



Correlation of serum fructosamine, erythrocyte $\text{Na}^+ - \text{K}^+$ ATPase and glutathione peroxidase with HbA1c levels

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Glycated hemoglobin is the frequently used test in the diagnosis of diabetes mellitus. However, because this test is affected by various factors and may not be accurate for patients of certain health conditions, the present study aims to explore the association between potential and cost-effective markers like serum Fructosamine, Erythrocyte $\text{Na}^+ - \text{K}^+$ ATPase, and Glutathione peroxidase with altering levels of established marker HbA1c (Glycated haemoglobin). The study showed that serum Fructosamine has a statistically significant ($P = <0.0001$) association with increasing levels of HbA1c as well as blood glucose. There was a 100% sensitivity and specificity for serum fructosamine test against HbA1c in ROC analysis, however, the erythrocyte glutathione peroxidase and erythrocyte membrane $\text{Na}^+ - \text{K}^+$ ATPase activity was not affected by increasing HbA1c levels.

Keywords: Diabetes mellitus, Fructosamine, Glycated hemoglobin, Sodium potassium ATPase

Glycated haemoglobin (HbA1c) is a product of a non-enzymatic glycation pathway. It occurs when haemoglobin is exposed to blood glucose. Measuring the HbA1c level provides a three-month average blood glucose concentration since the life span of red blood cells is of approximately 90-120 days (4 months). Due to frequent improvement in the quality of the test, the HbA1c test is being considered more and more in the diagnosis of diabetes. A person is said to be diabetic if their HbA1c level is $\geq 6.5\%$ ¹. However, the HbA1c level is affected by conditions such as hemolytic anemia, chronic renal failure, and the presence of variant haemoglobin. Their levels are also underestimated in patients with poor glycemic control as the survival of red blood cells is shortened

albumin, lipoproteins, *etc.* is 10 fold higher than that of human hemoglobin. Therefore, they exhibit a broader fluctuation and allow early detection of rapid changes in blood glucose¹.

However, it was found that glycated proteins (fructosamine) formed as a product of non-enzymatic glycation cause oxidative stress in conditions like diabetes⁴. Increased levels of glycated proteins and oxidative stress have a combined role in modifying various ion channels and transporters⁵. Erythrocyte membrane $\text{Na}^+ - \text{K}^+$ ATPase is one such transporter/pump which is affected. It is an enzyme present on the cell membranes of various cell types. It is necessary for the generation and maintenance of the Na^+ and K^+ gradients across the cell membrane and for cellular

measurement of HbA1c levels is costly which requires expensive reagents and instruments³. Alternatively, fructosamine, a ketoamine, synthesized by the non-enzymatic glycation of serum protein molecules (Albumin, lipoproteins, globulins *etc.*) to carbohydrate molecules (Glucose) provides a measure of average blood glucose concentration over a period of 1-2 weeks, when compared to HbA1c level. Also, the rate of glycation of serum proteins such as

diabetic conditions has a significant effect on the metabolism of erythrocytes which may cause decrease in the activity of $\text{Na}^+ - \text{K}^+$ ATPase and further decreasing the life span of erythrocytes in hyperglycaemic condition⁶⁻¹⁰. Diabetes-induced metabolic changes such as increase in oxidative stress can also down-regulate the enzyme activity in erythrocytes¹¹.

Glutathione peroxidase is one of the antioxidant enzymes found in erythrocytes. Oxidative stress can be defined as a disruption of the balance between the free radicals production and the defense mechanism brought about by the antioxidant system. Recent

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studies have found out that uncontrolled hyperglycemia is associated with oxidative stress¹² as it causes an increase in the production of superoxide anion and other reactive oxygen species¹³ as well as impairs the activity of antioxidant enzymes including glutathione peroxidase¹⁴.

The current study was designed to evaluate the association between the glycosylated protein Fructosamine, erythrocyte enzymes Na⁺-K⁺ ATPase, and Glutathione peroxidase with altered levels of HbA1c. This will help to further investigate whether an increase in the level of HbA1c causes a decrease in the activity of Erythrocyte Na⁺-K⁺ ATPase and Glutathione peroxidase. There have been studies conducted previously that have found a significant association between HbA1c and fructosamine³, HbA1c, and Glutathione peroxidase activity in erythrocytes¹⁵ and slightly lesser significant association between HbA1c and Na⁺-K⁺ ATPase¹⁶. But there has been no attempts made to study the simultaneous association of the three parameters (serum fructosamine, erythrocyte Na⁺-K⁺ ATPase and glutathione peroxidase) with different levels of HbA1c *i.e.* Normal, high and extremely high levels of HbA1c.

Material and Methods

The study protocol was approved by the Institutional Ethics Committee, Kasturba Medical College, Manipal, India. A total of 191 whole blood samples that were referred for the HbA1c test and blood glucose test were collected from the Clinical Laboratory of Biochemistry, KMC, Manipal after proper anonymization. The samples were collected based on their HbA1c levels and age (ranging from 35-65 years old) of the patient. Based on HbA1c levels the samples were categorized into 5 groups – 4 cases (ranging from 6.5%-14%) and 1 control (4.7%-5.7%). The control group was defined based on the normal range described by the said laboratory. HbA1c and blood glucose values for the samples were obtained from the laboratory after the regular analysis. Whole blood was centrifuged at 2000 rpm to separate the serum. Serum was stored separately at -20°C for the estimation of fructosamine and erythrocyte pellet was used for the estimation of Na⁺-K⁺ ATPase and Glutathione peroxidase.

Serum fructosamine was estimated by Nitroblue Tetrazolium chloride (NBT) method¹⁷.

Estimation of erythrocyte membrane Na⁺-K⁺ ATPase activity was done by first extracting the erythrocyte membrane. Further, the reaction was

initiated by adding ATP to the assay medium. Inorganic phosphate released in the reaction was measured using Fiske- Subbarow¹⁸ method and membrane protein estimation was done by Lowry method¹⁹. The enzyme activity was expressed as micromoles of Pi liberated/15 min/ug of protein.

Estimation of erythrocyte glutathione peroxidase activity was done by first extracting the enzyme from the erythrocyte pellet. Enzyme activity was estimated using an assay medium containing Hydrogen peroxide as a substrate along with glutathione reductase to complete the reaction. The data collected was compiled and analyzed to result in the conclusion of the study. To determine the association between the parameters and HbA1c as well as blood glucose, Pearson's correlation was used. $P < 0.05$ was considered to indicate the statistical significance. The optimal sensitivity and specificity of the fructosamine test to predict the respective HbA1c levels were examined using receiver operating characteristic curve (ROC) analysis.

Results

Sample distribution into groups based on HbA1c levels is illustrated in (Table 1).

A steady increase in serum fructosamine levels was observed in each group with an increase in HbA1c levels (Fig. 1). A significant correlation observed with $P = < 0.0001$, between HbA1c levels and serum fructosamine levels when all cases and controls were combined. (Fig. 2) A significant correlation with $P = < 0.001$ was observed between serum fructosamine levels and blood glucose levels when all cases and controls were combined. (Fig. 3).

The largest AUC in ROC analysis was obtained for Group 4 with specificity and sensitivity at 100%. (Fig. 4). Whereas, for Group 3 sensitivity was 98.6% and specificity was 100% under ROC analysis (Fig. 5).

Figure 6 represents an association of erythrocyte membrane Na⁺-K⁺ ATPase activity with HbA1c levels between the groups. The activity of the enzyme was found to be lower in groups 2 & 3 when compared to control and a sudden increase in group 4 was observed.

Table 1 — Distribution of samples into control and cases based on their HbA1c levels. 72 samples in each group were obtained

Groups	HbA1c(%)	HbA1c(mmol/mol)
Control	4.5-5.7	27.9-35.34
Group 1	6.5-7.5	40.3-46.5
Group 2	7.6-8.5	47.12-52.7
Group 3	8.6-9.5	53.32-58.9
Group 4	9.6-14	59.52-86.8

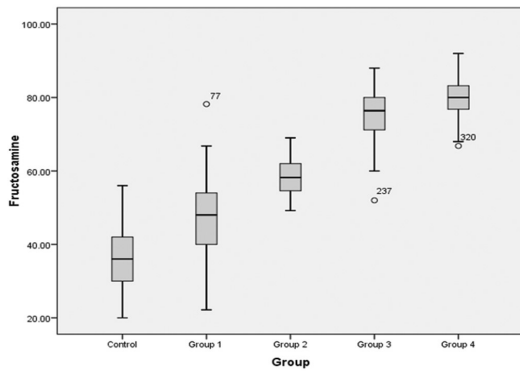


Fig. 1 — Box plot diagram for measured serum fructosamine (mg/dL) levels between the groups

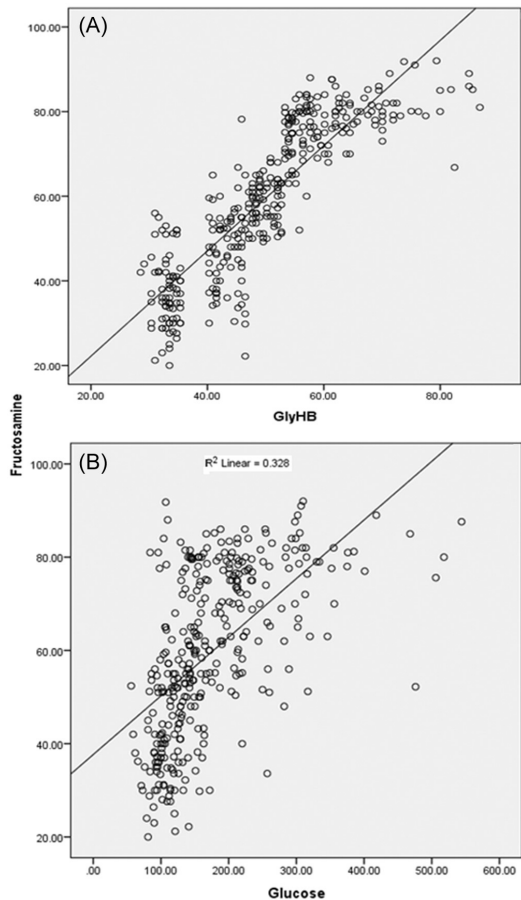


Fig. 2 — Correlation between (A) HbA1c (mmol/mol); and (B) blood glucose (mg/dL) levels with measured serum fructosamine (mg/dL) levels. Pearson's Correlation; N= 370; $r= 0.858$; $P= <0.0001$

We will further look for the reason behind this sudden increase.

There was no significant change observed in the activity of Glutathione peroxidase enzyme with an increase in the HbA1c levels.

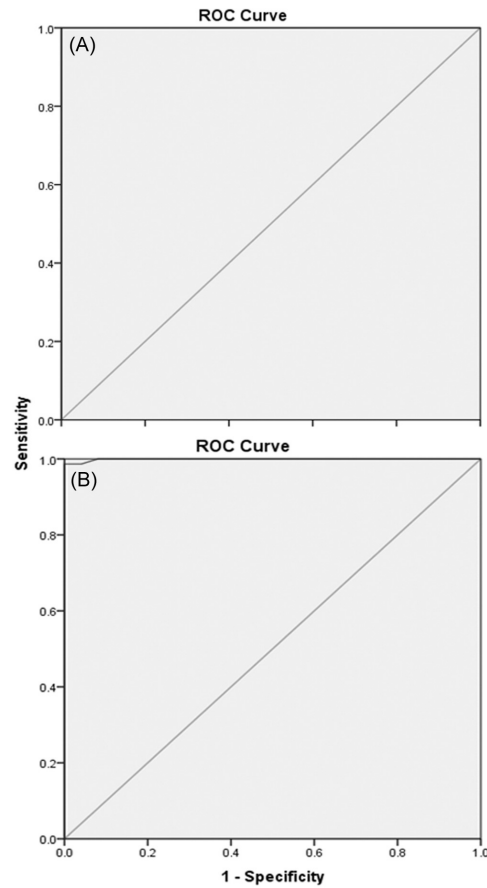


Fig 3 — ROC curve for the reliability of Serum Fructosamine test when compared to HbA1c test (A) (Group 4); and (B) (Group 3). Pearson's correlation; N= 370; $r = 0.573$; $P= <0.0001$. AUC 0.999, 95% CI, 0.997-1.000; sensitivity 100%; specificity 100%; Cut off 61.4

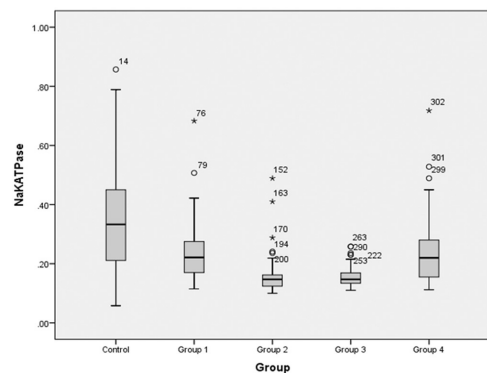


Fig 4 — Box plot diagram for measured erythrocyte membrane Na^+-K^+ ATPase activity levels between the groups. AUC 0.999, 95% CI, 0.997-1.000; sensitivity 98.6% ; specificity 100%; Cut off 58

Discussion

Previous studies³ have shown a significant association between serum fructosamine and HbA1c, similar to the results obtained in the current study, where a steady increase in serum fructosamine was

observed in each group. Also, when the groups were dissolved, serum fructosamine levels were observed to have a significant ($P = <0.001$) association with HbA1c levels. Similarly, blood glucose levels showed a significant ($P = <0.001$) association with serum fructosamine levels as well. The ROC curve obtained show that serum fructosamine test has an excellent sensitivity and specificity to the HbA1c levels. These results all-in-all indicate an increase in the formation of serum fructosamine as there is an increase in blood glucose levels and that it can be considered for its use as an alternative marker to HbA1c, as it is cost-effective, requires lesser time and can detect rapid changes in blood glucose levels compared to HbA1c.

As we know, $\text{Na}^+\text{-K}^+$ ATPase is a membrane protein, which has a vital role in maintaining the resting membrane potential. Based on the previous studies, activity of erythrocyte $\text{Na}^+\text{-K}^+$ ATPase is affected in conditions like diabetes due to the increase in blood glucose levels. But, the current study showed no association between the erythrocyte $\text{Na}^+\text{-K}^+$ ATPase activity and the HbA1c levels when compared between control and the four diabetic groups (Group 1-4). There are only scarce data on the correlation between erythrocyte $\text{Na}^+\text{-K}^+$ ATPase and HbA1c levels. A study, who obtained similar results as ours, stated that in diabetic condition an increase in blood glucose level does not cause the impairment in the activity of erythrocyte $\text{Na}^+\text{-K}^+$ ATPase²⁰. Contradictory to this, a Japanese study on type II diabetic patients revealed a slight reduction in the activity of erythrocyte $\text{Na}^+\text{-K}^+$ ATPase, but only in those with microalbuminuria²¹. Further, an elaborate study needs to be done to clarify the differences in the results obtained by us and previous studies and to find out the reason behind the sudden decrease in the activity of erythrocyte membrane $\text{Na}^+\text{-K}^+$ ATPase in group 2 and group 3 (Fig. 6)

Diabetes has been suggested to produce abnormally high levels of reactive oxygen species *via* mitochondrial electron transport chain and glucose autoxidation. The increased levels of free radicals formed during such conditions together with products of non-enzymatic glycation, glucose oxidation, and lipid peroxidation cause damage to enzymes, cell functioning, and insulin resistance due to oxidative stress¹²⁻¹⁴. Antioxidant enzymes on the other hand, such as erythrocyte glutathione peroxidase counteract

against free radicals formed during oxidative stress^{22,23}. In our attempt to evaluate its association with HbA1c levels, we observed that there was no association between erythrocyte glutathione peroxidase with increasing levels of HbA1c. There was no difference in the activity of erythrocyte glutathione peroxidase when compared between the control and diabetic groups (Group 1-4). Different studies have reported variations in the activity of antioxidant enzymes in different tissues like liver, kidney, muscle, erythrocytes *etc.*, in normal and diabetic conditions and an increase in the glutathione peroxidase activity was observed in erythrocytes^{24,25}. Whereas, there are studies imply there was a significant decrease in the activity of erythrocyte glutathione peroxidase when compared between diabetic and non-diabetic groups²⁶. At the same time, there are studies that found no significant difference in activity of glutathione peroxidase in erythrocytes between the two groups, similar to the current study²⁷. There is discrepancy among the results from previous studies and the present study. There could be various reasons for this which is yet to be cleared. Although, a recent study has shown that erythrocyte glutathione peroxidase activity begins to improve as insulin treatment is started. This could be one of the reasons for variations in the results.

Conclusion

The findings from the present study suggest that serum fructosamine has similar susceptibility to an increase in blood glucose levels as HbA1c. We observed that as there is an increase in HbA1c level and blood glucose level there is an increase in serum fructosamine levels. The fructosamine test is a cost-effective, time-saving, and more sensitive to changes in blood glucose levels. On the other hand, erythrocyte glutathione peroxidase and erythrocyte membrane $\text{Na}^+\text{-K}^+$ ATPase activity were found to be not affected by increasing HbA1c levels (a non-enzymatic glycated protein).

Conflict of interest

All authors declare no conflict of interest.

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