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# In Vitro Measurements of Glucose Consumption and Glycated Hemoglobin in Whole Blood Exposed to Glucose and Various Temperatures

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## Abstract

**Background:** *There is a proportionate relationship between glycosylated hemoglobin (HA1c), a key marker for diabetes and mean blood glucose. An HA1c value of 6.5% and 10% translates respectively to an estimated average blood glucose of 140 and 240 mg/dL. However, it remains unclear how quickly hemoglobin interacts with glucose to significantly alter HbA1c value, especially when red cells from non-diabetics are exposed to high glucose concentration.*

**Methods:** *In this study, we exposed whole blood from healthy donors to high glucose concentration in vitro and assayed HA1c levels at varying intervals. Using a glucose strip and A1CNow Plus test kit, blood glucose and HbA1c value of the specimen were measured and recorded*

**Results:** *We measured an increase in HA1c value within 3 days of exposing whole blood specimen to high glucose at ambient temperature. Further increases in HA1c were measured at day 4 of incubation, although a trace amount of hemolysis was observed in the specimen. We also found that the rate of glucose depletion in whole was low temperature was slower.*

**Conclusion:** *Glucose measurements decrease in stored whole blood. The rate of decrease was temperature dependent, fastest at 37 0C compared to 4 0C. Glycosylation of hemoglobin changed by as much as 15±5% within three days when red cells were exposed to high glucose concentration, hence increasing one's risk of developing diabetes and its associated complications.*

**Abbreviations:** *HA1c - hemoglobin A1c (glycosylated hemoglobin); ADA - American Diabetic Association; OGTT - oral glucose tolerance test; eAG – Estimated Average blood Glucose*

**Keywords:** *Whole blood; Diabetes; glucose; the A1CNow PLUS*

## 1. INTRODUCTION

Blood glucose and glycated hemoglobin A1c (HA1c) are measured to screen, diagnose and/or monitor a patient's response to the management of diabetes. The American Diabetes Association (ADA) uses the following criteria to diagnosis diabetes mellitus (1, 2): (i) fasting plasma glucose of or greater than 126 mg/dL, (ii) random plasma glucose of or greater than 200 mg/dl along with diabetic associated symptoms (polydipsia, polyuria, polyphagia etc.), (iii) when the patient's HA1c value is equal to or greater than 6.5%, or (vi) the Oral Glucose Tolerance Test (OGTT when plasma glucose after 2-hour plasma glucose is equal to or greater than 200 mg/dL.

The measurement of HA1c levels result from the irreversible binding of glucose to the N-terminal valine of the beta chain of hemoglobin and correlates directly with plasma blood glucose level. Plasma glucose can be estimated from HA1c using the equation [eAG (mg/dL) = 28.7 x HbA1c – 46.7] (3). An HA1c value of 6.5 (diagnostic of diabetes), for example translates to an average blood glucose of ~140 mg/dL.

In addition to diagnosing diabetes, HA1c value is measured to monitor a patient's compliance with medication and the management of their disease. Unlike the other diagnostic criteria, HA1c measurements reflect the average blood glucose level during the preceding 3 to 4 months. It is stable

and less subjected to changes in biological or analytical variables (4). HA1c provides also a better indication of patient's compliance with medication and predicts more accurately the patient's clinical outcomes such as risk of stroke and heart disease (1). Proper control of blood glucose and HA1c levels can significantly reduce the patient's risk of developing diabetic complications such as retinopathy, nephropathy, and neuropathy (5).

As the main and preferred energy source of cells, newly synthesized red cells are constantly exposed to ambient blood plasma glucose of 70-100 mg/dl under normal conditions. At higher ambient glucose levels, more glucose enters the red cell via membrane transporters and interact with hemoglobin to produce an elevated HA1c value. We investigated how quickly and under what conditions hemoglobin glycosylation occurs to significantly alter HA1c value when whole blood is exposed to high glucose in vitro.

## 2. MATERIALS & METHODS

Whole blood specimen was collected by venipuncture from a healthy donor into an EDTA vacutainer and gently mixed with back-and-forth inversion five times to ensure homogeneity of the specimen. Using a glucose strip and A1CNow Plus test kit, blood glucose and HbA1c value of the specimen were measured and recorded. Institutional Review Board (IRB) approval #22/03-0031 is filed with Morgan State University IRB Compliance Office of Sponsored Programs and Research.

To determine the effect of increased plasma glucose on HA1c, a glucose the concentration dependent studies was setup. Blood specimen were aliquoted by placing 200 µL of the uniform mixture into a 2 ml Eppendorf tube and brought to a total volume of 300 µL with a stock solution for a final glucose concentration ranging from 0 to 400 mM). The same set up was prepared with fructose concentration as a negative control. The mixtures were incubated at room temperature on a gently rocking platform and assayed at various time (24, 48, 72, and 96 hours) intervals for HbA1c levels using the A1CNow PLUS test kit obtained from Fisher Scientific. During each measurement, the specimen was observed for hemolysis. A peripheral blood smear was also prepared for microscopic observation.

To determine the effect of temperature on glucose consumption, 1 ml of whole blood was aliquoted into 2 ml Eppendorf tube and incubated at various temperatures [0 0C, 4 0C, ambient (24 0C) and 37 0C]. After day 1 (24 hours) incubation, glucose was measured. The measurement was repeated after day 2 and after day 3. HA1c levels were also measured after day 3 of incubation.

All glucose and HA1c measurements were performed respectively, using the True Metrix Pro-McKesson Professional Monitoring Blood Glucose Meter and the A1CNow PLUS test kit obtained from Fisher Scientific. During each measurement, the specimen was observed for hemolysis. Peripheral smears were also prepared

## 3. RESULTS

The HA1c levels increased in whole blood exposed to high concentration of glucose. Figure 1 shows an elevated HA1c level when whole blood was exposed to increasing glucose concentration within 3-days. The increase was concentration dependent and rose by as much as 15% of baseline by day three. Fructose, a structural isomer of glucose, served as a negative control and had no impact on the HA1c value. A time dependent study in figures 2, also show an increase in measured HA1cby as much as 15 ±5% from baseline control when cells were exposed in 100 mM glucose for up to 72 hours at room temperature. At 96 hours of incubation, hemolysis was evident a with further elevation in HA1c value by and additional~10% from previous day (72 hours) (indicative of the irreversibility of the interaction).

HbA1c values below 5.7 is considered normal, between 5.7 and less that 6.5 is prediabetic and 6.5 and above is diagnostic of diabetes. The result indicates that someone with HbA1c is close to the high-end of normal is going to be prediabetic, and a prediabetic is likely to diabetic if their blood is exposed to high sugar.

Because of the irreversibility of the interaction and the duration of the red cell life span, these individuals are likely to remain in their new condition for approximately four months. Any meaningful drop in HbA1c will only occur when glucose levels fall and replacement of older with newly synthesized red cells from the bone marrow. The peripheral smears in figure 3, show intact red cells, but smaller in size after three days of incubation with glucose at room temperature. In figure 4, glucose depletion was

measured over time in whole blood incubated at various temperatures. The rate of decreased was temperature dependent and slowest at 0 $^0$C.

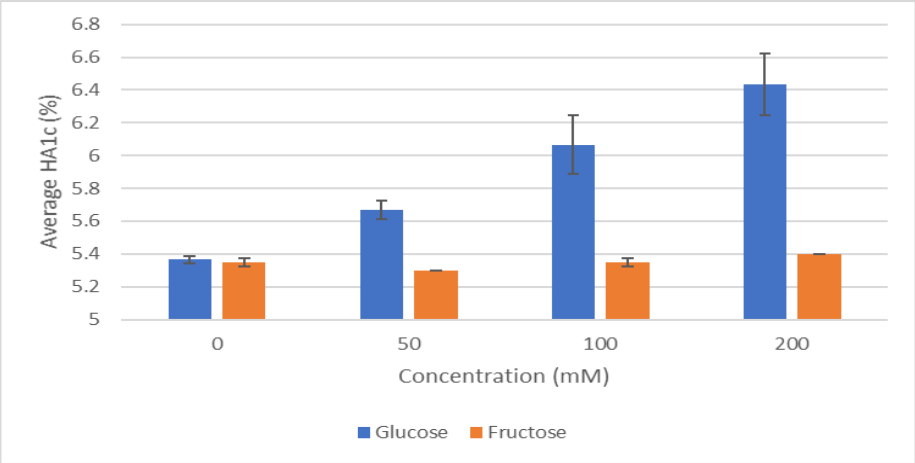


**Figure 1.** *HA1c values (%) from glucose-hemoglobin concentration dependent interaction, average from triplicates following a three-day (72 hours) incubation at room temperature. Fructose (negative control) is an isomer of glucose. Blood glucose measurements were within reference range (70-100 mg/dL) in all collected samples from healthy donors prior to setup of the experiment.*

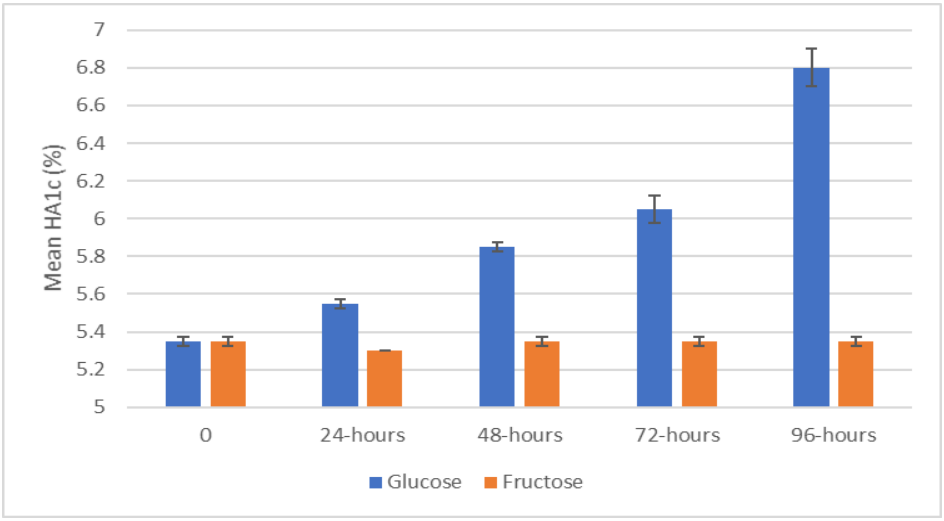


**Figure 2.** *Time dependent study of glucose –hemoglobin interaction. Specimen were incubated at room temperature with 100 mM (1800 mg/dl) glucose and assayed at various intervals for HA1c levels. Average from triplicates. Fructose (negative control) is an isomer of glucose.*

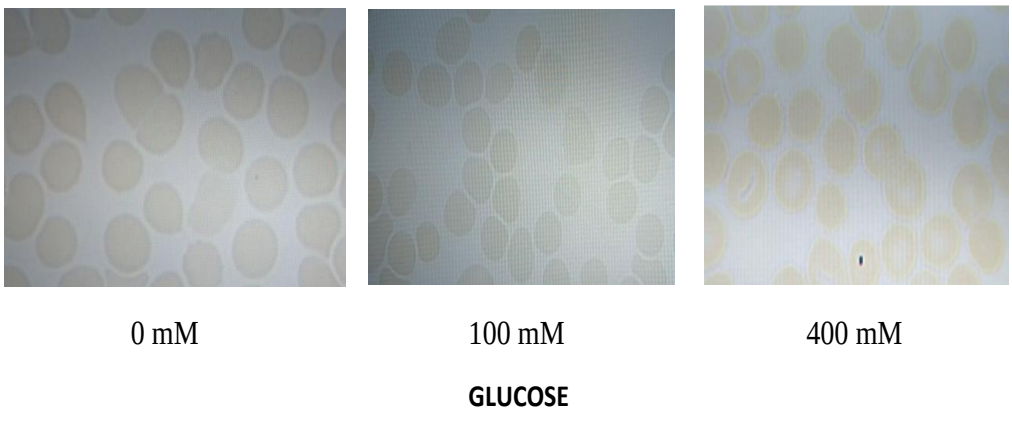


**Figure 3.** *Peripheral smear shows intact red cells post 3 days of incubation at room temperature. The decreased cell size is not unexpected with increased plasma tonicity due to the elevated glucose content.*

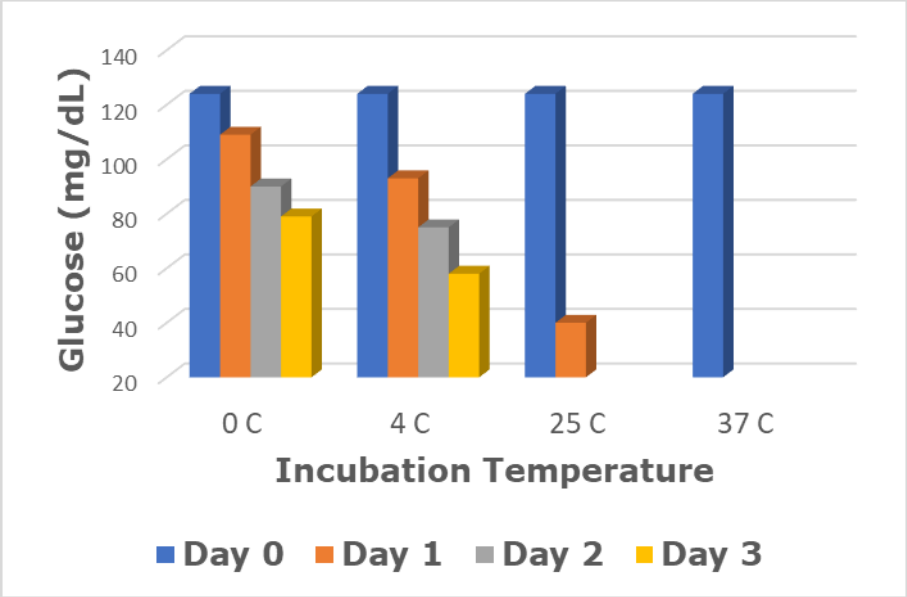


**Figure 4.** *Effect of temperature on the rate of glucose depletion in stored whole blood. Rate of depletion (increased consumption by red cells) was faster with increasing temperature. Glucose level below 20 mg/dl in samples was not measured, below the limit of detection for the analyzer.*

## 4. DISCUSSION

Hemoglobin A1c increased by as much as 15±5% on exposure of whole blood to high glucose in vitro. The elevated measurement correlated with increasing glucose concentration by day 3 (Fig.1). In agreement with the observation of Hughes et al (6), the cells were intact after three days of incubation (Figure 3). Although an additional increase in measured HA1c value (Figure 2) was measured by day 4 (96 hours) of incubation, specimen hemolysis was observed. This suggests a direct interaction, unperturbed by the red cell membrane occurred between glucose and the leaked hemoglobin from the ruptured cells. In intact red cells, the cell membrane serves as a barrier - regulating, via specific glucose transporter 1 (Glut 1) the entry of glucose into the cytosol ().

HA1c level reflects a patient's diabetic status. An increase of 15±5% would push the HA1c value for a healthy, non-diabetic (4.0-<5.7) or prediabetic (5.7-<6-5) individuals respectively into either the prediabetic or diabetic (>=6.5) classification. With appropriate blood glucose control [70-100 mg/dL (3.5-5.5 mM)], it will take a prediabetic at least four months, which is lifespan of a red cell to get their HA1c level back to normal. Because diabetes is chronic disease, appropriate glucose control and management necessary to keep HA1c value below 7.0, thus reducing the chances of developing complications associated with diabetes (5). As red cells age, they become smaller and denser, with higher HA1c content compared to the younger population (7). Also, the older cells are more likely irreversibly glycosylated because they have been more exposed to glucose longer than new synthesized young cells. If glucose level in the blood is properly controlled, HA1c levels should decrease with the removal and sequestration of older cells with higher HA1c content from blood.

The binding of glucose to the N-terminal amino acids of the beta chain of hemoglobin A is a nonenzymatic process dependent on blood glucose concentration and the age of the red cells. Glucose reacts nonenzymatically with the NH2-terminal amino acid of the beta chain of human hemoglobin A by way of a ketoamine linkage, resulting in the formation of hemoglobin AIc. Other minor components appear to be adducts of glucose 6-phosphate and fructose 1,6-diphosphate. This hemoglobin is formed slowly and continuously throughout the 120-day life-span of the red cell. There is a two- to threefold increase in hemoglobin AIc in the red cells of patients with diabetes mellitus. By providing an integrated measurement of blood glucose, hemoglobin AIc is useful in assessing the degree of diabetic control. Furthermore, this hemoglobin is a useful model of nonenzymatic glycosylation of other proteins that may be involved in the long-term complications of the disease. The older the erythrocyte, the higher the amount of glycosylation. Our result is consistent with the report of Viskupicova J. et al (8). where high glucose led to increase overall glycation A1) of the erythrocytes, a reliable indicator of mean blood

glucose levels for 8-12 weeks prior to the measurement. In addition to glycation, Viskupicova J. et al also noted changes in cellular parameters such as, loss of glutathione S-transferase and glutathione reductase activities. However, the increase in A1c resulting from the exposure of whole blood to high demonstrates how quickly one can become prediabetic or diagnosed as diabetic when red cells are exposed to high glucose. As a marker of diabetes, A1c represents a unique fraction of glycated hemoglobin that is measured to determine an individuals' average blood glucose (diabetic status) over a three-to-four-month period and diabetic status.

Glycosylation of hemoglobin is dependent on the age of the red cells and glucose concentration in blood plasma. In diabetics and well as older red cells, the proportion of glycosylation is greater. Viskupicova reported a time-dependent increase in hemoglobin glycosylation when cells were incubated in high glucose concentration. There is a correlation between a specific fraction of glycosylated hemoglobin, also known as hemoglobin A1c (HA1c) and diabetic complications. HA1c value of 6.5 or greater is consider diabetic. As HA1c values increases, the relative risk associated with micro/macrovascular complications increases (5). Diabetics are at increased risk of developing blindness, renal failure, loss of sensation and amputation if their HA1c value exceed 7.0. Because this fraction of hemoglobin glycosylation directly correlates with diabetes/complications, this study seeks to investigate how quickly HA1c values change when red cells (whole blood) is incubated with different concentrations of glucose.

Glucose enters the cell via glucose transporters by facilitated diffusion, a passive process or by an ATP-dependent active transport mechanism. In human erythrocytes, glucose transport is mediated passively by GLUT1 (in humans) and/or an insulin sensitive GLUT4 transporters. Glut 1 has a high affinity for glucose and helps maintain the basal rate (~ 5mM) of glucose uptake. In vitro studies show that phosphorylation of serine 226 in GLUT1 leads to rapid increase in glucose uptake (9).

Because the formation of HA1c is not reversible, HA1c values can only change after about four months with appropriate control of blood glucose levels, thus minimizing further interaction with newly synthesized red cells. As a chronic condition, it remains however unclear why HA1c level does not drop to below 6.5 (prediabetic or normal reference ranges) following proper control and management blood glucose once a patient is diabetic. Glycosylated hemoglobin alters red cell membrane properties, which perhaps favors further glycosylation even when blood glucose is well managed. Altered membrane properties leads to an increase in reactive free radicals in the cells and red cell aggregation. Aggregation results in increased blood viscosity, reduced flow, and hypertension (10). Increased free radicals decreases phagocytic index and one's ability to fight infections (11).

HA(1c) levels also reported to increase with age, by as much as 0.76 mmol/mol (0.07%) per 10 years in subjects with normal glucose tolerance (12). Besides the diagnostic capability associated, HbA1c is valuable for monitoring and managing of the disease. Hence age should be considered when using HA1c for diagnosis and management of diabetes. In managing diabetes, the goal is to keep the HbA1c level to below 7.0 to minimize the chances of developing nephropathy, neuropathy, retinopathy, and other cardiovascular diseases. Skyler (1996) reported a significant increase in diabetic complications when HA1c levels exceed 7.5 (5). Medications and lifestyle changes are critical to managing glucose levels and HA1c in diabetics. In healthy individuals, lifestyle changes such as diets, exercise and weight control are recommended to control blood glucose and reduce the risk of developing diabetes. Because HA1c levels can significantly change within 72 hours of exposure of red cells to high glucose levels in vitro, it is imperative to maintain blood glucose levels at the recommended reference range of 70 -110 mg/dL. Successive measurements of at least 126 mg/ml for fasting blood glucose is diagnostic of diabetes. That is likely to translated to an HbA1c measurement of at least 6.5 considering, it takes just 72 hours to significantly increase the HbA1c value in vitro by exposing whole blood to high glucose.

Unlike blood glucose, HbA1c measurement is free of the day-to-day fluctuations and unaffected by recent activities such as exercise, stress and/or food intake. Changes in HbA1c level are dependent on the lifespan of the erythrocyte and blood glucose concentration. With high plasma glucose, the osmolality around the red cells is significantly altered. The cells lose volume and size as water leave the cells but remain intact (figure 3). Also, the stability and integrity of the cells stored at ambient temperature ($25^0$C) or $4^0$C remained intact for up to three or more days respectively. Notwithstanding,

the rate of glucose depletion increases with storage temperature (figure 4), consistent with consumption by the cells.

We conclude that glucose measurements decrease in stored whole blood. The rate of decrease is temperature dependent, fastest at 37 $^0$C compared to 4 $^0$C. When whole blood is exposed to high glucose concentration, hemoglobin glycosylation changed significantly within three days by as much as 15±5%. Elevated HA1c increases one's risk of developing diabetes and its associated complications. Perhaps, in vivo animal studies should provide greater insight into the kinetics of glucose-hemoglobin interaction in red cells. The information may guide intervention to either limit or prevent the amount of glucose that traverses the red cells membrane, binds to hemoglobin to alter HA1c levels.

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