How To Understand The Relationship Between Oxidative Stress , Non Enzymatic Antioxidant And Trace Elements In Diabetic Patients .

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Absract:
The correlation between free radicals produced by diabetic patients and non enzymatic antioxidants in addition to trace elements have been undertaken in this study to evaluate the risky of such effects on different biological parameters in such patients .The results appears in this study state that anew information have been released to consider these effects and how to neutralize it.

الخلاصة
اوضحت هذه الدراسة العلاقة الجديدة بين االجذور الحرة الناتجة لدى مرضى السكري وبين مضادات الاكسدة غير الانزيمية من جهة والعناصر النزرة من جهة اخرى ، وبذا حدد معامل الخطورة في هذه العلاقة بين المتغيرات البايولوجية والتي عن طريقها يمكن معادلة اثار الجذور الحرة.

Introduction

The old criteria for diagnosis of DM recommended by the World Health Organization (1) were:
1. Fasting plasma glucose (FPG) ≥ 140 mg/dL (7.8 mmol/L).
2.Two-hours post load glucose (2-hPG) ≥ 200 mg/dL (11.1 mmol/L).
According to the new American Diabetes Association (ADA) recommendations to allow for earlier detection of the disease, all adults older than age 45 years should have a measurement of fasting blood sugar every 3 years unless the individual is otherwise diagnosed with diabetes (2).
The modified criteria for diagnosis of DM recommended by an expert committee to allow earlier detection of the disease were:
1. FPG (Fasting Plasma Glucose) < 110 mg/dL (6.1 mmol/L) = normal fasting glucose.
2. FPG ≥110 mg/dL (6.1 mmol/L) and < 126 mg/dL (7.0 mmol/L) = impaired fasting glucose (IFG).
3. FPG ≥ 126 mg/dL (7.0 mmol/L) = provisional diagnosis of diabetes.
The corresponding criteria when the oral glucose tolerance test (OGTT) is used are the following:
1. Two-h PG < 140 mg/dL (7.8 mmol/L) = normal glucose tolerance.
2. Two-h PG ≥140 mg/dL (7.8 mmol/L) and < 200 mg/dL (11.1 mmol/L) = impaired glucose tolerance.
3. Two-h ≥ 200 mg/dL (11.1 mmol/L) = provisional diagnosis of diabetes.
Glycosylated hemoglobin (GHb) is the term used to describe the formation of a glucose reacts with the amino group of hemoglobin (2).

Classification and Etiology of DM

In 1979, the National Diabetes Data Group (NDDG) developed a classification and diagnosis scheme for diabetes mellitus. This scheme provides dividing diabetes into two broad categories: type 1, insulin-dependent diabetes mellitus (IDDM), and type 2, non-insulin-dependent diabetes mellitus (NIDDM).

Free Radicals

A compound becomes a free radical through either oxidation i.e., the loss of an electron or through reduction i.e., the gain of an electron, however, a free radical is unstable and highly reactive in nature and may react with other nearby molecule also converting that molecule to a free radical, which can then initiate another reaction (3).

Free Radicals and Pathogenesis of DM

The cause of diabetes mellitus is not fully understood. Recently, increasing evidence suggest that free radicals formation are involved in the pathogenesis of diabetes and the development of diabetic complications (4,5).

In type 1: β-cells are prone to be destroyed by free radicals because of the low antioxidant enzyme nature. Immune-effect cells such as macrophages, T-cells, nature killer cells and B-cells are believed to produce free radicals that cause damage to β-cells. There are two mechanisms of free radicals in β-cells destruction (6):

1. Infiltration macrophages produce superoxide as primary source of free radicals. (4).

2. Cytokines are released by T-cells, macrophages, NK cells in the insulates and induce the formation of intracellular free radicals causing selective damage to β-cells. (7).

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In type 2 : is a heterogeneous disorder, that is, very different pathologic events result in the same clinical symptom (8). Genetic abnormalities, or environmental factors, or obesity, which may induce β-cells malfunction and/or insulin resistance, can cause mild hyperglycemia which further develops to type 2 DM.

Some studies |indicate that there are alterations in free radicals generation and antioxidant enzymes. The free radicals activity in type 2 DM patients was increased as measured by the markers of free radicals activity (9).

Oxidative Stress Syndrome

Oxidative stress, an imbalance between the generation of free radicals and antioxidant defense capacity of the body. This resulted in the alteration of the cellular components in term of; DNA break down, protein and lipid Peroxidation, cytotoxicity etc... (10).

Antioxidant Defense System

Antioxidants can be defined as substances whose presence in relatively low concentrations significantly inhibits the rate of oxidation of lipids, proteins, carbohydrates, and DNA. Antioxidants act as potent electron donors, they donate hydrogen atoms to pair up with unpaired

electrons on free radicals. Thus they convert reactive free radicals to inactive substances (11).One of antioxidant defense of the body consists of non-enzymatic antioxidants which are:

Glutathione (GSH)

GSH is γ-glutamylcysteinylglycine, the most abundant non-protein thiol compound in mammalian cells, is biosynthesized from amino acid precursors (12)**.**Tissue glutathione plays a central role in antioxidant defense.

The hydrogen atom of SH group abstraction is one of the most important aspects of GSH reactivity considering its function as an antioxidant.
Many recent studies have found decrease plasma GSH levels in different disease states especially those associated with oxidative stress like DM (13,14)

Vitamin E (a-Tochopherol)

Vitamin E is a fat-soluble powerful antioxidant and is the primary defense against potentially harmful oxidation that causes disease and aging (2).Vitamin E is the collective name for eight compounds, four tochopherol and tocotrienols, found in nature. It is a fat-soluble substance present in all cellular membranes and is mainly stored in adipose tissue, the liver and muscle (11).

Vitamin C (Ascorbic Acid)

Vitamin C is a water-soluble antioxidant vitamin. It neutralizes free radicals in the plasma, cytoplasm and extracellular fluid. Vitamin C is not synthesized in the body. Vitamin C is taken from the diet and absorbed from small intestines and it is transported through leukocytes and platelets (12).

Vitamin A (Retinol)

Vitamin A is often used as a collective term for several related biologically active molecules (retinoids). They are retinol, retinal, retinoic acid (all trans), and 11-cis-retinal (formed by photoisomerization of all trans-retinal). Some carotenoids (provitamin A) like β-carotene act as a precursor of vitamin A; which are synthesized by all plants and by some animals, but not by man (12).

Minerals

Minerals may be divided into two groups: (1) macro-minerals, which are required in amount greater than 100 mg/dL, and (2) micro-minerals (trace elements), which are required in amount less than 100 mg/dL.Trace elements appear to play a key role in most biological functions. The effect of these elements cannot be replaced by another element. (2) **.**Some of the trace and ultra trace elements have a scavenging property as antioxidant to free radical generation, thus the alteration in their levels may be considered a biochemical marker of free radical generation, such as zinc, selenium, copper, iron, and chromium.

Iron

Iron is an essential trace element. Most iron in the body is incorporated into heme of hemoglobin and a smaller proportion as myoglobin, heme-containing enzyme like catalase, and iron-sulfur proteins (2).Iron is a first-line prooxidant, it contributes to regulate the clinical manifestations of numerous systemic diseases, including diabetes, atherosclerosis, and deoxyribonucleic acid (DNA) damage (15).
Iron is intimately linked to oxidative stress. It participates through the Fenton reaction, in the formation of highly toxic free radicals such as OH. and O2.- and causes oxidative damage (16). Oxidative stress influences both glucose and iron metabolism (by decreasing internalization of insulin and increased ferritin synthesis) (17).

Copper (Cu)

Copper is an essential trace element, a small amount of circulating copper is bond to albumin, whereas most is incorporated into ceruloplasmin (12).An important role for copper is as a component of enzymes or proteins involved in redox reaction (18) like:

1. Cytochrome c oxidase catalyzes the reduction of oxygen to water in the last step of the electron transport chain (12).
2. Cytosolic SOD, containing copper and zinc, catalyzes the conversion of $2O_2^{\cdot-}$ to O_2 and H_2O_2 .
Copper deficiency associated with low concentration of ceruloplasmin and leads to accumulation iron in the liver and causes increase free radical production (19), and lack of the copper-containing antioxidant enzyme related to the shortened life of erythrocytes and neutrophils (20), and excess copper can cause free radicals production and damage tissues (2).

Zinc (Zn)

Zinc is an essential trace element. It is cofactor for more than 300 enzymes, and zinc-containing enzymes are found in every enzyme class. Alkaline phosphetase, alcohol dehydrogenase, carbonic anhydrase, DNA polymerase, and SOD are containing zinc (12).Zinc enzymes are essential to growth, wound healing, reproductive function, the immune system, and protection from free radicals damage (21).Oxidative stress syndrome in human with DM results in disturbance of zinc. Zinc has an important role in modulating the immune system and its dysfunction in DM may be related in part to the status of zinc (12).

Chromium (Cr)

Trivalent chromium (Cr^{3+}) is an essential trace element for animals and humans. Its deficiency in organisms causes e.g. disturbance of carbohydrate and lipid metabolism, hypoglycemia, impaired glucose tolerance (22).
Cr^{3+} may improve insulin sensitivity, which can modify the risk of diabetes and cardiovascular disease (CVD), therefore, when chromium supplementation is beneficial for preventing CVD among people with diabetes (23).

Selenium (Se)

Selenium is an essential trace element. The essentiality of selenium results from its present as a necessary component to form the active center, seleno group (-SeH), of GPx, thioredoxin reductase and of other seleno-enzymes (24). Selenium is widely distributed in nature and found in combination with sulfide and other minerals (25). Selenium is a component as a cofactor of the enzyme GPx, which protects polyunsaturated fatty acid in the cell membrane from oxidative damaging effect of peroxide by decomposing these oxidizing compounds. GPx catalyzes the reduction of H_2O_2 and organic hydropeoxides in the presence of GSH (26), Thus selenium deficient cells are less capable of scavenging intracellular peroxides after exposure to exogenous H_2O_2 (27). Therefore, selenium was suggested as antioxidant acting as a free radicals scavenger (28).

Material and Methods:

All the chemical used have been imported with highly purified from fluka company.

Place of work

This research was conducted in Al-Qadisiya Governorate, Al-Diwaniya General Hospital; Department of Biochemistry, College of Medicine, Al-Nahrain University, and Department of Chemistry, College of Sciences, Babylon University.

Subjects

The study samples included 50 Patients suffering from type 2 of diabetes (28 males, and 22 females) aged between 35 and 65 years; and 50 patients with type 1 diabetes (32 males and 18 females) aged between 13 and 65 years, controlled with 50 healthy individuals (38 males and 12 females) aged between 15 and 65 years. The practical work was expended 15 months period, beginning in October 2003 and ending in December 2004.

Samples

The study samples of patients were collected from Al-Diwaniya General Hospital between October 2003 to December 2004, and the blood was drawn from venous of fasting patients recently diagnosed with diabetes mellitus (type 1 and type 2) and healthy subjects were used as control. All tests were performed on serum, some of the blood was allowed to clot on crushed ice, and then centrifuged (450 X g for 10 minutes) to be used serum samples immediately for detection of variable in this study, and others was stored at deep freezing (-20°C) until using.

Determination of Serum Glutathione (30)

Principle; -

5,5-Dithiobis (2-nitrobenzoic acid) (DTNB) is a disulfide chromogen that is readily reduced by sulfhydryl group of GSH to an intensely yellow compound. The absorbance of the reduced chromogen is measured at 412 nm and is directly proportional to the GSH concentration .

Calculation: -

The calibration curve of glutathione was drawn between absorbance and different concentration of glutathione, then concentration of serum glutathione of sample was calculated from it.

Determination of Serum Antioxidant Vitamins

1-Determination of Serum Vitamin (E) by HPLC using the technique described by (31).

2-Determination of Serum Vitamin A by HPLC using the technique described by (32).

3.Determination of Serum Vitamin C by HPLC using the technique described by (33).

Determination of Serum Trace Element

1-Determination of Serum Iron and copper by Using Commercially Available Kit (Randox - UK).

2-Determination of Serum Zinc,chromium and Selenium by Flameless Atomic Absorption Spectrophotometer (FAAS)by Atomic Absorption Technique (30) .

Statistical Analysis

The data were analyzed by using student's T-test taking $P < 0.05$ as the lowest limit of significant of difference and simple linear correlation between two quantitative parameters, and correlation considered significant at $P< 0.05$.

Results and Discussion

Non-Enzymatic Antioxidants

Table (1): Levels of several non-enzymatic antioxidants in serum of patients with type 1 or type 2 DM and control.

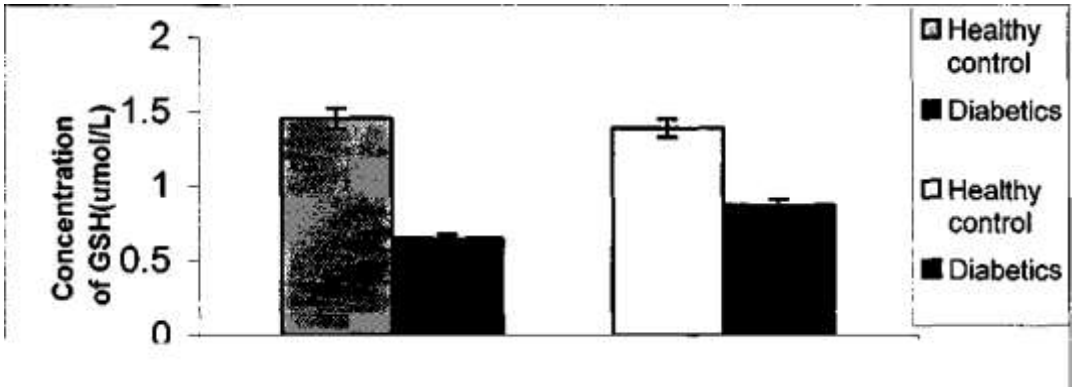
			GSH (µmol/L)	Vitamin E (µmol/L)	Vitamin A (µmol/L)	Vitamin C (µmol/L)
Type 1 DM No.=50	Mean±SE		0.653±0.027	3.16±0.82	1.212±0.087	3.452±0.165
	SD		0.193	0.58	1.212	1.170
	Range		1.24 – 0.17	4.225 – 1.98	2.35 – 0.23	5.45 – 1.2
	%95C.I.	Lower	0.607	3.023	1.066	3.175
		Upper	0.698	3.297	1.358	3.729
Control No.=	Mean±SE		1.456±0.070	6.551±0.222	2.163±0.140	6.534±0.242
P-Value			S	S	S	S
Type 2 DM No.= 50	Mean±SE		0.872±0.041	3.312±0.106	1.381±0.080	3.642±0.148
	SD		0.296	0.752	0.571	1.053
	Range		1.9 – 0.17	2.67 – 1.567	2.67 – 0.245	5.719 – 1.7
	% 95C.I.	Lower	0.803	3.143	1.246	3.393
		Upper	0.940	3.49	1.516	3.891
Control No.=25	Mean±SE		1.392±0.060	6.834±0.239	2.470±0.124	6.984±0.248
P-Value			S	S	S	S

Glutathione (GSH) Level

It was found an inverse correlation between GSH level and the presence of DM complications in type 1 and type 2 DM patients(34).

However, most studies have also found decreased level of serum GSH in diabetic patients (10,13,35,36).

In this work, serum GSH level was significantly decreased in patients with type 1 and type 2 DM when it is compared with control, $P \leq 0.05$. Hence, serum GSH level is approximately 45% than that of control in patients with type 1 DM, while serum GSH level in patients with type 2 DM was approximately 62% than that of control, figure (1), table (1).



Type 1 DM **Type 2 DM**
Figure (1): Level of glutathione (GSH) in serum of healthy control and patients with type 1 and type 2 diabetes mellitus (DM) disease. The values are mean ± SE.

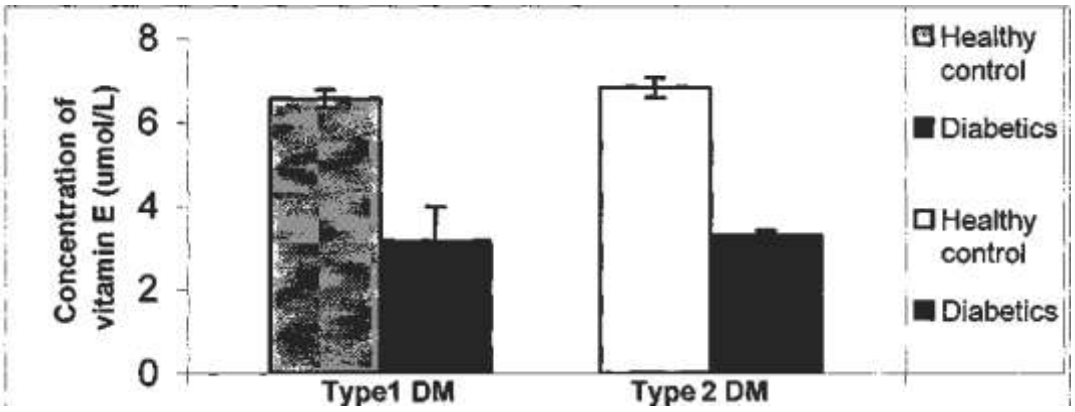
Decreased level of serum GSH in diabetic patients could represent an adaptive response to increased oxidative stress and free radicals generation that oxidized thiol group of GSH and decline reduced glutathione level, therefore it is a good indicator to diabetic complications.

These results probably were due to the following:
1. Depletion serum GSH levels lead to slightly accumulation of intracellular total homocysteine, which induces endothelial cells damage and slightly increases risk of cardiovascular disease in diabetic patients. These results go with the results of the study of (37).

2. Serum GSH has an important role in the protection of cells from the perooxidation injury, therefore decreases the degree of lipid perooxidation via end products; MDA in diabetic patients, these results are in agreement with the results of (38).

3. Presence of high level of toxic compounds in diabetic patients, especially with type 2 DM (treatment with of hypoglycemic oral such as tablets of daonil, glucophage...etc) induced accumulation of GST enzyme and altered GSH metabolism, therefore toxic compounds increase ROS production that decreases levels of GSH, while increases synthesis of GST enzyme to play a protective role (39,40).

Vitamin E (a-tocopherol) Level
Numerous studies have shown decrease in vitamin E level in diabetic tissue and blood (42) . This study indicated that serum vitamin E levels was significantly reduced in diabetic patients compared with control P ≤0.05, figure (2), therefore the mean ±SE of serum vitamin E levels in patients with type 1 DM was (3.16 ± 0.82 umol/L *vs* 6.551 ± 0.222µmol/L of control), while in patients with type 2 DM was (3.312 ± 0.106umol/L *vs* 6.834 ± 0.239µmol/L of control), table (1).

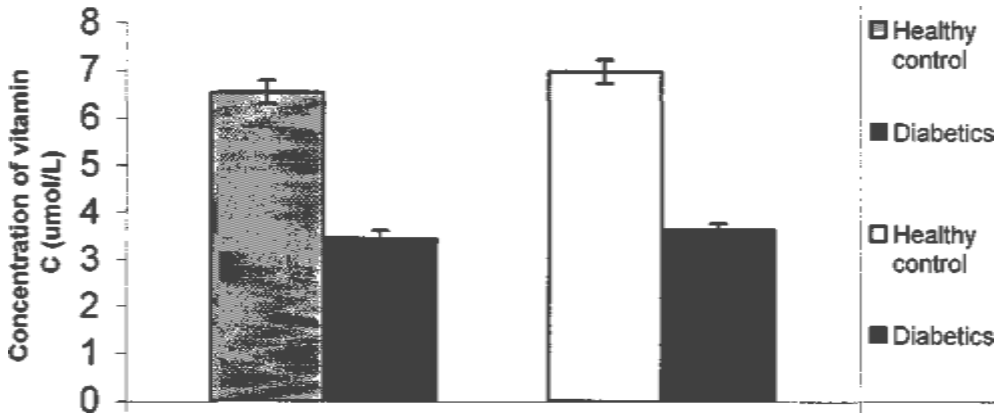


Figure(2): Level of vitamin E in serum of healthy control and patients with type 1 and 2 diabetes mellitus (DM) disease.

Reduced level of serum vitamin E possibly is due to increased utilization of this antioxidant in neutralizing free radicals in diabetic patients, thus supplementation of vitamin E to diabetic patients could reduce level of oxidative stress and diabetic complications.

Vitamin C (Ascorbic Acid) Level

The results of this study show the mean ±SE of serum vitamin C in patients with type 1 DM, which was (3.452 ± 0.165µmol/L *vs* 6.534 ± 0.242µmol/L of control), while in patients with type 2 DM, it was (3.642 ± 0.148µmol/L *vs* 6.984 ± 0.248µmol/L of control), table (3-5). There was a significantly decreased vitamin C levels in serum of diabetic patients than that of control, $P \leq 0.05$, figure (3).

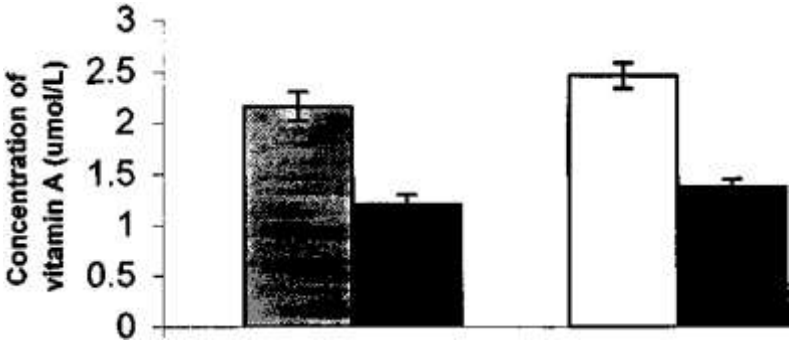


Figure(3): Level of vitamin C in serum of healthy control and patients with typed and 2 diabetes mellitus (DM) disease.

This decline in levels of serum vitamin C in diabetic patients may be due to (1) reduce renal re-absorption of vitamin C induced by hyperglycemia, (2) the competition between glucose and vitamin C for the uptake into certain cells and tissues, (3) the activity of polyol pathway that inhibited affectivity by utilizing high level of vitamin C to avoid hyperglycemia, (4) and possible secondary depletion due to increased oxidative stress have been proposed (43,44)

Vitamin A (Retinol) Level

The present study indicates a significant decrease in serum vitamin A levels in diabetic patients than that of control, P≤0.05, figure (4). The mean ±SE of serum vitamin A in patients with type 1 DM was (1.212 ± 0.087µmol/L *vs* 2.163 ± 0.140µmol/L of control), while in patients with type 2 DM was (1.381 ± 0.080µmol/L *vs* 2.47 ± 0.124µmol/L of control), table (1).



Figure(4): Level of vitamin A in serum of healthy control and patients with type 1 and 2 diabetes mellitus (DM) disease.

These results may be due to the increased utilization of this vitamin in the neutralization of free radicals induced by hyperglycemia, thus depletion of serum vitamin A in diabetic patients is due to the increased oxidative stress and diabetic complications. These results are in agreement with the singings of many studies *(*45).

Trace Elements (Iron, Zinc, Copper, Chromium, and Selenium) Levels.

Some serum trace elements act as antioxidants and prevent membrane peroxidation. Others act directly on glucose metabolism. Ceruloplasmin, the major plasma copper transporting protein, possesses a potent oxidant property. Chromium trivalent (Cr^{3+}) plays an important role in insulin-receptor activation (46).

The function of zinc (Zn) in the body metabolism is based on its enzymatic affinity, way of a Zn-enzyme complex or Zn metalloenzyme. Iron (Fe3+) is stored in the ferittin and iron is released from it by the action of reducing agents that convert Fe^{3+} to Fe^{2+}, thus ferrittin can act as a source of iron, which induces oxidative stress, and as a mechanism that protects against iron toxicity (48). Selenium (Se) plays important role in antioxidant defense system (49). present study indicates a significant decrease in levels of serum iron, Zinc, chromium, and selenium in diabetic patients with type 1 DM by about 51%, 33.7%, 50%, and 33% respectively from that of control, P ≤ 0.01, figure (5).

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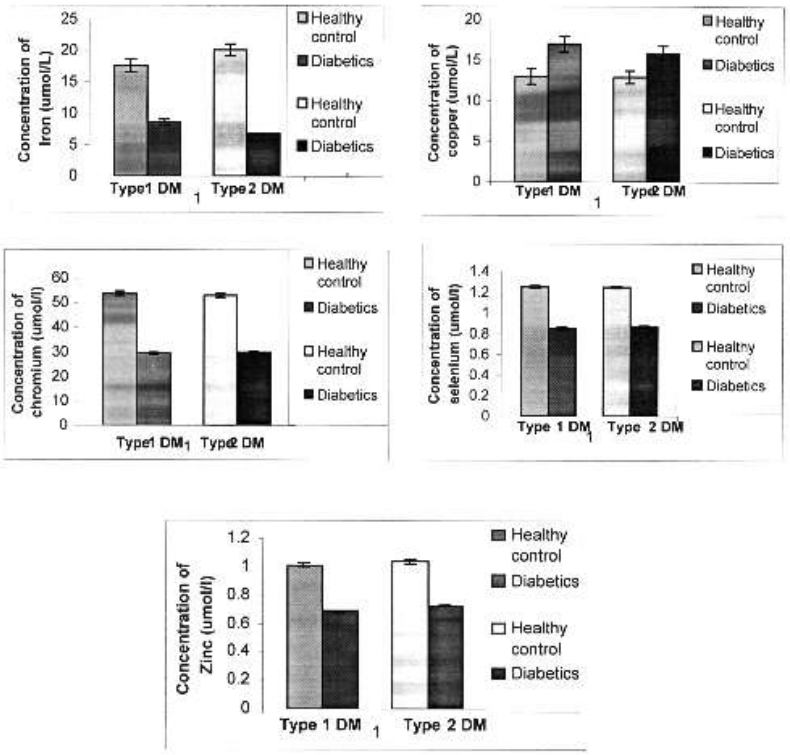

Figure (5):

level of some trace elements in serum of healthy control and patients with type 1 and 2 diabetes mellitus (DM) disease

However, these results may be due to:

1.All studies found increased level of serum iron in diabetic patients but in this work pointed out decreased levels of serum iron in diabetic patients than control may be due to increased drinking tea in Iraq that inhibits iron absorption (50), thus tea and its phenolic constituent are

reported to have antioxidant activity in vitro, the antioxidant action of tea was dependant on the ability of their constituent phenolic compounds to scavenger free radicals and to chelate iron (reduce the utilization of dietary iron), (51)

2. Decreased levels of serum zinc in diabetic patients may be due to reduced level antioxidant enzyme alkaline phosphatase or due to excessive urinary output especially in patients with diabetic nephropathy, gastrointestinal malabsorption or genetic factors or signs of infection during which Zn will act as defense mechanism (52).

3. Decreased levels of serum chromium in diabetic patients may be due to increase in glucose and insulin concentration, which increases urinary Cr output , These results go with the results of (53).

4. Decreased levels of serum selenium in diabetes mellitus disease may be due to increased oxidative stress that requires increased utilization of selenium as antioxidant by binding with vitamin E and as a cofactor of glutathione perooxidase (GPx) to scavenge free radical, which acting to detoxify tissue peroxidation , These results agree with the results of (49)

5. Increased levels of serum copper in diabetic patients may be due to the presence of oxidative stress by hyperglycemia, which leads to the glycation of ceruloplasmin enzyme, whereas most of copper is incorporated into it, and releases high level of serum copper that is associated with low concentration of ceruloplasmin and iron in the liver and causes increased free radical production in diabetic patients. These results go with the results of the study of (52,54).

From about obscuration the percentage of non enzymatic antioxidant and trace elements that assessment in this study for diabetic patients (with type I or Π DM) one appears in following data :-

Antioxidant	Type 1 DM	Control	Type 2 DM	Control
GSH(µmole/ L)	14.93	33.30	19.94	31.83
Vit.E(µmole /L)	15.91	32.99	16.68	34.42
Vit.A(µmole / L)	16.77	29.94	19.11	34.18
Vit.C(µmole / L)	16.75	31.70	17.67	33.88
Zinc(µmole / L)	19.79	29.25	20.98	29.98
chromium(µmole / L)	17.76	32.37	18.00	31.87
Selenium(µmole / L)	20.16	29.67	20.56	29.61

From above results the clear relationships can understand now to show the effect of antioxidant in Diabetic patient

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