Bengal, India



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Corresponding Author

Pinaki Saha

spinaki1973@gmail.com

Department of Biochemistry, KPC
Medical College & Hospital,
Kolkata, West Bengal, India

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ABSTRACT

Background and objective: Diabetes mellitus is a metabolic disorder with diverse etiological factors. Imbalances between oxidative stress and antioxidant defenses play critical roles in the pathogenesis and progression of diabetes. Peroxidase, an antioxidant enzyme similar to superoxide dismutase and catalase, is involved in the etiology and progression of diabetes. This study aimed to elucidate the relationship between peroxidase activity of hemoglobin and glycated hemoglobin with fasting blood glucose levels in patients with type 2 diabetes mellitus.

Methods: Sixty-four patients with type 2 diabetes and an equal number of healthy controls were recruited for the study. Peroxidase activity of hemoglobin and glycated hemoglobin was assayed using L. Goth's method for serum catalase assay, with further modifications and standardization.

Results: Peroxidase activity of hemoglobin was significantly reduced ($p < 0.0001$) in patients with type 2 diabetes compared to healthy controls, as was peroxidase activity of glycated hemoglobin ($p < 0.001$). In both patients and controls, peroxidase activity of glycated hemoglobin was significantly higher than normal hemoglobin. In patients with type 2 diabetes, glycated hemoglobin showed a significant negative correlation ($r = -0.308$, $p = 0.013$) with its peroxidase activity, indicating increased activity is associated with poorer glycemic control. Furthermore, peroxidase activity of normal hemoglobin positively correlated with peroxidase activity of glycated hemoglobin (mainly HbA_{1c}) ($r = 0.305$, $p = 0.014$).

Conclusions: Peroxidase activity of glycated hemoglobin increases with glycated hemoglobin levels, indicating deteriorating glycemic control in patients with type 2 diabetes.

Keywords Fasting Blood Glucose, Glycosylated Hemoglobin A, Hemoglobins, Peroxidase, Type 2 Diabetes Mellitus

INTRODUCTION

Diabetes mellitus is a metabolic disorder of multiple etiologies leading to impaired normal glucose metabolism, resulting in the common feature of hyperglycemia. While type 1 diabetes results from an absolute deficiency of insulin, type 2 diabetes is characterized by a marked decrease in insulin function, primarily manifested as insulin resistance along with defects in glucose utilization. In type 2 diabetes, insulin resistance increases either due to receptor or post-receptor cascade defects, as target tissues gradually become insensitive to insulin, resulting in chronic high plasma glucose and insulin levels.¹ In both types of diabetes, oxidative stress is generated during disease development, and as a compensatory mechanism, antioxidant enzymes such as catalase, peroxidase, superoxide dismutase, and glutathione peroxidase are utilized to neutralize the oxidizing effects of reactive oxygen species.^{2,3} Even patients suffering from diabetes for a short period, and yet to develop complications, exhibit reduced antioxidant plasma capacity and uric acid levels, indicating that oxidative stress occurs early in diabetes.⁴

Hemoglobin is a hemoprotein that can act as an enzyme peroxidase with high oxidizing potential when present with oxidizing equivalents like hydrogen peroxide (H_2O_2).⁵ In red blood cells, this dangerous oxidizing tendency is regulated by the reducing environment, primarily reduced glutathione and the deficiency of oxidizing equivalents. Methemoglobin is the ferric form of hemoglobin and is short-lived, being effectively converted back to ferro-Hb (deoxy-Hb) by methemoglobin reductase.^{2,6} Typically, in humans, less than 2% of total Hb exists as methemoglobin because the rate of hemoglobin reduction far exceeds its oxidation.⁷⁻⁹ For Hb appearing outside RBCs in hemolysis, the intracellular protective phenomenon is lost, and Hb exhibits higher peroxidase activity. This is significantly increased in inflammation (e.g., sepsis, diabetes) by the generation of superoxide radicals. The superoxide radicals may be transformed into hydrogen peroxide (H_2O_2), which fuels Hb peroxidase activity.¹⁰

Peroxidases in the body can be either: (1) true peroxidases, which generate free radicals in the peroxidase cycle like myeloperoxidase, eosinophil peroxidase, and lactoperoxidase; or (2) pseudo-peroxidases, enzymes that behave like peroxidases under external conditions, such as hemoglobin, myoglobin, and cytochrome C.¹¹ Enhanced peroxidase activity has been observed in oxidative stress and may initiate various human diseases like diabetes mellitus.¹¹ Therefore, regulating peroxidase activity is important. Not only do structurally different peroxidases differ in properties and location, but there are also specific mechanisms to control peroxidase activity for different hemoproteins.¹¹

The study aims to determine if there is any relationship between the peroxidase activity of hemoglobin and glycosylated hemoglobin in patients with type 2 diabetes mellitus.

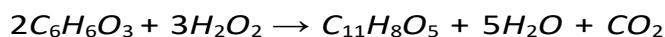
MATERIALS AND METHODS

Participants

Sixty-four patients with type 2 diabetes (30 males and 34 females) and a similar number of healthy controls (35 males and 29 females) from the same age group were selected for the study. Patients aged between 25 to 75 years with type 2 diabetes mellitus diagnosed and confirmed by clinical and biochemical tests were included. However, the following patients were excluded: those with other endocrine disorders, type 1 diabetes, pregnancy, polycystic ovarian disease, renal failure, malignant diseases, or hemolytic anemias like thalassemia. The study was undertaken in 2014-2015 after approval by the Institutional Ethics Committee of N.R.S. Medical College, Kolkata, India.

Peroxidase activity

There are various methods to assay the peroxidase enzyme, with the pyrogallol method being especially notable. In the past, peroxidase activity of a cell was expressed in units based on the oxidation rate of pyrogallol, a method introduced by Willstatter and Stoll in 1917. This is not in accordance with later studies (Maehly and Chance 1954). Willstatter and Heiss (1923) studied the method of oxidizing pyrogallol to purpurogallin. According to their assay:



Thus, the formation of 1 mg purpurogallin should yield 102 μ l of CO₂ (J. Etori, 1948). Sumner and Gjessing (1943) devised a new method conducting the reaction in 20 ml phosphate buffer. They found the resulting peroxidase activity values were somewhat higher than Willstatter's method. Later, the effects of dilution and buffer concentrations on enzyme activity were investigated for different preparations, comparing Willstatter's method to Sumner and Gjessing's. Colorimetric assay methods have also been compared to the monomeric assay based on CO₂ formation when pyrogallol was oxidized with a catalyst. Various hydrogen donor molecules have been used to assay peroxidase, including potentially carcinogenic compounds like o-dianisidine. Some researchers report improved assays using 4-aminoantipyrine as the hydrogen donor (Trinder 1966). Reaction rate is determined by measuring increased absorbance at 505 nm from hydrogen peroxide decomposition.¹² Based on Goth's serum catalase assay method, a modified method to estimate hemoglobin peroxidase activity was developed.¹³ In the original Goth method, serum was used to measure serum catalase activity. In this method, diluted whole blood containing only red blood cells was used to assess hemoglobin peroxidase activity.

The hemolysate prepared from patients' whole blood contains the enzyme peroxidase and supernatant of the resin tube used to estimate glycosylated hemoglobin. Added hydrogen peroxide acts as the substrate. The enzymatic reaction was stopped with 1.0 ml of 32.4 mmol/l ammonium molybdate ((NH₄)₆Mo₇O₂₄, 4H₂O heptamolybdate tetrahydrate), and the yellow molybdate-hydrogen peroxide complex was measured at 405 nm against B₃.

Preparation of buffers and reagents (check careful)

Buffer for peroxidase assay: 60mmol/L Sodium/Potassium Phosphate Buffer, pH 7.4
 32.4mmol/L ammonium molybdate solution
 60mmol/L hydrogen peroxide in 60mmol/L Na-K-Phosphate Buffer, pH 7.4
 Normal saline

Procedure

Hemolysate was prepared by mixing 20 microliters of ethylene diamine tetra acetic acid (EDTA) blood with 180 microliters of normal saline. 1 milliliter of substrate (hydrogen peroxide) was added to 0.02 ml of hemolysate containing hemoglobin peroxidase, and the reaction was stopped by adding 1 ml of ammonium molybdate. Absorbance (B₁) was measured spectrophotometrically at 405nm. Two blanks were calculated - substrate blank (B₂) and true blank (B₃). The substrate blank (B₂) was determined spectrophotometrically by taking the absorbance of 1 ml of substrate and 0.02 ml of buffer with 1 ml of ammonium molybdate. The true blank (B₃) was calculated from the absorbance of 1 ml buffer, 1 ml ammonium molybdate, and 0.02 ml of buffer at the same wavelength.¹³

$$\text{Hemolysate peroxidase}_{(KU/L)} = \frac{(B_1 - B_3) \times 271}{(B_2 - B_3)}$$

For glycosylated hemoglobin, the resin tube supernatant was used instead of hemolysate, and B₁ was calculated with dilution factors adjusted in both cases.

Standardization of peroxidase activity assay

Different volumes of pooled hemolysate were used to construct a curve relating absorbance (B₁) at 405nm (Figure 1).

Other parameter estimation

Fasting blood glucose was estimated by the glucose oxidase peroxidase (GOD-POD) method. Glycosylated hemoglobin was measured by ion-exchange chromatography with standardized kits. Total hemoglobin was estimated by the cyanmethemoglobin method using Drabkin's reagent.

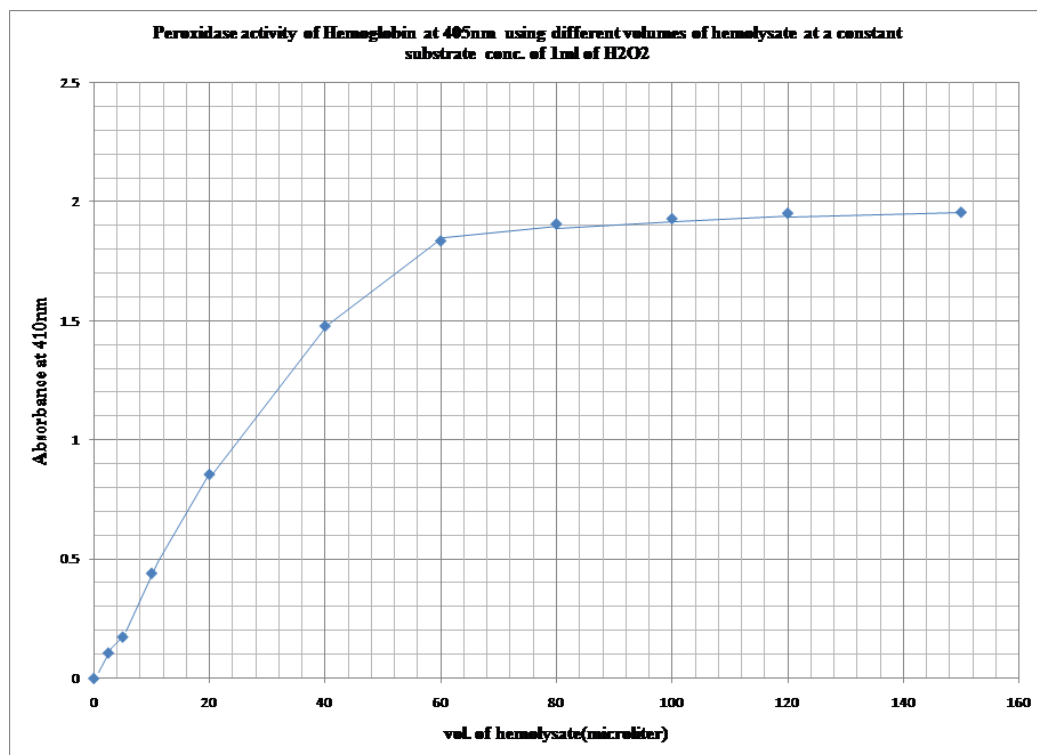


Figure 1 Calibration curve of hemoglobin peroxidase activity.

Statistical Analysis

Data was entered into MS Excel and cleansed. It was then transferred to IBM SPSS version 20 and analyzed. Data normality was checked and descriptive statistics like mean and standard deviation were generated. Analytical statistics like the student's t-test were used for comparisons. A p -value <0.05 was considered significant.

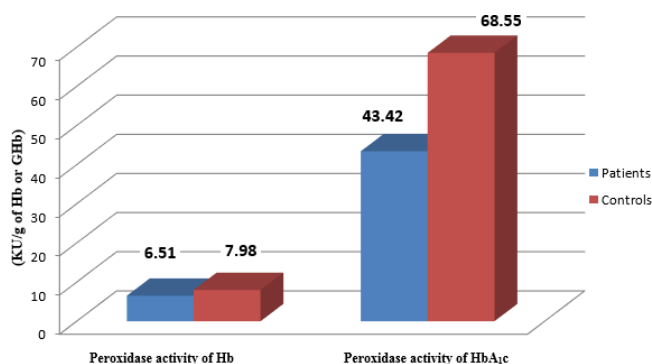
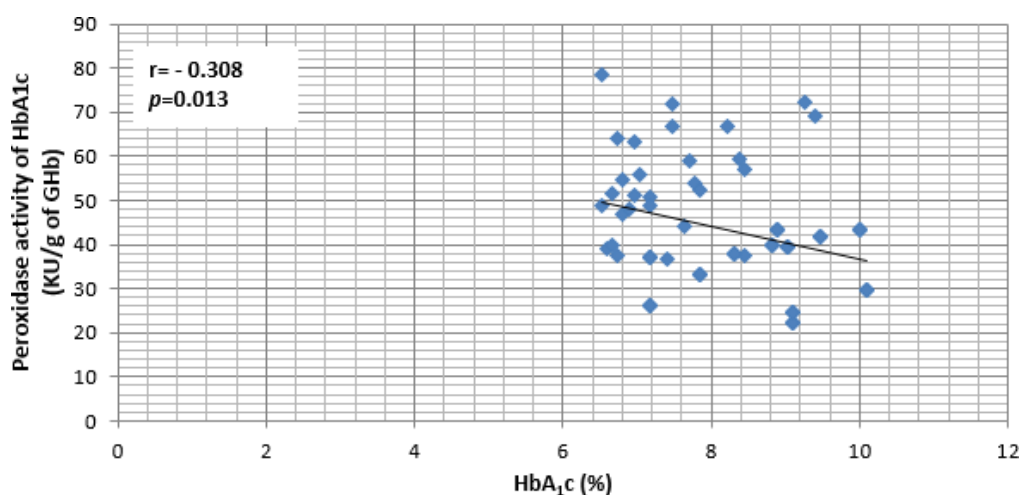
RESULTS

In this case control study, it was found that peroxidase activity of hemoglobin was significantly decreased ($p < 0.0001$) in patients with type 2 diabetes compared to healthy controls, as was peroxidase activity of glycosylated hemoglobin in these patients ($p < 0.001$, Table 1). Additionally, peroxidase activity of glycosylated hemoglobin was significantly higher than peroxidase activity of normal hemoglobin in both patients and controls (Figure 2). Glycosylated hemoglobin showed a significant negative correlation ($r = -0.308$, $p = 0.013$) with its peroxidase activity in patients with type 2 diabetes (Figure 3), indicating that increased activity is associated with poorer glycemic control. Furthermore, peroxidase activity of normal hemoglobin positively correlated with peroxidase activity of glycosylated hemoglobin (mainly HbA_{1c}) ($r = 0.305$, $p = 0.014$).

Table 1. Demographic and biochemical parameters of patients & controls.

Variables	Patient (N=64)	Control (N=64)	P-value
Age (years)	47.910.7	53.710.9	Insignificant
Sex (M/F)	30/34	35/29	-
Bodymass index (BMI)	23.0±2.7	23.6±2.5	Insignificant
Fasting blood glucose (mg/dl)	171.733.9	96.317.2	<0.001*
Glycosylated haemoglobin (%)	8.2±1.1	5.60. 6	<0.001*
Total hemoglobin (g/dl)	12.1±1.4	12.4±2.0	<0.001*
Peroxidase activity of hemoglobin (KU/g of Hb)	6.5±1.8	8.0±2.1	<0.001*
Peroxidase activity of glycosylated hemoglobin (KU/g of GHb)	43.4±13.5	68.6±21.2	<0.001*

Define the abbreviations included in the table rows please

**Figure 2 Comparison of mean peroxidase activity of hemoglobin and glycosylated hemoglobin in patients with type 2 diabetes and healthy controls.****Figure 3 Correlation scatterplot of HbA_{1c} and peroxidase activity of glycosylated hemoglobin in patients with type 2 diabetes.**

DISCUSSION

It has been well established that like many other chronic diseases, type 2 diabetes is also associated with increased levels of oxidative stress and reduced antioxidants *in vivo*.¹⁴⁻¹⁶ In our study, peroxidase activity of both hemoglobin and glycosylated hemoglobin was significantly reduced in patients with type 2 diabetes compared to healthy controls. In an experimental study with diabetic rats, Wu *et al.* (1999) reported that in diabetic rat kidney tissue, superoxide dismutase, peroxidase and catalase activities significantly declined at weeks 8 and 16.¹⁷ In 2014, Kresyun *et al.* reported that after modeling streptozotocin-induced diabetes in Wistar rats, antioxidant enzyme activities of superoxide dismutase and catalase decreased.¹⁸ In previous studies, it has been reported that peroxidase activity in plasma is decreased in patients with chronic diseases like type 2 diabetes, where increased oxidative stress plays an important role from the early stages. In one similar study, glutathione peroxidase levels decreased in patients with type 2 diabetes with or without complications like coronary artery atherosclerosis, which supports our findings.¹⁹ This decrease in the peroxidase antioxidant enzyme has been attributed to poor glycemic control (decreased glycosylated hemoglobin). The peroxidase activity of hemoglobin depends on the pH of the solution.²⁰ However, at the same pH, the peroxidase activity of glycosylated hemoglobin is higher than that of normal hemoglobin for individual subjects in our study.

There are certain limitations in this study. As a pilot study, this case control study had a small sample size. In the future, the researchers plan to recruit more patients with type 2 diabetes as cases, and more healthy volunteers as controls. In this study, there is no scale exhibiting the increase in peroxidase activity of glycosylated hemoglobin corresponding to the percentage increase in glycosylated hemoglobin. This again requires recruiting a large number of study subjects.

CONCLUSIONS

The peroxidase activity of glycosylated hemoglobin increases with levels of glycosylated hemoglobin, indicating deteriorating glycemic control in patients. If spectrophotometric assays of glycosylated hemoglobin peroxidase activity using inexpensive reagents could be developed, this may allow assessment of patients' glycemic control.

DECLARATIONS

Authors' contributions

Conceptualization: UKB, PS, PG. Data curation: PS, PG, SM. Formal analysis: PS, UKB, PG, SS, UBB. Funding acquisition: N/A. Investigation: PS, UKB, PG, SS. Methodology: PS, PG, UBB, SM. Project administration: UKB, SS, PG. Resources: UKB, PS, PG, SS, UBB. Software: PS, UKB, UBB. Supervision and visualization: UKB, PS, SS. Validation: UKB, PS, PG. Writing-original draft: PS, SS, UBB, IBB. Writing-review & editing: UKB, PS, SS, IBB.

Conflict of interest

None.

Data availability

The data that support the findings of this study are available from the corresponding author, PS, upon reasonable request.

Ethical approvals

The study was ethically approved by the Institutional Ethics Committee of N.R.S. Medical College, Kolkata, India.

Funding resources

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