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Original Article:

Correlation of Lipid Peroxidation with Glycated Haemoglobin Levels in Diabetes Mellitus

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Abstract: Reactive oxygen species are crucial to normal biological processes; they are potentially dangerous and are commonly referred to as prooxidants. The reactive oxygen intermediates can cause direct cellular injury by including lipid and protein peroxidation and damage to nucleic acid. The polyunsaturated fatty acids present in the cells are vulnerable to free radicals causing lipid peroxidation. Determination of Malondialdehyde (MDA) by using thiobarbituric acid is used as an index of the extent of lipid peroxidation. This study was done to know if lipid peroxidation correlated with the glycated haemoglobin levels. Diabetic status was assessed by estimating fasting blood sugar and glycated haemoglobin level while oxidant stress was evaluated by estimating erythrocyte MDA levels. The lipid peroxidation in erythrocyte lysates was significantly increased in diabetic individuals compared to controls ($p<0.001$). The result of this study indicates that in diabetic individuals are more prone to oxidative stress and glycated haemoglobin is a marker in evaluating the long term glycemic status in diabetic individuals.

Key Words: Oxidative stress; Glycated haemoglobin; Lipid peroxidation; Malondialdehyde

Introduction:

Cells can tolerate mild oxidative stress, which often results in up regulation of the synthesis of antioxidant defence systems in an attempt to restore the balance, but when severe, cause derangement in all metabolism causing cell injury and death. In most human diseases, oxidative stress is secondary phenomenon, a consequence of the disease activity. There is a growing awareness that oxidative stress plays a role in various clinical conditions e.g. malignant diseases, diabetes, atherosclerosis etc.

Diabetes mellitus, a common metabolic disorder resulting from defects in insulin secretion or action or both, is characterized by hyperglycemia often accompanied by glycosuria, polydipsia, and polyuria.(1) During diabetes, persistent hyperglycemia causes increased production of free radicals especially reactive oxygen species (ROS), for all tissues from glucose auto-oxidation and protein glycosylation.(1) In addition, superoxide is generated by the process of glucose autoxidation that is associated with the formation of glycated proteins in the plasma of diabetic patients. Many factors are responsible for this like polyol pathway, prostanoid synthesis and protein glycation which disturbs the antioxidant defence system thereby increasing the amount of reactive oxygen species.(2) The increase in ROS production contributes to the development of diabetic complications.

Monitoring blood glucose control as performed by patients and health care providers is considered as the cornerstone of diabetes care. Carbohydrates such as glucose can bind non enzymatically to proteins such as hemoglobin in a process known as glycation. The human erythrocytes are freely permeable to glucose and within each erythrocyte glycated hemoglobin is formed continuously from hemoglobin. The formation of glycated hemoglobin is dependent on the ambient glucose concentration. Individuals with higher levels of blood glucose will have higher levels of glycated hemoglobin.(3) The glycation process is slow and continuous that occurs over days to 3-4 months in vivo. In a normal person about 3-6% of HbA is glycated; in a diabetic patient the percentage of HbA may double or triple depending on the degree of hyperglycemia.(4-6) Glycated hemoglobin is the best surrogate marker for setting the treatment goals. Nonenzymatic glycation is a spontaneous chemical reaction between glucose and the amino groups of proteins in which reversible Shiff bases and more stable Amadori products are formed.(7) Advanced glycation end products (AGEs) are then formed through oxidative reactions and cause irreversible chemical modifications of proteins(7).

Free radicals are very reactive chemical species, which can cause oxidation injury to the living beings by attacking the macromolecules like lipids, carbohydrates, proteins and nucleic acids.(8) The significant targets for injury are mainly proteins and DNA than lipids. Lipid peroxidation occurs late in the injury process. An increased concentration of end products of lipid peroxidation is the evidence most frequently quoted for the involvement of free radicals in human disease. It is likely that increased oxidative damage occurs in most, if not all human diseases and plays an significant pathological role in them.(9) Lipid peroxidation end-products very commonly detected by the measurement of thiobarbituric acid reactive substances (TBARS).(10)

Free radicals are produced as a result of glycosylation of several proteins including hemoglobin (Hb) by non-enzymatic mechanisms.(11) Subsequently free radicals change lipid/protein ratio of membranes by affecting polyunsaturated fatty acids and lipid peroxidation causes functional irregularities of several cellular organelles.(11) Lipid peroxidation is a free radical-related process, which is potentially harmful because its uncontrolled,

self-enhancing process causes disruption of membranes, lipids and other cell components. (12) Lipid peroxidation is the oxidative deterioration of polyunsaturated fatty acids. The free radicals steal electrons from the lipids in the cell membrane, resulting in cell damage. Lipid peroxidation is a late event accompanying rather than causing final cell death. The end products of lipid peroxidation process are aldehydes, hydrocarbon gases and chemical residues including malondialdehyde. MDA is an important reactive carbon compound which is used commonly as an indicator of lipid peroxidation (11). Abnormally high levels of lipid peroxidation and the simultaneous decline of antioxidant defence mechanisms can lead to damage of cellular organelles and lead to oxidative stress.(12) Diabetes mellitus is characterised by hyperglycaemia together with biochemical alterations of glucose and lipid peroxidation.(12) Significantly higher values of thiobarbituric acid-reactive substances (TBARs) in serum, which provide an indirect measurement of lipid peroxidation and decreased erythrocyte antioxidant enzyme activities, have been observed in adult diabetic patients. (7) Some complications of diabetes mellitus are associated with increased activity of free radical-induced lipid peroxidation and accumulation of lipid peroxidation products.(12) Diabetic red blood cells (RBC) s were shown to be more susceptible to lipid peroxidation as measured by TBARS in rats and humans.(10) In erythrocytes from diabetic patients increased membrane lipid peroxidation may lead to abnormalities in composition and function.(13) Diabetes erythrocytes have higher malondialdehyde levels.(13) Diabetes produces disturbances of lipid profiles, especially an increased susceptibility to lipid peroxidation.(1) An enhanced oxidative stress has been observed in these patients as indicated by increased free radical production, lipid peroxidation and diminished antioxidant status.(1)

The objective of the present study is to evaluate the oxidant stress in diabetes mellitus and its association with glycated hemoglobin levels in diabetes mellitus. The diabetic status was assessed by estimating the fasting blood sugar and glycated hemoglobin while the oxidant stress was evaluated by estimating erythrocyte malondialdehyde in terms of thiobarbituric acid reacting substance.

Materials and Methods:

Sample collection:

The study group comprised of nondiabetic individuals and diabetic patients attending the Kasturba hospital, Manipal. Informed consent from the patients was obtained for the study. Patients were selected at random and no distinction was made between those with insulin dependent or non- insulin dependent diabetes. The diabetic status was assessed by estimating the fasting blood sugar (FBS) using glucose oxidase method. Test group consisted of fifty diabetic individuals; whose fasting glucose level was more than 126mg%. Blood (2ml) was collected by venepuncture into tubes containing 3.6mg EDTA and stored at 4°C. The mean age of controls was 54±12.1 and that of cases was 52 ± 12.1.

Erythrocyte malondialdehyde was estimated within 24 hours of blood collection. The hemolysates prepared from the above blood samples were stored at -25°C.

Estimation of glycated haemoglobin by affinity chromatography: (14)

Affinity gel columns (Glycogel B)were used to separate bound, glycosylated haemoglobin from the non-glycosylated fraction. The gel contains immobilized m- amino-phenylboronic acid and cross linked beaded agarose. The boronic acid reacts with the cis- diol groups of glucose bound haemoglobin to form a reversible 5- membered ring complex, thus relatively holding the glycosylated haemoglobin on the column. The non- glycosylated haemoglobin is eluted. The complex is next dissociated by sorbitol, which permits elution of glycosylated haemoglobin. Absorbances of the bound and unbound fractions, measured at 415nm are used to calculate the percent of glycosylated haemoglobin.

Estimation of malondialdehyde (MDA):

Erythrocyte MDA concentration was determined using the method described by Jain et al.(15)

At low pH and elevated temperature, MDA readily participates in nucleophilic addition reaction with 2- TBA generating a red fluorescent 1:2 MDA- TBA adduct. The absorbance was read at 532 and 600nm using a spectrophotometer. Butylated hydroxyl toluene is added to the assay mixture in order to prevent lipid peroxidation during heating. A standard graph was prepared by taking concentration of standard in moles/ ml along the x- axis and absorbance (532-600nm) along the y- axis. TBARS values were calculated from the standard graph and expressed as nanomoles/ gram of haemoglobin. Estimation of haemoglobin was done by the method of Drabkin.(16)

Results:

The erythrocyte malondialdehyde levels was determined in the erythrocytes taken from both individuals with diabetes mellitus (test group) and normal healthy individuals (control group). Erythrocyte malondialdehyde levels was higher in cases (4.7±1.7 nmoles/gHb) than the controls (3.3±2.2 nmoles/gHb). The glycated hemoglobin level was higher in cases (8±2.9) than the controls (6.1± 2.2). The fasting blood sugar did not correlate with the erythrocyte malondialdehyde levels but did correlate with glycated hemoglobin i.e. $p<0.05$. Among the cases the erythrocyte MDA did not correlate with glycated hemoglobin. Thus the lipid peroxidation in the diabetic erythrocytes were significantly higher when compared to the control group ($p=0.001$) (Table 1, Figure 1-4).

Table 1: FBS, MDA and Glycosyltaed Hemoglobin levels

Parameters	Mann- Whitney 'u' test	'p'value
Fasting blood sugar (mg %)	8.61	0.001
MDA (nmoles/gHb)	4.20	0.001
Glycated hemoglobin (% of Hb)	8.0	0.001

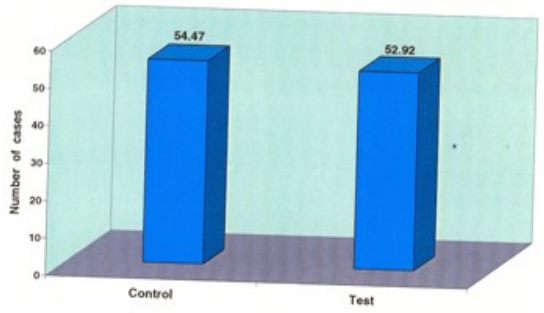

Figure 1: Age Distribution

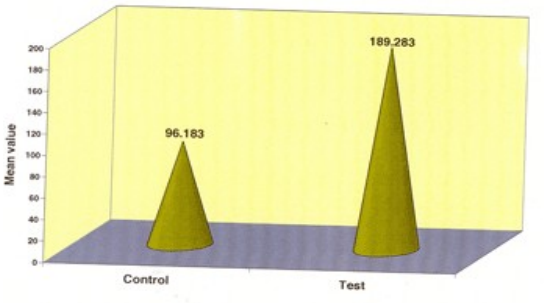

Figure 2: Comparison of Mean FBS Between Cases and Controls

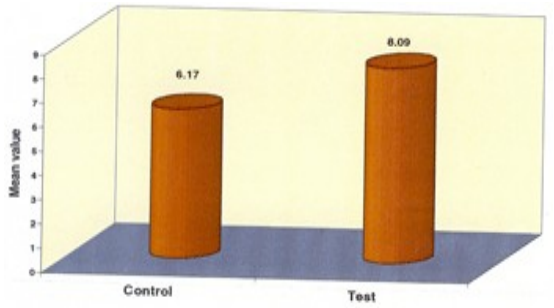

Figure 3: Comparison of Mean Glycated Hb Between Cases and Controls

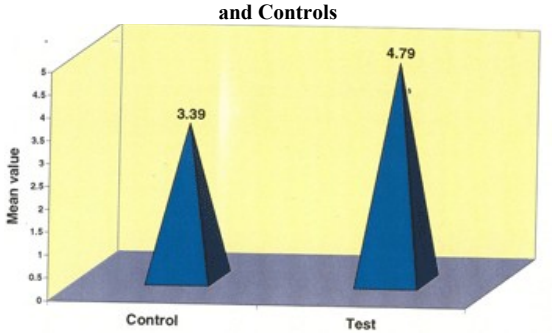

Figure 4: Comparison of Mean MDA Between Cases and Controls

Discussion:

Oxidative stress depicts the existence of products called free radicals and reactive oxygen species (ROS) which are formed under normal physiological conditions but become deleterious when not being quenched by the antioxidant system. There are convincing experimental and clinical evidence (1) that generation of reactive oxygen species is increased in both types of diabetes mellitus and that the onset of diabetes is closely associated with oxidative stress. Free radical mediated cytotoxic process of lipid peroxidation appears to have a role in the development of multifactorial disease, diabetes mellitus. Possible sources of elevated free radicals in type 2diabetes include increased production of radical oxygen species, especially from glycation or lipoxidation processes, auto-oxidation of glucose and oxidizing of glucose and decreased antioxidant defense systems.(17)

In the present study the individuals with diabetes mellitus showed statistically significant levels of lipid peroxidation as indicated by the levels of erythrocyte MDA. The increased levels of thiobarbituric acid-reactive substances (TBARS) suggest a net increase in the levels of oxygen free radicals which could be due to their increased production and/or decreased destruction. This increased level of MDA could be because of increased glycation of proteins in diabetes mellitus. The glycated protein may themselves act as a source of free radicals. There is a clear association between lipid peroxide and glucoses concentration which also could be thought to play a role in increased lipid peroxidation in diabetes mellitus. The exact mechanism by which the elevated blood glucose leads to membrane lipid peroxidation is not known. Some studies have shown that glucose can enolise and then reduce molecular oxygen to give α- keto aldehydes, hydrogen peroxide and ROS. Hydrogen peroxide formed by superoxide dismutation regenerates the catalytic metal oxidation state and produces hydroxyl radicals. The ROS formed causes peroxidative breakdown of phospholipid fatty acids and accumulation of MDA.(15) Elevated levels of MDA could also be due to alteration in the function of erythrocyte membrane.

The present study was carried out to know the relation of fasting sugar with glycemic control i.e. by determining the glycated haemoglobin levels. In the present study there was an increase in the level of glycation of haemoglobin in diabetic patients. Results of the present study suggest that increased production of high levels of free oxygen species is linked to glucose oxidation. Several studies have reported similar results. The glycated haemoglobin as a marker of glycemic status over last 2-3months and malondialdehyde was taken as an oxidative marker of diabetes mellitus. The glycation induced structural modification of hemoglobin.

The present study results show that lipid peroxidation might not contribute to glycation of haemoglobin. Martin and others (18-21) have reported similar results, whereas Jain et al have found a positive correlation.

In conclusion, the estimation of lipid peroxide along with lipid profiles in diabetes mellitus would serve as a useful monitor to judge the prognosis of the patient. Improvement of glycemic control appears to be a beneficial factor in decreasing lipid peroxidation in patients with diabetes. Prevention of lipid peroxidation may help to delay the development of diabetic complications. The detection of the risk factor in the early stage of the disease helps to improve and reduce the mortality rate.

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