The Relationship between Erythrocytes Membranes Variables, Conductivity of Erythrocytes Suspension and Activity of Purified Erythrocyte Glutathione-S-Transferase Enzyme GST in Diabetic Patients

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Abstract

This study was included 69 persons (26 patients with insulin dependence diabetes mellitus IDDM with mean ages (49.5±7.66) years , and 43 patients with non- insulin dependence diabetes mellitus NIDDM with mean ages (46.16±7.22) years , for patients admitted to Medical city of Margan in Hilla, Babylon Governorate, Iraq. and 25 healthy person with mean ages (30.72±4.46) years, as control group. The blood glucose, sera malondialdehyde (MDA), sera glutathione (GSH), glycated hemoglobin (HbA1c) and conductivity for erythrocyte suspension are measured and compare with control group. The purified enzyme activity glutathione S-transferase (GST) from erythrocyte using anionic exchanger was measured at 340 nm with using 1-Chloro-2,4-Dinitrobenzene (CDNB) as a substrate at 37 °C . The specific activity of enzyme was calculated .The levels of variables are contacted together with these relationships to the erythrocyte cell wall by comparing with control group.

Keywords: Diabetes Mellitus, Erythrocyte conductivity, Glutathione-S transferase

العلاقة بين متغيرات جدار خلية الدم الحمراء مع فعالية انزيم كلوتاثايون –اس ترانسفيريز GST المنقى من كريات الدم الحمراء للمرضى المصابين بداء السكري

الخلاصة

اشتملت هذه الدراسة على 94 شخصا , 26 منهم مصابين بداء السكري من النوع الأول IDDM اعمارهم بين (37-61)سنة و 43 شخصا من المصابين بالسكري من النوع الثاني NIDDM اعمارهم بين (29-59) سنة ، للمرضى الراقدين في مدينة مرجان الطبية في محافظة بابل .العراق ، و 25 شخصا سليما يمثلون مجموعة السيطرة وكانت اعمارهم بين (22-38) سنة ، تم قياس مستويات كل من كلوكوز الدم و المالون ثنائي الديهايد MDA والكلوتاثايون GSH في مصل الدم وكذلك النسبة المئوية للهميوكلوبين المتسكر HbA1c وقيست التوصيلية لعالق كريات الدم الحمراء للمرضى ومقارنتها بمجموعة السيطرة . تم تنقية الإنزيم كلوتاثايون اس – ترانسفيريز (GST) glutathione –S transferase من كريات الدم الحمراء تنقية جزئية باستخدام مبادل ايوني سالب، (قيست الفعالية الإنزيمية عند 340 nm باستخدام المركب 1-Chloro-2,4-Dinitrobenzene كمادة أساس عند درجة حرارة 37 °C وتم حساب الفعالية النوعية للإنزيم)، وتم الربط بين مستويات هذه المتغيرات بعلاقتها بغشاء كرية الدم الحمراء من خلال مقارنتها بمجموعة السيطرة .

Introduction

Diabetes Mellitus is define as a family of metabolic disorders of multiple etiology that is characterized by chronic hyperglycemia accomplished with metabolism disturbances of

carbohydrates, fats and proteins resulting from insulin secretion, insulin action or both or insulin resistance by cells , the common symptoms of diabetes is polyuria ,polydipsia and polyphagia. [1] As a result for continuous of high levels of glucose in the blood especially in patients with poor control for Diabetes Mellitus that caused glycation of hemoglobin [2] and increase production of free radicals as a by-product of auto-oxidation of glucose [3] free radicals can be defined as any species capable of independent existence that contain one or more unpaired electrons that including reactive oxygen species, nitrogen and chlorine reactive species which responsible for more than 50 diseases [4] that also produced at normal levels in the most metabolism processes that have highly reactivity according their chemical properties which causes damage for cells if they concentration increased with low levels of antioxidants [5] that reach between 1 billion and 3 billion reactive species are generated in one cell per day [6] ,so the antioxidant defense systems is become very important to minimize the high levels of these species and decreased the tissue damage , these antioxidants including glutathione (Master antioxidant), ascorbic acid, vitamin E , vitamin A, uric acid and minerals including Se, Zn, Mn are trace elements that form an essential part of various antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) serve as primary line of defense in scavengers of free radicals[7]. The enzymes of glutathione S-transferase (GSTs) (E.C 2.5.1.18) are dimeric enzymes that is consider antioxidant indirectly because its action against exogenous and endogenous xenobiotics [14,15]. Some of these xenobiotics are produced free radicals[16].These enzymes are found in insects, plants and nearly in all mammalian tissues in different isoenzymes that are divided into three main classes including basic, near neutral and acidic , in human body there are about 200 different kinds of specialized cells reach in total about to more than 10 [14] cells[8] it thought that nearly all of these cells have the GST enzymes such as kidney[9], liver [10], prostate [11], ovary [12], lenses [13]. The cells of all kinds share in their structural membranes are a fluid structure composed from two opposite bi-layer of lipids, immersed in these bi-layer some proteins in not covalently linked.[25] The function of cell membranes are enclosure and isolation of cells and organelles, regulation transport of ions and has receptors on its surface to regulate biomolecules transportation [26].

Material and Methods

Chemicals and reagents, acetone and absolute ethanol were purchased from BDH and chloroform, EDTA, DEAE-Cellulose (DE-52), GSH, and 1-Chloro-2,4-dinitrobenzene (CDNB) obtained from Sigma K_2HPO_4 , KH_2PO_4 and Tris-Base purchased from Merk company.

Blood samples: Blood samples were obtained under fasting conditions .

Purification of human Glutathione S-Transferase erythrocyte GST enzyme [17]

Hemolysis of blood

The 2.5 ml fresh venous blood sample in EDTA – tube was centrifuged at 600xg for 10 minutes and the plasma and buffy coat was removed and washed the erythrocyte cells by cold 0.9 % NaCl three times with centrifugation, then added 3 volumes of cold de-ionized water to packed cells for hemolysis the RBCs with stirring in vortex at least 20 minutes and freeze.

Protein precipitation

The precipitation of proteins done by adding the cold acetone (-20 °C) four volumes to one volume of hemolysate and using ultra-centrifuge at 14000xg for 20 minutes at 4°C in eppendorf tubes 1.5 ml, the suspension was removed and re-suspended the precipitate in 0.05 M potassium phosphate buffer pH 7.5 and stirring until the precipitate completely dissolved then re-centrifugation process at 14000xg for 15 minutes at 4°C and the precipitate was removed and treat the suspension with ethanol-chloroform mixture.

Ethanol-Chloroform Treatment

one ml of ethanol-chloroform mixture (1:3) was added for each one ml resultant from protein precipitation process then stirred vigorously for one minute, then centrifugation to remove the denatured hemoglobin, and then the mixture is allowed to evaporated in vacuum.

DEAE-Cellulose Chromatography

The solution resulted from the last step is applied into DEAE-Cellulose Column (1.9 x 40 cm) previously equilibrated with 10 mM of Tris-chloride buffer pH 7.5 containing 0.1 mM EDTA and 0.1 mM of reduced glutathione .The eluent solution was contained 10 mM of Tris-chloride buffer pH 7.5 , 0.1 mM EDTA, and 1 mM of GSH and gradient salt of NaCl (0- 0.1-0.5-1) M. Collected of 1.5 ml fractions at flow rate equal to 0.4 ml/ min and measure the total proteins at 280 nm. The fractions that contain enzyme activity was took and calculated the specific activity for each tube.

Activity of human erythrocyte Glutathione S-Transferase [18]

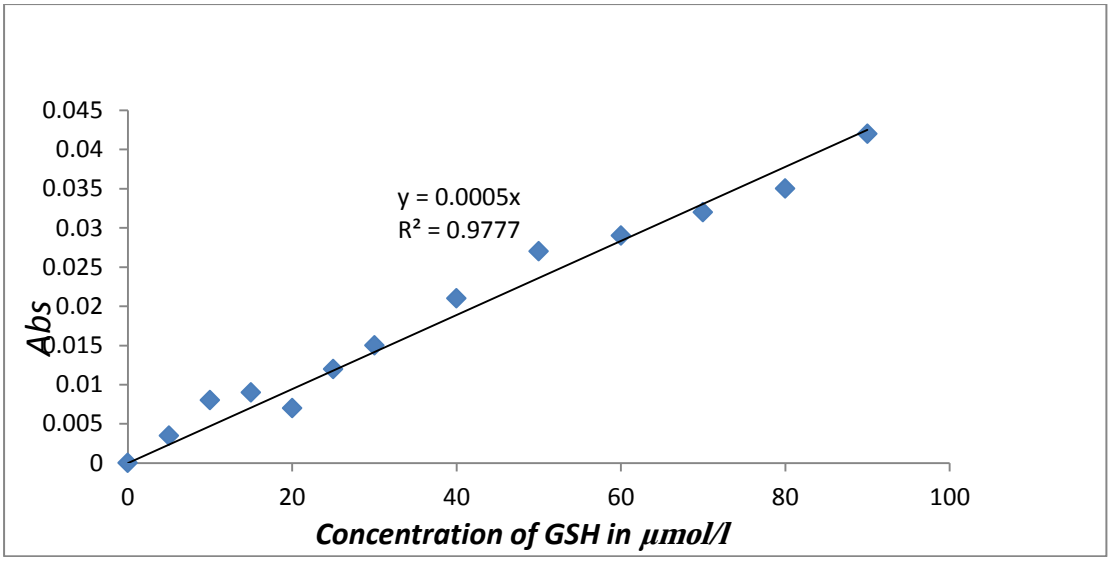
The activity of human erythrocyte GST can be determine by using 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate at 37 °C.

Reaction of CDNB with GSH in presence of GST enzyme to produce Dinitrophenyl glutathione that absorbed at 340 nm.

The specific activity was also calculated in each step of purification , the total protein measured according to Bradford method[21].

Determination of reduced glutathione GSH [19]

Principle: the compound 5,5-Dithiobis (2-nitrobenzoic acid) DTNB is consider as disulfide chromogen can be readily reduced by the thiol group of free glutathione to gives yellow color compound that absorbed at 412 nm .The diagram below show the calibration curve for glutathione.



The calibration curve of glutathione GSH.

Determination of sera Malondialdehyde [20]**:** The colorimetric procedure was used to measure the malondialdehyde in serum, thiobarbutaric acid (TBA) was used at low pH and elevated temperature. It is most common method that used to estimation the lipid peroxidation process ,in this procedure the MDA is participate with TBA in nucleophilic addition reaction to produced red color compound that absorbed at 532 nm.

Conductivity measurement: [22]

The apparatus of conductivity was detected with WTW lnolab 740 by immersed the probe into solution contained 0.5 ml of packed RBCs with 25 ml of normal saline , aginst blank solution (0.5 ml distal water + 25 ml normal saline)

HbA1c Measuring:

Glycated hemoglobin HbA1c was calculated according to Stanbio Glycohemoglobin kit .

Results and Discussion

Table -1 explain the levels of serum glucose and percentage values of glycated hemoglobin for patients and control there are significant increase in these levels between three groups , and for the reduced glutathione GSH that represented as a component of antioxidant defense system, there are significant decrease in the levels, between the control group 3.21±o.16 , the diabetic groups for IDDM 0.73±0.08 ,for NIDDM 1.47±0.15 . The levels of malodialdehyde that represented of the lipid peroxidation process there are higher significant increase in their levels between the control group 1.89±0.15 , the IDDM 7.84±0.29 , the NIDDM 8.32±0.28, the mean value for patients with type -2 have value higher than the patients with type 1 because the obesity is caused the insulin resistance which is consider major cause for diabetic patients with type 2 [23,24], this means high levels of lipid in serum that produced high rate of lipid peroxidation. In other hand the most patients with type 1 loss their body fats contains in the first stage of diabetes.

The oxidative stress produced free radical and reactive oxygen species ROS even in normal conditions but they quenched by antioxidants [27], if these levels are increase in cases of diabetes mellitus causes damages on the cell wall and the conductivity of the erythrocytes are increase, and

enhanced the erythrocyte enzyme glutathione-S transferase levels as shown in table- 3 for control and table -4 for diabetic (IDDM & NIDDM) . The clinical parameters were summarized in table-1 .

Table 1 The biochemical data for healthy and diabetic patients, the data are expressed as mean ±St. Error of mean.

Variable	Control	IDDM	NIDDM
Glucose mmol/l	4.64±0.14	13.18±0.81	11.49±0.46
HbA1c %	4.89±0.14	9.15±0.3	7.72±0.18
GSH µmol/l	3.216±0.16	0.73±0.08	1.47±0.15
MDA µmol/l	1.89±0.15	7.84±.029	8.32±0.28

The demographic parameters of sixty nine patients with Diabetes Mellitus (26 with type 1 and 43 with type 2) and 25 persons as control group that including in table -2 .

Table 2 The Demographic data for patients and control.

Parameter	Control N=25	IDDM N=26	NIDDM N=43
Gender M/F	16/9	14/12	23/20
Age(mean±S.D)	30.72±4.46	49.5±7.66	46.16±7.22
Duration of Disease mean±S.D	-------	10.26±3.06	7.97±4.29

The diagram show the conductivity for red blood cells suspension in ms/cm . There are significant increase in conductivity of erythrocyte suspension in diabetic patients (IDDM & NIDDM) when compared with control group. (fig-1)

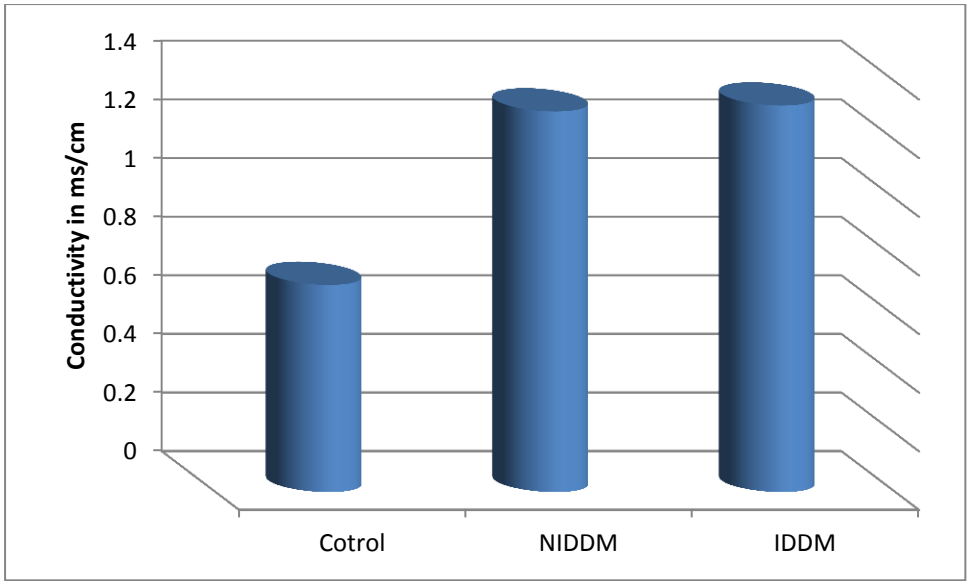


Figure 1 The conductivity for red blood cells suspension in ms/cm.

The diagram show the levels of glycated hemoglobin in red blood cells HbA1c %. There are significant increase in glycated hemoglobin HbA1c in diabetic patients (IDDM & NIDDM) when compared with control group. (fig-2).

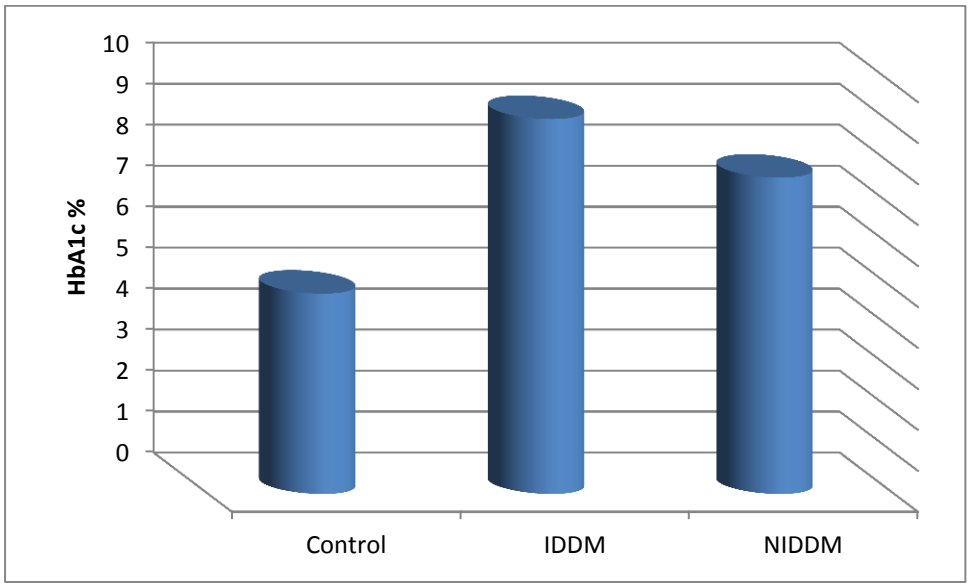


Figure 2. The glycated hemoglobin in red blood cells HbA1c % .

Table 3 Purification steps for human erythrocyte GST for control .

Purification step	Activity (U)	Total protein (mg)	Specific Activity (U/mg)	Purification Fold *	Yield** %
Hemolysis Blood	150	708	0.21	1.00	------
Acetone Precipitation 30 %	73.75	73.5	1.11	5.26	49.15
Ethanol- CCl_4 Mixture	37.5	17.92	2.09	9.90	25.16
DEAE-Cellulose	12.822	3.33	3.85	18.33	8.54

*A measure of how much more pure protein is after purification step in comparison to the crude.

** A measure of the percentage of the protein activity in each purification step.

Table 4 Purification steps for human erythrocyte GST for type-1 diabetes (**I**) and type-2 diabetes (**II**).

Purification steps	Activity (U)		Total protein (mg)		Specific Activity (U/mg).		Purification Fold.		Yield %	
	I	**II**	**I**	**II**	**I**	**II**	**I**	**II**	**I**	**II**
Hemolysis Blood	183	169	699	721	0.26	0.23	1.00	1.00	------	-----
Acetone Precipitation 30 %	80.1	77.4	72.1	74	1.11	1.04	4.26	4.52	43.7	45.7
Ethanol-CCl_4 Mixture	42.5	35.7	17.9	16.9	2.37	2.11	9.11	9.17	23.22	21.1

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