

Evaluation of Oxidative Stress in Juvenile Diabetes.

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Abstract: Background:-Juvenile Diabetic patients are claimed to be under oxidative stress because of hyperglycemia. The Influence of free radical production by this hyperglycemic induction may involve cardiovascular complications in diabetes.

Aim:-The present study aimed to compare the glutathione (GSH) level and glutathione peroxidase (GPx) activity in Juvenile and a normal healthy group.

Materials and method: - Twenty normal healthy subjects and twenty juvenile diabetic subjects of 6 to 15 years were selected. Venous blood samples for the successive biochemical determinations were drawn at fasting. Blood biochemistry determinations were performed using the enzymatic reagent kit.

Results: - Red cell GSH was significantly lower in Juvenile ($p = 0.011$) but red cell GPx activity was significantly increased ($p = 0.003$) when compared to age-matched normal control. The correlation between FPG and GSH in Juvenile diabetic patients compared with healthy normal subjects was also observed and it was found that there was a negative correlation, but not found between FPG and GPx activity.

Conclusion:-The present finding suggested that juvenile diabetic patients were susceptible to oxidative stress and higher blood glucose level had an association with free-radical-mediated lipid peroxidation. Therefore, any means that can reduce oxidative stress may be beneficial for slow progression of cardiovascular complication in juvenile diabetic patients.

Keywords: Juvenile diabetes, Oxidative stress, cardiovascular complication, FPG, GPx, GSH.

INTRODUCTION

The development of Juvenile diabetes required genetic susceptibility [30]. Rather, several genetic alterations, most but probably not all of which are located on chromosome six within the major histocompatibility complex, result in an increased likelihood of β -cell damage [35,18, 36, 28, 5, 41]. The mechanism of β -cell damage and destruction is thought to be the result of an organ-specific autoimmune process in which the immune system reacts abnormally against the body's own insulin secreting β -cells in the pancreatic islets [1,11]. Substantial evidence suggests that antigens released from the β -cell are seen as foreign proteins by the macrophage or antigen presenting cell (APC) that presents the altered antigen to a highly specialized HLA-linked receptor in a helper T cell. This process then initiates an active cellular and humoral response involving antibody production and lymphokine release [8, 19]. The final biochemical mediator of this toxic process is probably nitric oxide (NO) [17]. NO will react with O_2 to produce peroxynitrite (ONOO-) which can initiate the destruction of β -cells through the lipid peroxidation process [12].

The epidemiologic studies of Juvenile diabetes have shown that a high incidence was found in the developing countries, intermediate levels in much of the West, and very low levels in the Far Eastern countries, including Japan, China, Korea, and Thailand [14, 16, 40, 43]. This distribution has an extraordinary similarity to that seen in the worldwide distribution of atherosclerosis and of morbidity and mortality from coronary heart disease (CHD) [29]. The atherosclerotic risk and Diabetes mellitus incidence may correlate with the oxidative stress [10]. Several studies have suggested that chronic hyperglycemia and oxidative stress may associate with diabetes and its complications (18). The oxidative stress results from increased free radical formation and/or decreased antioxidants in the body. The free radicals or sometimes specifically called reactive oxygen species (ROS) can be influenced by exogenous agents, radiation, trauma, drug activation, oxygen excess, or by endogenous mechanisms such as oxidative metabolism, transition metals catalyzed reactions, microbial killing by phagocytic cell, and inflammation. Moreover, there are more mechanisms that induce oxidative stress in diabetic patients than in normal individuals; glucose autooxidation, non-enzymatic glycation of protein, and polyol pathway [39]. These pathways enhance the generation of ROS that lead to tissue damage and cause several complex syndromes in diabetic patients such as cataracts, renal dysfunction, nerve damage, and atherosclerosis. Especially, the atherosclerosis leads to the CHD, which is the major cause of death among diabetics [6, 22]. However, the

action of ROS can be prevented by the antioxidant defense system. In the human body, there are antioxidant enzymes such as superoxide dismutase (SOD), the enzyme which dismutates superoxide (O_2^-) to hydrogen peroxide (H_2O_2), and the other two H_2O_2 -scavenging enzymes - glutathione peroxidase (GPx) and catalase (CAT) - that convert H_2O_2 to water.

In addition, there are endogenous non-enzymatic antioxidants like glutathione (GSH) and exogenous antioxidant vitamins from the diet such as fruits and vegetables. These non-enzymatic antioxidants act as terminators of free radicals' chain reactions caused by lipid peroxidation [12]. The GSH has the advantage of antioxidant property in that it is a cofactor of GPx enzyme and is involved in the detoxification of ROS, whereas, exogenous antioxidant vitamins acts only as detoxification of ROS. GSH precursor has been successfully used in diabetes to attenuate renal damage and peripheral neuropathy in experimental models [25, 33] but its effects on cardiac damage remain unknown. Therefore, the aim of the present study was to investigate the level of glutathione and GPx activity in juvenile diabetes compared to the normal subjects to be the knowledge base for understanding the progression of pathogenic mechanisms of cardiovascular complication in diabetes.

MATERIALS AND METHOD:-

Twenty normal healthy subjects were the control group with fasting plasma glucose (FPG) < 110 mg/dL. The ages ranged from 6 to 15 years. Twenty juvenile diabetic subjects whose FPG > 140 mg/dL. The ages ranged from 6 to 15 years. Venous blood samples for the successive biochemical determinations were drawn at fasting in the morning. Blood biochemistry determinations were performed using the enzymatic reagent kit. Normal eligible subjects were recruited from subjects who attended for a routine medical check at the Pravara Institute of Medical Sciences, Loni. They were defined as healthy by physical examination (weight, height, and blood pressure measurements, respiratory, examination) and laboratory tests [complete blood count, blood urea nitrogen, creatinine, uric acid, fasting blood glucose, liver function tests, total cholesterol, triglyceride, and high density lipoprotein cholesterol (HDL-C)]. Subjects with hypertension, diabetes mellitus, cardiovascular diseases, renal or hepatic diseases, inflammation, injury, or trauma in the previous month were excluded from the present study. Juvenile diabetic subjects were selected from the medical outpatient department and I.P.D in Hospital of Pravara Institute of Medical Sciences, Loni. All patients with any renal dysfunction, (i.e. raised blood urea and serum creatinine levels), with coexistent illness (i.e. infections), liver diseases, respiratory tract diseases,

congestive heart failure, acute myocardial infarction, proliferative retinopathy, with diabetic macroangiopathic complications (i.e. coronary artery disease, peripheral vascular disease and stroke: diagnosed by clinical history and examination) were excluded from the present study. All patients had been on insulin treatment for more than 6 months. The control group and patients supplemented with antioxidants were also excluded. Oral Inform Consent form the patients were taken and the study protocol was based on with the Helsinki Declaration of 1975 which was revised in 2000 and approved by the local ethical committee.

Erythrocyte reduced glutathione assay

The reduced glutathione (GSH) in red cells was performed as described by [2]. In brief, 3% TCA was added to the 0.1 ml of packed red cells and mixed, 0.25 ml followed by 0.9 ml of cold distilled water. The suspension was centrifuged at 1870 x g for 5 min and supernatant was assayed glutathione by adding 1 ml of 0.3 M phosphate buffer, pH 7.0. Then, 0.125 ml of 40 mg% DTNB solution was added and the mixture was left to stand at 4°C for 5 min. The optical density was read at 412 nm against an unknown blank. The GSH level was obtained from a standard curve that was prepared by using 1.25, 25, 50, 100, and 200 mg/dL of standard reduced GSH from Sigma in distilled water.

Erythrocyte glutathione peroxidase assay

This enzyme was assayed in the hemolysate according to the method of [31] with modifications.

A mixture containing 0.4ml of tris-HCl buffer, 0.2 ml of Glutathione, 0.1ml of sodium azide 0.1 ml of water, 0.1 ml of peroxide and 0.1ml of haemolysate was incubated at 37°C for 15 minutes. The reaction was terminated by the addition of 0.5ml of 10% TCA. After sedimenting the precipitated proteins, 0.5ml of the supernatant was taken and 2.0 ml of disodium hydrogen phosphate was added, followed by 0.5ml of Ellman's reagent. The absorbance of the resulting solution was immediately read at 420nm. Suitable aliquots of standard Glutathione solution were treated in a similar manner to obtain a standard curve. This provided the basis for measuring the reduced Glutathione remaining after the reaction.

STATISTICAL ANALYSIS

Values expressed as (Mean + SEM). Unpaired t-test was performed to compare juvenile diabetic patients with healthy normal groups. Pearson correlation coefficient was used to assess relationship among oxidative stress and FPG of juvenile diabetic patients and healthy subjects. Differences when $p < 0.05$ considered as significant.

OBSERVATION

Table 1. General Parameters of age, gender, FPG, hematological data and lipid profiles in normal Healthy subjects and Juvenile diabetic patient (Mean \pm SEM).

	Healthy Subjects	Juvenile Diabetes
Age	12.45 \pm 3.60	14.25 \pm 3.40
Gender (M/F)	12/8	11/9
FPG (mg/dL)	95.71 \pm 3.30	212.58 \pm 20.35*
Hb (g/dL)	15.87 \pm 0.45	13.80 \pm 0.60
Hct (%)	45.79 \pm 0.86	45.69 \pm 1.30
Total cholesterol (mg/dL)	188.81 \pm 9.61	216.48 \pm 13.28*
LDL cholesterol (mg/dL)	95.80 \pm 8.51	135.81 \pm 10.65*
VLDL cholesterol (mg/dL)	20.15 \pm 2.74	29.37 \pm 4.63*
Triglyceride (mg/dL)	106.73 \pm 13.65	145.89 \pm 23.14*
HDL cholesterol (mg/dL)	72.86 \pm 4.09	54.30 \pm 3.57*

* $p < 0.05$

Table 2. Erythrocyte GSH and GPx activity in Healthy subjects and Juvenile diabetes (Mean \pm SEM)

	Healthy Subjects	Juvenile Diabetes
GSH (mg/gHb)	2.27 \pm 0.07	1.73 \pm 0.06*
GPx (u/gHb)	28.18 \pm 1.55	38.83 \pm 2.97*

* $p < 0.05$

RESULTS

Table 1 shows the database of age, gender, FPG, and lipid profile between the normal healthy group and juvenile diabetic patients. This table shows that FPG in juvenile diabetic patients was significantly higher than that in the normal group. The study of plasma lipid profile showed total cholesterol, VLDL-cholesterol and triglycerides in juvenile diabetic patients were not significantly different from the normal healthy group. This may be due to the treatment of cholesterol-lowering drug to maintain the blood lipid level in diabetic subjects. However, the significant increase of LDL-cholesterol and significant decrease of HDL-cholesterol were also found in juvenile diabetic subjects. The increasing of and decreasing of HDL-cholesterol may be the major risk factors to exacerbate the cardiovascular complication in this group of diabetes. In addition, hemoglobin (Hb) concentration was also significantly lower in juvenile diabetic compared to normal control subjects, whereas, hematocrit (Hct) was not different.

Red blood cell GSH level and GPx activity

The mean level of red cell GSH that functions as an antioxidant and a cofactor of enzyme GPx and GPx activity are summarized in Table 2. The level of GSH was significantly lower in juvenile diabetes comparing with normal subjects ($p = 0.011$). Conversely, the red cell GPx activity was significantly higher than the normal group ($p = 0.003$). The demographic data for Pearson correlation analysis among FPG, GSH and GPx activity in juvenile diabetic patients and normal subjects were also performed. There were negative correlations between FPG and GSH in juvenile diabetic subjects and in total subjects ($r = -0.589$, $p = 0.006$; $r = -0.460$, $p = 0.001$, respectively) whereas normal subjects showed no correlation ($r = 0.344$, $p = 0.138$) and have a trend to increase the GSH level. No correlation was observed with FPG and GPx in normal subjects, juvenile diabetic patients and total subjects ($r = -0.417$, $p = 0.068$; $r = -0.318$, $p = 0.171$; $r = 0.110$, $p = 0.538$, respectively).

DISCUSSION

Chronic hyperglycemia and oxidative stress in juvenile diabetic patients may be associated with long-term damage, dysfunction, and failure of various organs. These chronic complications include retinopathy with potential loss of vision; nephropathy leading to renal failure; peripheral neuropathy with risk of foot ulcers, amputation, and Charcot joints; and autonomic neuropathy causing gastrointestinal, genitourinary, and cardiovascular symptoms. In addition to these microvascular diseases

(microangiopathies), the macrovascular disease (macroangiopathy) such as atherosclerosis was also found. The atherosclerotic vascular disease is the cause of mortality and significant morbidity in diabetes. This chronic hyperglycemia can initiate the $O_2^{\cdot -}$ production by glucose autooxidation, non-enzymatic glycation of proteins, and polyol pathway as described earlier. The $O_2^{\cdot -}$ can be converted to H_2O_2 and OH^{\cdot} , respectively. The hyperactivity of these ROS is directed against lipids and proteins as well as nucleic acid [44] and resulting in structural modification and fragmentation that cause cell damage in diabetes [2]. Antioxidant defense system, however, in the blood can protect tissues and organs against the ROS. The antioxidant systems are composed of enzymatic and non-enzymatic antioxidants. There are three main antioxidant enzymes such as SOD, CAT, and GPx. Study in the erythrocyte GPx activity in juvenile diabetic showed that GPx activity was markedly increased corresponding to the other reports with type 2 diabetes [27, 37, 20]. The increment of GPx activity may be explained by which the GPx is an inducible enzyme and was stimulated by ROS [21]. The increased production of ROS may promote the GPx activity for adaptive process of combating excessive peroxidative damage. In another mechanism, GPx is a selenium-dependent enzyme; diabetic tissue may retain selenium in the cells and thereby increases GPx activity [3,9, 20]. The thalassemic patients are also known to be under the oxidative damage similar to the diabetic patients. However, an increased level of plasma selenium in diabetic children was observed by [9,32]. Therefore, a selenium turnover study is required to confirm whether a compensatory mechanism sets in for saving tissue selenium levels in diabetes. Another factor that increases GPx activity is dietary factors of the PUFA (polyunsaturated fatty acid) intake because PUFA within the cells are prone to peroxidative damage by ROS [23]. The increasing of hydroperoxide, the product of lipo- peroxidative damage by ROS, is also known as GPx activation [3]. GSH is a ubiquitous tripeptide that presents in red cells and participates in GPx reaction. When H_2O_2 is detoxified by GPx, the GSH is simultaneously converted to the oxidized form (GSSG). In the present study, the authors found that GSH levels in juvenile diabetic patients were significantly lower than that in their same age-matched control subjects. These results are in good agreement with other studies [10, 4, 34]. As already mentioned GSH serves as an essential cofactor for the enzyme GPx and formed oxidized glutathione (GSSG) during the enzyme processes. Thus, increasing in GPx activities imply higher consumption of GSH. Other mechanisms that may explain the GSH reduction in diabetes are that the GSH is regenerated by the enzyme glutathione

reductase, using reducing equivalents from NADPH. The NADPH is generated in red blood cells through the pentose phosphate pathway, which is stimulated by insulin [42], and in Diabetes mellitus NADPH production may be sluggish, probably resulting in lowered glutathione reductase activity and reduced GSH recycle. The enzyme glutathione reductase was found to be decreased in type 2 diabetic patients was reported [7]. Moreover in diabetes mellitus, the increased sorbitol synthesis via the polyol pathway occurred. This elevated sorbitol production caused the NADPH depletion that was required by aldose reductase enzyme in this pathway. This deficiency will also limit the GSH recycle [7]. To study the association between FPG and parameters of oxidative stress in juvenile diabetic and normal subjects, The Pearson product moment correlation was performed. From the value of correlation coefficient (r) between FPG and GSH, the authors found that there was a negative fair correlation ($r = -0.46$, $p = 0.001$) reflecting some association of the GSH with increased oxidative stress. However, the association between FPG and GPx activity showed no correlation, this implied that the GPx enzyme might be glycated by

hyperglycemic state [15] resulting in fragmentation of this enzyme [39, 26]. This indicated that the glycated form of GPx may be less active. As already described, Wolff et al [45] reported that glycated protein produced ROS. There was also increased glycation of proteins may produce ROS and accelerate atherogenesis because of oxidative modifications of vascular membrane lipids [24, 38, 13].

CONCLUSION:

The production of ROS by the glycated proteins, on the one hand, and the inactivation of GPx and GSH activity by glycation, on the other hand, may enhance the accumulation of ROS leading to the serious complications in diabetes

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