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Glutathione and Glutathione Peroxidase in Type 2 Diabetic Patients

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Abstract :

Objectives : Hyperglycemia is claimed to cause oxidative stress in diabetic (DM) patients. The influence of free radical production by hyperglycemia may exacerbate the cardiovascular complications in diabetes. However, the effects of glycemic control and cardiovascular complications in diabetes on oxidative stress parameters have been not fully studied. This study compared the red cell glutathione (GSH) level and glutathione peroxidase (GPx) activity in fairly controlled type 2 DM (Fasting plasma glucose [FPG] ≤ 180 mg/dl), poorly-controlled type 2 DM (FPG >180 mg/dl) and type 2 DM complicated with coronary heart disease (CHD) with that in a normal healthy group (FPG <110 mg/dl).

Materials & Methods : GSH level and GPx activity were determined in the red cells of 19 subjects with poorly controlled type 2 DM, 26 subjects with fairly controlled type 2 DM, and 20 subjects with type 2 DM complicated CHD with that of 20 healthy subjects with normal plasma glucose level matched for age and gender who served as a control group. In all groups of DM these oxidative stress parameters were compared to a control group by one-way ANOVA test. The association between these parameters and FPG was determined using the Pearson product moment correlation.

Results : The red cell GSH levels were significantly lower in all types of diabetes compared with those of age-matched normal control subjects ($p < 0.05$). Red cell GPx activity was significantly increased only in the poorly controlled type 2 DM and type 2 DM with CHD ($p < 0.05$). The decrement of red cell GSH may be due to the higher consumption of GSH for the increasing of GPx activity or there is a reduction of the pentose phosphate pathway that is stimulated by insulin resulting in lowered GSH recycling. The association

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between FPG and GSH or GPx activity in all subgroups of type 2 diabetic patients compared with normal healthy subjects showed no correlation.

Conclusion : These findings suggested that type 2 DM patients were susceptible to oxidative stress and higher blood glucose level in poorly controlled type 2 DM and type 2 DM complicated with CHD had an association with free radical-mediated lipid peroxidation. Therefore, any means that can reduce oxidative stress may be beneficial for the prevention or slowing of progression of cardiovascular complication in these types of diabetic patients.

Key words : Type 2 diabetes, oxidative stress, glutathione, glutathione peroxidase, Cardiovascular complication.

เรื่องย่อ : ระดับกลูตาไธโอนและเอนไซม์กลูตาไธโอนเปอร์ออกซิเดสในผู้ป่วยเบาหวานชนิดที่ 2
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วัตถุประสงค์ : ภาวะน้ำตาลในเลือดสูงมักเป็นสาเหตุสำคัญที่ก่อให้เกิดภาวะออกซิเดทีฟสเตรสในผู้ป่วยเบาหวาน ซึ่งภาวะออกซิเดทีฟสเตรส หรืออนุมูลอิสระนั้นเป็นปัจจัยสำคัญในการก่อให้เกิดโรคแทรกซ้อน โดยเฉพาะโรคหลอดเลือดหัวใจตีบในผู้ป่วยเบาหวาน อย่างไรก็ตามผลของการควบคุมระดับน้ำตาลและการเกิดโรคแทรกซ้อนในผู้ป่วยเบาหวานต่อตัวแปรของภาวะออกซิเดทีฟสเตรสยังไม่มีการศึกษามาก่อน ดังนั้นวัตถุประสงค์ของการศึกษานี้เพื่อเปรียบเทียบระดับของกลูตาไธโอนและการทำงานของเอนไซม์กลูตาไธโอนเปอร์ออกซิเดสในผู้ป่วยเบาหวานชนิดที่ 2 ที่ควบคุมระดับน้ำตาลในเลือดได้ดีปานกลาง (ระดับน้ำตาลในพลาสมาในระยะอดอาหารน้อยกว่าหรือเท่ากับ 180 มก./ดล.) ผู้ป่วยที่มีการควบคุมระดับน้ำตาลในเลือดไม่ดี (ระดับน้ำตาลในพลาสมาในระยะอดอาหารมากกว่า 180 มก./ดล.) และผู้ป่วยเบาหวานที่มีโรคหัวใจแทรกซ้อนกับคนปกติที่มีระดับน้ำตาลในพลาสมาในระยะอดอาหารน้อยกว่า 110 มก./ดล.)

วิธีการศึกษา : ทำการวิเคราะห์ระดับกลูตาไธโอนและการทำงานของเอนไซม์กลูตาไธโอนเปอร์ออกซิเดสในเม็ดเลือดแดงของผู้ป่วยเบาหวานชนิดที่ 2 ที่ควบคุมระดับน้ำตาลในเลือดได้ดีปานกลาง จำนวน 26 คน ผู้ป่วยที่มีการควบคุมระดับน้ำตาลในเลือดไม่ดี จำนวน 19 คน และผู้ป่วยเบาหวานที่มีโรคหัวใจแทรกซ้อนจำนวน 20 คน กับคนปกติจำนวน 20 คน ที่มีจำนวนของเพศและอายุใกล้เคียงกันจัดเป็นกลุ่มควบคุม ข้อมูลที่ได้จะถูกวิเคราะห์โดย one-way ANOVA (one-way analysis of variance) และสหสัมพันธ์ของค่าเหล่านี้กับระดับน้ำตาลในพลาสมาระหว่างกลุ่มของผู้ป่วยเบาหวานชนิดที่ 2 และคนปกติจะถูกวิเคราะห์โดย Pearson product moment correlation

ผลการศึกษา : จากการศึกษาพบว่าระดับกลูตาไธโอนในเม็ดเลือดแดงของผู้ป่วยเบาหวานชนิดที่ 2 ทุกประเภทมีระดับต่ำกว่าปกติอย่างมีนัยสำคัญ ($p < 0.05$) แต่การทำงานของเอนไซม์กลูตาไธโอนเปอร์ออกซิเดสจะมีค่าเพิ่มขึ้นอย่างมีนัยสำคัญเฉพาะในผู้ป่วยเบาหวานที่ควบคุมระดับน้ำตาลได้ไม่ดีและผู้ป่วยเบาหวานที่มีโรคหัวใจ

แทรกซ้อนเท่านั้น ($p < 0.05$) การลดลงของระดับกลูตาไธโอนนี้อาจจะเกิดขึ้นได้ เนื่องจากการใช้กลูตาไธโอนในการเพิ่มการทำงานของเอนไซม์กลูตาไธโอนเปอร์ออกซิเดส กอปรกับการทำงานของเอนไซม์ในวิถีเพนโทสฟอสเฟตที่ลดลงอันเนื่องมาจากการลดลงของอินซูลินในการกระตุ้นวิถีนี้ ทำให้การสังเคราะห์กลูตาไธโอนลดลง เมื่อศึกษาความสัมพันธ์ของระดับน้ำตาลในพลาสมาในระยะอดอาหารกับค่าตัวแปรเหล่านี้ระหว่างกลุ่มของผู้ป่วยเบาหวานชนิดที่ 2 ทุกชนิดกับคนปกติไม่พบว่ามีความสัมพันธ์ต่อกัน

สรุป : การศึกษาครั้งนี้ชี้แนะว่าผู้ป่วยเบาหวานชนิดที่ 2 อยู่ภายใต้ภาวะออกซิเดทีฟที่ฟอสเตอร์ และระดับน้ำตาลในเลือดที่เพิ่มสูงขึ้นในผู้ป่วยเบาหวานที่ควบคุมระดับน้ำตาลไม่ดี และผู้ป่วยเบาหวานที่มีโรคหัวใจแทรกซ้อนจะมีความสัมพันธ์กับการเกิดอนุมูลอิสระ ซึ่งจะไปทำลายเซลล์โดยกระบวนการ lipid peroxidation ดังนั้นวิธีการใดๆ ที่สามารถลดภาวะออกซิเดทีฟที่ฟอสเตอร์ในผู้ป่วยเบาหวานชนิดที่ 2 ก็อาจจะเป็นประโยชน์ในการป้องกันหรือลดภาวะแทรกซ้อนของการเกิดโรคหลอดเลือดหัวใจตีบในผู้ป่วยได้

INTRODUCTION

Type 2 diabetes is a chronic systemic disease, involving altered relationships among the liver, muscle, adipose tissue, brain, kidneys, and most other tissues of the body¹. The genetic and lifestyle factors related to the risk of developing type 2 DM include obesity, body fat distribution, physical inactivity, and insulin resistance. Insulin resistance is a usual contributor, but not the only cause of diabetes². Strong evidence exists for an additional pancreatic β -cell defect³, which is difficult to detect because of overall system compensations⁴. Also, the disease is presumably heterogeneous, and different causes may well contribute to single symptomology¹.

Epidemiological studies of type 2 diabetes have shown that type 2 DM is a disease found worldwide⁵. After the discovery of insulin and the clinical use of drugs in diabetes, mortality from acute diabetic complications such as ketoacidosis showed significant decrease in contrast to the increasing prevalence of long-term complication which became the major causes of mortality in diabetes⁵. Although diabetic patients usually suffer from microvascular disease, they often die from macrovascular disease and the major cause of mortality is CHD^{6,7}.

Several studies suggest that chronic hyperglycemia and oxidative stress may be associated with diabetes and its complications⁸. 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 such as 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, such as glucose autooxidation, non-enzymatic glycation of protein, and the polyol pathway⁹. These pathways enhance generation of reactive oxygen species (ROS) that lead to tissue damage and cause several complex syndromes in diabetic patients such as cataracts, renal dysfunction, nerve damage, and atherosclerosis. Atherosclerosis in particular leads to coronary heart disease (CHD) which is the major cause of death among diabetics^{10,11}.

However, the action of reactive oxygen species (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 ($O_2^{\bullet-}$) to hydrogen peroxide (H_2O_2), and 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 a GSH and exogenous antioxidant vitamins from the diet found in fruits and vegetables. These non-enzymatic antioxidants act as terminators of free radicals' chain reactions caused by lipid peroxidation¹².

Glutathione has an advantageous antioxidant property in that it is a cofactor of the GPx enzyme and is involved in the detoxification of ROS, whereas exogenous antioxidant vitamins act only in the detoxification of ROS. Therefore, the aim of this study is to compare the level of glutathione and GPx activity in poorly controlled type 2 DM, fairly controlled type 2 DM, and type 2 DM complicated with CHD with that of normal subjects. This information can serve as a knowledge base for understanding the progression of pathogenic mechanisms of cardiovascular complication in type 2 diabetes.

MATERIALS AND METHODS

Subjects

Twenty normal healthy subjects with FPG < 110 mg/dl were a control group. Their ages ranged from 35 to 70 years old. Type 2 diabetic subjects were subdivided into three groups, as follows: 26 subjects were fairly-controlled type 2 DM whose FPG < 180 mg/dl, age range from 49 to 85 years old. 19 subjects were poorly controlled type 2 DM whose FPG > 180 mg/dl, age range from 39 to 83 years; 20 subjects were type 2 DM complicated with CHD, age range from 31 to 76 years old. All the subject gave their consent to this study which was approved by the ethical clearance committee on human rights, Faculty of Medicine Siriraj Hospital, Mahidol University. Venous blood samples for the successive biochemical determinations were withdrawn in the morning after fasting for 8 hours. Blood biochemistry determinations were performed using an enzymatic reagent kit is commonly done in our laboratory.

Erythrocyte reduced glutathione assay

The reduced glutathione assay (GSH) in red cells was performed as described by Beutler¹³. In brief, 3% TCA was added to 0.1 ml of packed red cells and mixed, then 0.25 ml followed and 0.9 ml of cold distilled water was added. The suspension was centrifuged at 1,870 g for 5 minutes 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 let to stand at 4°C for 5 minutes. The optical density was

read at 412 nm against an unknown blank. The GSH level was obtained from a standard curve which was prepared by using 12.5, 25, 50, 100 and 200 mg/dl of standard reduced GSH from Sigma in distilled water.

Erythrocyte glutathione peroxidase assay

The GPx activity in red cells was determined by a Erythrocyte glutathione peroxidase assay according to the instructions of glutathione peroxidase reagent kit (Ransel), No. RS504 from Randox Laboratories, UK.

Statistical analysis

Descriptive statistics were expressed as mean $\pm$ SEM (standard error of the mean). The SPSS for windows program was used for statistical analysis. Since the data obtained were normally distributed and more than two groups, the one way analysis of variance (one-way ANOVA) was performed to compare all subgroups of diabetes subsets with those in the normal healthy group. The Pearson product moment correlation coefficient was used to assess the relationship among these oxidative stress values and the FPG of type 2 diabetic patients and normal healthy subjects. Differences were considered significant when $p < 0.05$.

RESULTS

Database characteristics of type 2 DM

Table 1 shows the database characteristics of age, gender, fasting plasma glucose and lipid profile between normal healthy group subsets and type 2 diabetic patients. This table shows that all subgroups of type 2 DM had significantly higher FPG than did normal subjects. The study of plasma lipid profile showed that the total cholesterol, LDL-cholesterol were not significantly different from that of the normal healthy group subjects. This may be due to treatment with a cholesterol-lowering drug for maintenance the blood lipid level in these diabetic subjects. However, the increase of triglycerides and VLDL-cholesterol, and the decrease of HDL-cholesterol also found in type 2 DM was complicated with CHD. The lower level of HDL may be a risk factor that exacerbates the cardiovascular complications in this group of diabetes patients.

Table 1. Database of age, gender, FPG and lipid profiles in normal healthy subjects and type 2 diabetic patients (Mean $\pm$ SEM)

Parameter	Normal (n = 20)	Poorly controlled type 2 DM (n = 19)	Fairly controlled type 2 DM (n = 26)	Type 2 DM with CHD (n = 20)
Age (years)	56.35 $\pm$ 1.62	63.96 $\pm$ 1.69	62.3 $\pm$ 2.46	60.50 $\pm$ 2.56
Gender (M/F)	12/8	8/11	13/13	6/14
FPG (mg/dl)	105.10 $\pm$ 3.05	245.00 $\pm$ 22.52 ^a	128.50 $\pm$ 3.88 ^b	167.26 $\pm$ 13.26 ^b
Total cholesterol (mg/dl)	217.10 $\pm$ 9.57	255.65 $\pm$ 13.05	229.87 $\pm$ 9.02	225.56 $\pm$ 13.90
LDL-cholesterol (mg/dl)	134.24 $\pm$ 9.79	172.43 $\pm$ 12.38	156.65 $\pm$ 8.01	143.59 $\pm$ 13.55
Triglyceride (mg/dl)	133.31 $\pm$ 12.66	180.28 $\pm$ 22.59	153.58 $\pm$ 11.97	220.06 $\pm$ 20.32 ^b
VLDL-cholesterol (mg/dl)	27.05 $\pm$ 2.25	36.06 $\pm$ 4.51	30.44 $\pm$ 2.44	43.51 $\pm$ 3.78 ^b
HDL-cholesterol (mg/dl)	55.81 $\pm$ 2.69	47.16 $\pm$ 3.15	42.79 $\pm$ 2.42 ^c	43.25 $\pm$ 1.97 ^b

a = significance at $p < 0.001$ as compared with the normal healthy subjects

b = significance at $p < 0.01$ as compared with the normal healthy subjects

c = significance at $p < 0.05$ as compared with the normal healthy subjects

Red blood cell GSH level and GPx activity

The mean levels of red blood cell reduced glutathione (GSH) that functioned as an antioxidant and a cofactor of enzyme GPx and GPx activity were determined and are summarized in Table 2. The levels of GSH in all subgroups of type 2 DM were significantly lower when compared with that of normal healthy group subjects ($p < 0.05$) (Figure 1). However, there was no significant difference among type 2 DM subgroups.

The activity of red blood cell glutathione peroxidase (GPx) in poorly controlled type 2 DM and type 2 DM complicated with CHD were significantly higher than that in normal healthy subjects ($p < 0.05$) (Figure 2). However, no significant difference was shown when compared with the fairly controlled type 2 DM ($p = 0.92$). The comparisons were also observed among these type 2 DM subgroups and showed no significant differences.

Table 2. Red cell GSH and GPx activity in normal healthy subjects and type 2 DM patients (Mean $\pm$ SEM).

Subjects	GSH (mg/gHb)	GPx (U/gHb)
Normal (n = 20)	1.29 $\pm$ 0.08	23.59 $\pm$ 2.18
Poorly controlled Type 2 DM (n = 19)	0.99 $\pm$ 0.04 ^c	37.75 $\pm$ 3.65 ^c
Fairly controlled Type 2 DM (n = 26)	0.98 $\pm$ 0.06 ^c	26.59 $\pm$ 2.54
Type 2 DM with CHD (n = 20)	0.97 $\pm$ 0.06 ^c	35.93 $\pm$ 4.20 ^c

c = significance at $p < 0.05$ as compared with the normal healthy subjects

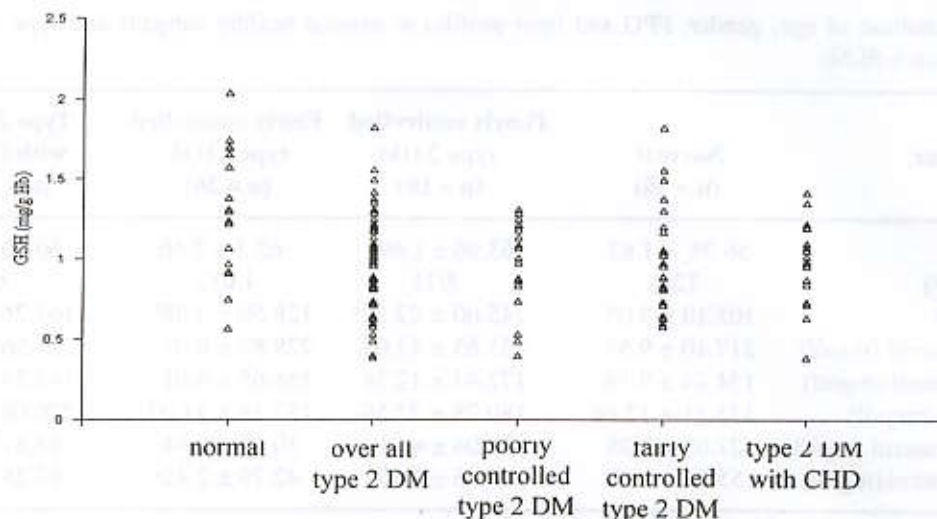


Figure 1. Erythrocyte reduced glutathione in normal healthy subjects (n=20) and type 2 diabetes (n=65) subdivided into poorly controlled type 2 DM (n=19), fairly controlled type 2 DM (n=26) and type 2 DM with CHD (n=20). Results are given in mg/gHb and expressed as the mean $\pm$ SD. The long horizontal bar represent the mean value and * shows the level of significance.

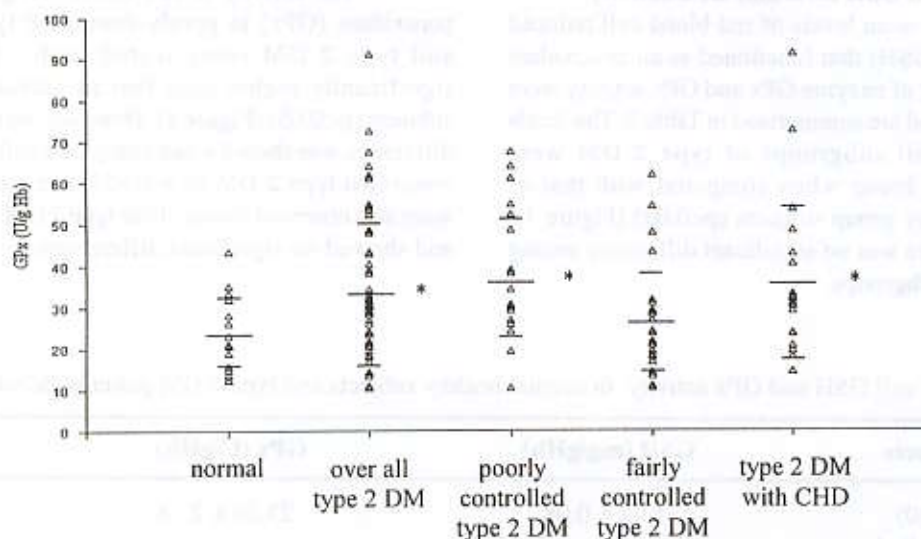


Figure 2. Erythrocyte glutathione peroxidase activity in normal healthy subjects (n=20) and type 2 diabetes (n=65) subdivided into poorly controlled type 2 DM (n=19), fairly controlled type 2 DM (n=26) and type 2 DM with CHD (n=20). Results are given in U/gHb and expressed as the mean $\pm$ SD. The long horizontal bar represent the mean value and * shows the level of significance.

The correlation among FPG, GSH, and GPx activity in all subgroups of type 2 diabetic patients and normal subjects was also calculated. No

correlation was observed between FPG and GSH or GPx activity among these subgroups of type 2 diabetes and normal healthy subjects (Figures 3, 4).

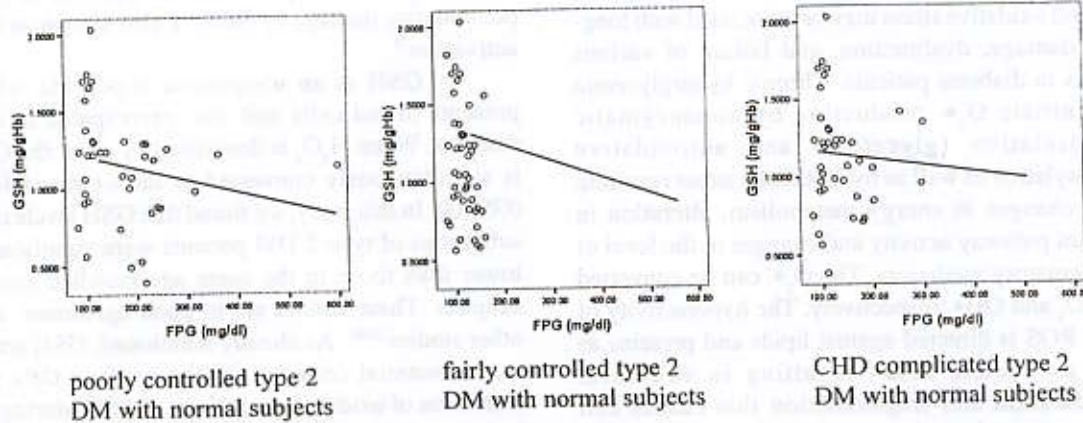


Figure 3. Correlation between fasting plasma glucose and reduced glutathione in type 2 diabetic patients and normal subjects ($r = -0.20$, $p > 0.05$ for poorly controlled type 2 DM; $r = -0.28$, $p > 0.05$ for fairly controlled type 2 DM; and $r = -0.08$, $p > 0.05$ for type 2 DM complicated with CHD).

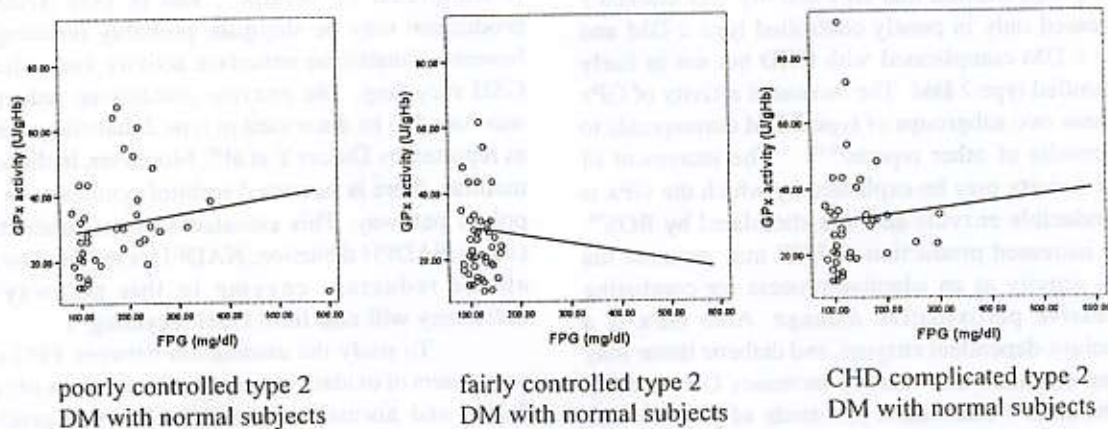


Figure 4. Correlation between fasting plasma glucose and glutathione peroxidase activity in type 2 diabetic patients and normal subjects ($r = 0.10$, $p > 0.05$ for poorly controlled type 2 DM; $r = -0.05$, $p > 0.05$ for fairly controlled type 2 DM; and $r = 0.05$, $p > 0.05$ for type 2 DM complicated with CHD).

DISCUSSION

As already mentioned, chronic hyperglycemia and oxidative stress may be associated with long-term damage, dysfunction, and failure of various organs in diabetes patients. Chronic hyperglycemia can initiate $O_2^{\bullet-}$ production by nonenzymatic glycosylation (glycation) and autooxidative glycosylation as well as by metabolic stress resulting from changes in energy metabolism, alteration in sorbitol pathway activity and changes in the level of inflammatory mediators. The $O_2^{\bullet-}$ can be converted to H_2O_2 and $OH^{\bullet}$, respectively. The hyperactivity of these ROS is directed against lipids and proteins as well as nucleic acid¹⁴ resulting in structural modification and fragmentation that causes cell damage in diabetes¹⁵.

However, the antioxidant defense system in the blood can protect tissues and organs against the ROS. These antioxidant systems are composed of enzymatic and non-enzymatic antioxidants. There are three main antioxidant enzymes, SOD, CAT, and GPx. This study of the erythrocyte GPx activity in type 2 DM showed that GPx activity was markedly increased only in poorly controlled type 2 DM and type 2 DM complicated with CHD but not in fairly controlled type 2 DM. The increased activity of GPx in these two subgroups of type 2 DM corresponds to the results of other reports¹⁶⁻¹⁸. The increment of GPx activity may be explained by which the GPx is an inducible enzyme and was stimulated by ROS¹⁹. The increased production of ROS may promote the GPx activity as an adaptive process for combating excessive peroxidative damage. Also GPx is a selenium-dependent enzyme, and diabetic tissue may retain selenium and thereby increases GPx activity in the cells¹⁸. The results of a study of patients with β -thalassemia/hemoglobin E as reported by Likidilid et al²⁰ demonstrated the higher level of selenium in red cells and lower level in plasma. The thalassemic patients are also known to be under oxidative damage similar to the diabetic patients. However, Gebre-Medhin et al²¹ observed an increased level of plasma selenium in diabetic children. 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 diet, the PUFA (polyunsaturated fatty acid) intake²², because PUFA within cells

is prone to peroxidative damage by ROS. The increasing of hydroperoxide, the product of lipo-peroxidative damage by ROS, is also known as GPx activation²³.

GSH is an ubiquitous tripeptide which presents in red cells and also participates in GPx reaction. When H_2O_2 is detoxified by GPx, the GSH is simultaneously converted to the oxidized form (GSSG). In this study, we found that GSH levels in all subgroups of type 2 DM patients were significantly lower than those in the same age-matched control subjects. These results are in good agreement with other studies²⁴⁻²⁶. As already mentioned, GSH serves as an essential cofactor for the enzyme GPx and formation of oxidized glutathione (GSSG) during the enzyme processes. Thus increasing in GPx activity implies higher consumption of GSH. Another mechanism which may explain the GSH reduction in diabetes is 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 insulin²⁷, and in DM NADPH production may be sluggish, probably resulting in lowered glutathione reductase activity and reduced GSH recycling. The enzyme glutathione reductase was found to be decreased in type 2 diabetic patients as reported by Dincer Y et al²⁸. Moreover, in diabetes mellitus, there is increased sorbitol synthesis via the polyol pathway. This elevated sorbitol production causes NADPH depletion. NADPH is required by the aldose reductase enzyme in this pathway. A deficiency will also limit GSH recycling²⁸.

To study the association between FPG and parameters of oxidative stress in all subgroups of type 2 DM and normal subjects, the Pearson product moment correlation was performed. We observed no correlation of GSH in all subgroups of type 2 DM was observed ($r = -0.28$, $p > 0.05$ for fairly controlled type 2 DM; $r = -0.20$, $p > 0.05$ for poorly controlled type 2 DM; and $r = -0.08$, $p > 0.05$ for type 2 DM complicated with CHD). This result suggested that the sluggish stimulation of insulin in type 2 DM to the pentose phosphate pathway was less as compared with normal subjects, resulting in the decrease of GSH synthesis. When comparing FPG and GPx activity, we also found no correlation in all subgroups of type 2 DM ($r = -0.05$, $p > 0.05$ for fairly controlled type 2

DM; $r = 0.10$, $p > 0.05$ for poorly controlled type 2 DM; and $r = 0.05$, $p > 0.05$ for type 2 DM complicated with CHD). This implied that the hyperglycemic condition in DM may glycate this enzyme²⁹ resulting in fragmentation^{15,30}, causing the enzyme to be less active, as already described. Wolff et al³¹ reported that glycated protein produced ROS. Mullarkey et al (32) also suggested that increased glycation of proteins may produce ROS and accelerate atherogenesis as a result of oxidative modifications of vascular membrane lipids. The production of ROS by the glycated proteins, on the one hand, and the inactivation of GPx activity by glycation. On the other hand, may enhance the accumulation of ROS leading to the serious complications of diabetes.

CONCLUSIONS

In conclusion, our study supported the hypothesis that hyperglycemia activates cellular and

tissue damage by oxidative stress. Although, there are compensatory mechanisms for defense against ROS, normalization of oxidative stress is not achieved in the diabetic patients. Thus, any means that reduces oxidative damage may be useful for treatment of diabetic patients in the future³³.

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