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INCREASED GLYCATED HEMOGLOBIN LEVEL IN NON-DIABETIC NEPHROTIC CHILDREN IS ASSOCIATED WITH OXIDATIVE STRESS

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Abstract : Glycation and lipid peroxidation are spontaneous reactions believed to contribute to the pathogenesis of nephrotic syndrome. Possible interrelations of glycated hemoglobin with reduced glutathione and malondialdehyde were evaluated in nephrotic syndrome patients. Eighteen nephrotic syndrome patients and 15 healthy controls were enrolled for this study. Glycated hemoglobin, reduced glutathione, malondialdehyde and fasting glucose were analyzed for their correlation in both the groups. In nephrotic syndrome patients, while glycated hemoglobin and malondialdehyde levels were found to be significantly increased, glutathione levels decreased significantly when compared with controls. Glycated hemoglobin was found to have a significant positive correlation with malondialdehyde and a negative correlation with glutathione. Erythrocytes depleted of glutathione, by pre-treatment with 1-chloro-2, 4-dinitrobenezene, were found to have higher glycated hemoglobin levels when compared with erythrocytes incubated with glucose alone. These data suggest that glycated hemoglobin levels are closely associated with malondialdehyde and glutathione in nephrotic syndrome patients.

Key words : glutathione, glycated hemoglobin, malondialdehyde, nephrotic syndrome

INTRODUCTION

The nonenzymatic reaction of glucose with proteins is widely recognized and it is thought to be an important component in the aetiology of the long-term complications of many pathological conditions like diabetes and chronic renal failure (CRF) (1). This non enzymatic modification of proteins alters not only the structure, but also the biological properties of protein. Glycation has been reported to alter the functional properties of several important matrix molecules like collagen IV and laminin (2). It is shown that glycation stimulates matrix production, and the glycated glomerular basement membrane

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is more resistant to digestion by protease (3). Glycation of proteins, a factor possibly contributing to tissue damage in nephrotic syndrome, has been extensively modeled by the exposure of proteins to glucose *in vitro* (4).

Glycated hemoglobin (HbA_{1C}) is being used with increasing frequency to monitor long-term blood glucose control in diabetes mellitus and its estimation provides an accurate index of the mean concentration of blood glucose during the preceding two to three months (1). Furthermore, hemoglobin has been considered as a model which has provided insights into the non-enzymatic glycation of other tissue proteins (5). Other factors which influence the rate of glycation of proteins include the prevailing concentrations of glucose and the half-life of the protein (1). However, evidences indicate that glycation reaction apart from these classical factors can be modulated by the levels of reduced glutathione and malondialdehyde (MDA) (6, 7).

Lower concentrations of glutathione in the erythrocytes of nephrotic syndrome patients has been reported (8). In addition, an increase in malondialdehyde concentration has also been reported in patients with nephrotic syndrome (9). Even though much effort has been spent studying the oxidative stress status in nephrotic syndrome, yet to our knowledge, the relationship of glycated haemoglobin with MDA and glutathione is not reported. As glycation can cause deleterious physiological effect on the function of renal tissue, it was of interest to investigate the association of glycated haemoglobin with MDA and glutathione in nephrotic syndrome patients, and to study the *in vitro* effect of glutathione on the glycation of hemoglobin.

MATERIALS AND METHODS

Eighteen children between the ages of 5 and 13 years with relapse in nephrotic syndrome from the outpatient clinics of Pediatrics Department of Jawaharlal Institute of Postgraduate Medical Education and Research, Pondicherry were recruited for this study. Participants were considered to be nephrotic based on the clinical symptom (edema) and associated proteinuria (>40 $mg/m^2/h$). The blood sample from nephrotic syndrome patients was collected before the initiation of steroid therapy when there was significant proteinuria and edema. The control group consisting of 15 healthy age and sex matched volunteers were enrolled for the study. Written informed consent was obtained from the parents or guardians of the participating subjects. The protocol was approved by the Human Ethics Review Committee of the Institute.

Blood samples (5 ml) from the subjects were collected after overnight fasting in EDTA tubes. An aliquot of 1.0 ml was used to determine the HbA_{1C}, whole blood reduced glutathione and hemoglobin levels. The rest of the sample was centrifuged at 2500 × g for 5 minutes, and the plasma obtained was used for the assay of glucose and lipid peroxides.

Measurement of glycated hemoglobin (HbA_{1C}): Glycated hemoglobin concentration was determined by using Haemoglobin A_{1C} micro columns (Bio-Rad, Hercules, CA) and expressed as the percent of total hemoglobin.

Assay of reduced glutathione : Reduced glutathione was determined spectrophotometrically using the Ellman's reagent (10). GSH estimation is based on the development of a yellow color when 5,5' dithio (2-nitrobenzoic acid) (DTNB) is added to compounds containing sulfhydryl groups.

Hemoglobin determination : Hemoglobin was assayed in whole blood by its oxidation to methemoglobin with alkaline ferricyanide reagent (Drabkin's reagent) giving intensely colored cynmethemoglobin, which is measured at 540 nm (11). The results are expressed as g/dl.

Determination of malondialdehyde (MDA) : Malondialdehyde was measured using the established thiobarbituric acid (TBARS) method (12). This assay is based on the formation of red adduct in acidic medium between thiobarbituric acid and malondialdehyde, a colorless product of lipid peroxidation, measured at 532 nm. The MDA values were calculated using the extinction coefficient of MDA-thiobarbituric acid complex (1.56×10^5 $l \times mol^{-1} \times cm^{-1}$) at 532 nm and expressed as nmol/ml.

Estimation of plasma glucose : Plasma glucose was estimated by glucose oxidase method using the commercially available kits (Dr. Reddy's laboratories, Diagnostic division, Hyderabad, India) adapted to 550 express plus auto-analyzer (Ciba Corning Diagnostics, Oberlin, Ohio, Canada).

In vitro incubation protocol : Blood was collected from normal healthy volunteers into tubes containing EDTA as approved by the Institutional Human Ethics Review Committee. The blood was centrifuged and the supernatant plasma including the buffy coat was discarded. The cells were washed with cold physiological saline and were suspended in phosphate-buffered saline (PBS containing 0.016 M Na_2HPO_4, 0.001 M NaH_2PO_4 and 0.14 M NaCl, pH 7.4) in the ratio of 15:85 v/v. GSH depletion of erythrocytes were carried out by treating erythrocyte suspension with 1 mM of 1-chloro-2,4-dinitrobenezene (CDNB) for 1 hour at 37°C. At the end of incubation the erythrocytes were washed with PBS and treated with either 5 or 50 mM of glucose for 24 hours. At the end of the incubation glycated hemoglobin was estimated.

Statistical analysis : All results are shown as mean ± S.D. The statistical significance of difference between groups was evaluated using Student's t-test. Correlation was assesed by the partial correlation analysis. The p value of 0.05 levels was selected as the point of minimal statistical significance.

RESULTS

All the parameters tested in both the groups are reported in Table I. The HbA_{1C} levels were significantly higher

TABLE I : Mean ± S.D of age, biochemical parameters in controls (n = 15) and nephrotic syndrome patients (n = 18).

	Control	*Cases*
Age (years)	8.21±2.89	8.39±2.57
Sex (M/F)	8/7	10/8
Fasting glucose (mg/dl)	75.07±6.42	79.06±7.80
HbA_{1C} (%)	5.12±0.54	7.04±1.02*
MDA (nmol/ml)	0.87±0.33	3.04±0.52*
Reduced GSH (mg/g Hb)	2.78±0.69	1.05±0.38*

*P<0.01 as compared to control subjects.

(P<0.01) among nephrotic syndrome patients as compared to controls. Levels of malondialdehyde were significantly increased in nephrotic syndrome group when compared to controls (P<0.01). The reduced GSH levels were found to be significantly low in the nephrotic group when compared to healthy control (P<0.01). A significant positive correlation (r = 0.54, P<0.03) was obtained between HbA_{1C} and MDA using partial correlation analysis controlling fasting glucose and reduced GSH. The linear regression analysis of glycated hemoglobin and MDA is depicted in (Fig. 1). Similarly a significant negative correlation (r = –0.49, P<0.05) was obtained between GSH and HbA_{1C} even after the effect of MDA and glucose on glycation was refuted by partial correlation analysis. The regression analysis of GSH and HbA_{1C} is shown in Fig. 2.

Blood samples from 4 healthy volunteers were used for the *in vitro* study to explore the possible role of GSH in modulating the glycation of hemoglobin. Table II shows the effect CDNB on the glycation of hemoglobin. A significant increase in HbA_{1C} levels were found in the erythrocytes pretreated with 1 mM of CDNB than when compared with erythrocytes incubated with 50 mM glucose alone. No significant effect of pretreatment with CDNB was found in erythrocytes incubated with 5 mM glucose.

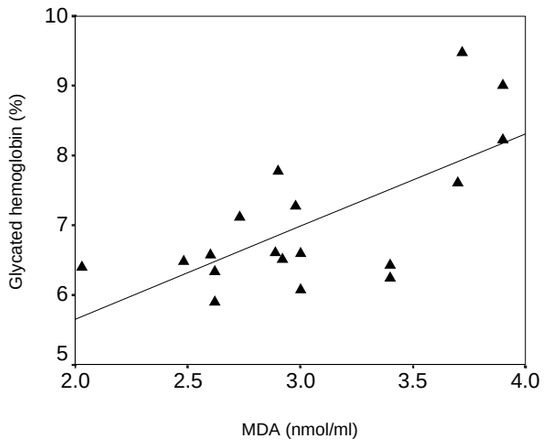

Fig. 1 : Relationship between MDA and glycated hemoglobin in nephrotic syndrome patients. A significant positive relationship between MDA and glycated hemoglobin was observed (r = 0.54, P<0.03). The regression equation is y = 2.99 + 0.68 x.

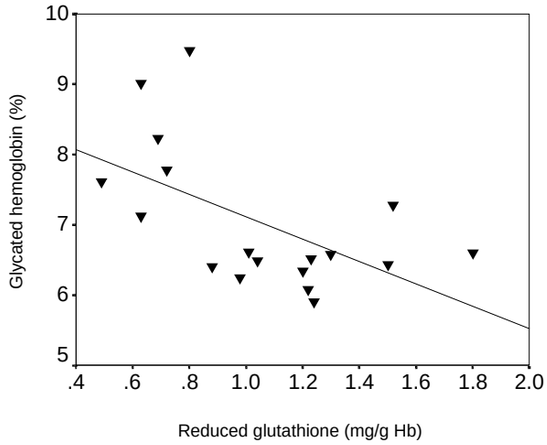

Fig. 2 : Linear regression analysis of reduced glutathione and glycated hemoglobin in nephrotic syndrome patients. A significant negative relationship between reduced glutathione and glycated hemoglobin was observed (r = –0.49, P<0.05). The regression equation is y = 8.71 – 0.56 x.

TABLE II : Effect of GSH depletion on glycation of hemoglobin in erythrocytes treated with glucose (5 or 50 mM). Values are mean±S.D from four experiments.

Treatment	*HbA_{1C} values (%)*
Glucose 5 mM	5.47±0.48
Glucose 5 mM + 1 mM CDNB	5.53±0.47
Glucose 50 mM	6.26±0.22
Glucose 50 mM + 1 mM CDNB	6.99±0.32*

*P<0.05 when compared with HbA_{1C} values of erythrocytes treated with 50 mM glucose alone.

DISCUSSION

Free radical oxidative stress has been implicated in the pathogenesis of a variety of diseases, and natural antioxidant defenses have been found to be defective in many diseases (13). In the present study, MDA levels were found to be significantly higher in nephrotic syndrome patients when compared with controls. We also found a significant decrease in GSH levels in the test group when compared with controls. In nephrotic syndrome patients, the average glycated hemoglobin was 7.28% and among the controls it was 5.8%. The magnitude of this difference in HbA_{1C} values between nephrotic syndrome patients and controls cannot be attributed to blood glucose as there was no significant difference in the glucose levels between the two groups. Increased HbA_{1C} levels have been previously reported in nephrotic syndrome patients (14). The elevated level of HbA_{1C} has also been reported in several non-diabetic clinical conditions like renal failure, myocardial infarction, hyperthyroidism, non diabetic smokers and in elderly people (15–19). *In vitro* studies have demonstrated that malondialdehyde *per se* can promote the glycation of hemoglobin (7, 20). The reduced glutathione have inhibitory effect on the process of non-enzymatic glycation (6). Thus, oxidative stress has been reported to be involved in the glycation of hemoglobin.

Several studies have shown increased lipid peroxidation in nephrotic syndrome (8, 9). However, there are no studies demonstrating a correlation between MDA and glycated haemoglobin. In this article, we address an important potential hypothesis in which glycation of hemoglobin may have a link with the increased levels of MDA and reduced level of GSH in children with nephrotic syndrome.

The mechanisms for increased HbA_{1C} in nephrotic syndrome patients are not clear. The present study found a significant relationship between HbA_{1C} and MDA levels in nephrotic syndrome patients even when the effects of glucose and GSH were refuted by partial correlation analysis. The mechanism by which MDA enhances the glycation process has not been clearly elucidated. MDA has been thought to enhance the process of protein glycation by acting as an anchor between sugar and hemoglobin moieties (22). It has also been suggested that oxidative stress can facilitate the autoxidation of glucose to dicarbonyl intermediates, an early step in the Maillard reaction (7).

Apart from MDA, GSH was also a significant factor that influences the glycation of hemoglobin in nephrotic syndrome patients. An inverse association between GSH and HbA_{1C} was seen, even after adjusting the effects of MDA and glucose by partial correlation analysis. To further examine the association between HbA_{1C} with GSH, we carried *in vitro* study to explore the role GSH on the glycation of hemoglobin. A significant increase in HbA_{1C} levels was found in erythrocytes pretreated with 1 mM of CDNB when compared with erythrocytes incubated with 50 mM glucose alone. Similar findings have been reported by Jain *et al* in healthy controls and in glucose-6-phosphate dehydrogenase (G-6-PD) deficient patients (6). Further, replenishment of GSH with either DTT or DTE in erythrocytes from G-6-PD patients decreases the glycation of hemoglobin (6). In accordance with their

hypothesis Jain *et al* have shown a significant correlation between reduced glutathione and glycated hemoglobin in diabetic patients (23). This indicates that GSH *per se* can modulate the glycation reaction. The mechanism by which GSH affects glycation is difficult to explain from the present study. One explanation for this finding is that GSH can prevent the tautomerism of sugar from enediol form to dicarbonyl intermediates which is thought to play an important role in the process of glycation (6). These observations support the notion that alteration in the levels of MDA and GSH in nephrotic syndrome patients, may be the basis for enhanced levels of HbA_{1C}.

In conclusion, present study documents increased glycated haemoglobin levels in nephrotic syndrome patients when compared with controls. Additionally, this study shows high MDA and low GSH levels as determinants of enhanced glycation in nephrotic syndrome.

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