Evaluation of Ceruloplasmin Oxidase Activity and C-Reactive Protein in the Sera of Patients With Diabetes Mellitus

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Abstract

The type two Diabetes Mellitus patients (independent DM2) was used to investigate the level of C-reactive protein CRP & ceruloplasmin oxidase activity CP. The study aimed to compare the effects of altering the glycemic index or the amount of carbohydrate (serum glucose) with C-reactive protein and CP activity in patients compare with control group. As a marker of systemic inflammation, raised CRP concentrations are still within the normal range have been associated with an increased inflammation of Diabetes Mellitus DM.

The our experimental data reveal that ceruloplasmin oxidase enzymatic activity is a serum non-specific factor acting during the early and late stages of DM.
Also this study aimed to compare the effect of raising blood sugar with levels of CRP & CP in DM2 patients.

The Glucose kit method & CRP latex were used in present study, CRP kit which is based on immunological reaction between CRP antisera bound to biologically inert latex particles and CRP in the test specimens of patients with DM (13Female, 17Male median age years, range=30-65)yr & in control subjects (7F,14M ,age range=30-45) yr.

The results are classified according to visible agglutination to:

1- A positive result / is indicated by the obvious agglutination pattern of the latex, in a clear solution.
2- A negative result: is indicated by no change in the latex suspension on the test slide.

Then the results of the precipitin test correlated with the quantitative data on CRP.

Ceruloplasmin oxidase activity measured by using the modified Rice method & phenylene diamine – 2HCl as a substrate .

This study has found that CRP concentrations in patients with DM increased very clearly than normal subjects, and established that CRP conc. in male was more than in female for patients specimens. An increase in serum ceruloplasmin (CP) oxidase activity has also been indicated in both sex, higher activity noted in female than male.

Abbreviations :CRP:C-reactive protein, mg/dl: milligram per diacelitter,
BS:blood suger, CP: ceruloplasm in, F: Female, M: Male.
DM2: Type Two Diabetes Mellitus

الخلاصة

تم التحري عن مستوى البروتين الفعال (سي) وفعالية انزيم السيرلوبلازمين اوكسيديز في النوع الثاني من مرضى السكري (غير المعتمدين DM2) حيث هدفت الدراسة لمقارنة تاثير التغيرات الحاصلة في المرجع السكري او كمية الكاربوهيدرات على مستويات كل من الكلوكوز والبروتين الفعال –سي وانزيم السيرلوبلازمين اوكسيديز في امصال المرضى مقارنة مع مجموعة السيطرة.

وكمؤشر للنظام الالتهابي، فان ارتفاع تركيز الـ C-reactive protein والذي يبقى ضمن المدى الطبيعي يرتبط مع زيادة الالتهاب في مرضى السكري.

واظهرت النتائج التجريبية ان الفعالية الانزينية للـ Ceruloplasmin oxidase هي عامل مصلي غير متخصص يعمل خلال المراحل الاولى والنهائية لمرضى السكري.

كما هدفت الدراسة ايضاً لمقارنة تاثير ارتفاع سكر الدم ومستويات CRP و CP لدى مرضى DM2.

استخدمت طريقة العدة الجاهزة لقياس الكلوكوز و CRP بمحاليل Latex حيث تستند طريقة CRP على تفاعل مناعي بين مضاد مصل CRP المرتبط بحبيبات (Latex inert) مع الـ CRP الموجود في امصال مرضى السكري (13 انثى و 17 ذكر) معدل اعمارهم (30-45) سنة ومجموعة السيطرة (7 انثى و 14 ذكر) معدل اعمارهم (30-45) سنة.

صنفت النتائج وفقا للتلازن المرئي الى:

1. نتيجة موجبة / واستدل عنها بوجود تلازن واضح للـ (Latex) في محلول رائق.
2. نتيجة سالبة / واستدلت بعدم وجود تغير في عالق الـ (Latex) في شريحة الاختبار.

ثم ربطت نتائج اختبار الترسيب المناعي مع قيم كمية للـ CRP .

اما فعالية انزيم CP فقد قدرت باستخدام طريقة Rice المحورة وبوجود مادة (الفنيلين ثنائي امين - 2 حامض الهيدروكلوريك) كمادة اساس.

خلصت الدراسة الى وجود ارتفاع في تركيز CRP لدى مرضى السكري بصورة واضحة مقارنة بمجموعة السيطرة، ووجد ان تركيز CRP في الذكور اعلى مما في الاناث بالنسبة لعينات المرضى. وكشف عن زيادة في فعالية CP في امصال الجنسين، ولوحظت الفعالية الاعلى لدى الاناث عن الذكور.

Introduction

The general term diabetes comes from a Greek word meaning "Siphon"; it refers to the frequent urination associated with this condition.[1]

The hyperglycemia of DM results from either the insufficient secretion of insulin by the beta cells of the islets of langerhans or the inability of secreted insulin to stimulate the cellular uptake of glucose from the blood .Diabetes mellitus, in short , result from the inadequate secretion or action of insulin.[1]

There are two forms of DM in insulin-dependent diabetes mellitus, also called type1 diabetes DM1, the beta cells are progressively destroyed and secrete little or no insulin; injections of exogenous insulin are thus required to sustain the person's life.

This form of the disease accounts for only about 10% of the known cases of diabetes. About 90%of the people who have diabetes have non-insulin-

dependent diabetes mellitus, also called type II diabetes DM2.[1]

Type I diabetes was onese known as juvenil –onset diabetes because this condition is usually diagnosed in people under the age of 20.Type II diabetes has also been called maturity – onset diabetes, since it is usually diagnosed in people over the age of 40[1].

The people at risk of diabetes mellitus are those with (over weight, do not exercise regularly, family history of diabetes,40 years old or older, women who have once had a body weight more than nine pounds at birth .

Diabetes considered as a silent killer, due to its complications like (Blindness, Kidney disease, Amputations, Heart disease and Stroke)[2].

In Iraq the eight cause in 2004 and the fourth cause in 2005 for death is diabetes millets for patients their age ≥5 years old[33].

In 1930 CRP was first described as a protein found in the blood of patients with pneumococcal pneumonia .It was named CRP because of its ability to react with and precipitate the C-poly saccharide of the pneumococcus. In the 1940 & 1950,CRP as one of the most frequently requested clinical laboratory test for initial evaluation of patients with acute inflammation of any origin[3] .

One of the most dramatic changes is an increase in blood serum levels of an inflammatory marker known as (CRP)[4], which is one of a group of substances known as "acute phase reactants" among all acute – phase reactants, CRP rises the fastest and is the most reliable indicator of clinical disease and its severity.

CRP is a normal plasma protein that belongs to the evolutionary ancient and stably conserved pentraxin family. It rises 100-1000 fold within 24-72 h in a cytokine-mediated response to most forms of tissue injury, infection and inflammation. In clinical studies elevated levels of CRP have been shown to be a risk factor for atherosclerosis and development of cardiovascular diseases such as peripheral artery diseases ,myocardial infraction and stroke.[5]

Causes of CRP elevations include acute bacterial, viral and other infections, pulmonary tuberculosis ,non infections illnesses such as rheumatic diseases rheumatoid arthritis, polymyalgia Rheumatica and Giant cell arthritis, heart attack , inflammatory bowel disease, and various malignant disease .Other causes include systemic lupus erythematosus, obesity, diabetes, uremia ,hypertension ,physical exertion, hormone replacement therapy, sleep disturbance , chronic fatigue, high levels of alcohol consumption, low levels of physical activity , and even depression[4].

But decreasing CRP level in addition to decreasing low-density lipoprotein (LDL) cholesterol may further decrease coronary heart disease risk (6).

It is important to reduce CRP to prevent and speed recovery from the diseases. A number of substances have been shown to effectively reduce C-reactive protein, from whatever cause (ex.Statins /Red yeast Rice Extract, Vitamin E, Vitamin B_6, Aspirin, Gugulipid, Proteolytic Enzymes).[5]

The clinical utility of CRP includes:

-Screening for inflammatory disease

-Monitoring the extent & activity of disease.

-Detection & management of intercurrent infection [5].

Recent study established some of the strongest predictors in type1 diabetes patients (age, body mass index–"BMI', duration of diabetes,

albumin excretion, serum triglyceride). Additionally, sex, race, a family history of cardiovascular disease & a high current glycated haemoglobin value are also related to rises in CRP[7].

Ceruloplasmin CP is an ancient multi-copper oxidase evolved to ensure a safe handling of oxygen in some metabolic pathways of vertebrates. The presently available knowledge of its structure provides a glimpse of it's plasticity, revealing a multitude of bending sites that point to an elaborate mechanism of multifunctional activity. CP represents an example of "Moon lighting " protein that overcomes the one gene–one structure function concept to fellow the changes of the organism in its physiological and pathological conditions [8].

The presently available knowledge of the CP structure provides a glimpse of its plasticity and the multitude of binding sites allude to elaborate mechanisms of multifunctional activity [9].

Although "one gene, one protein, one function" has been a paradigram of biochemistry.CP joins the escalating number of enlisted "Moon Lighting " protein [10]which present scientists with the challenge of identifying when , how and why the exert their multiple roles [11].The following have all been plausibly proposed as physiological functions of CP [12]:

- Plasma ferroxidase Activity: Iron homeostasis.
- Ascorbate Oxidase Activity .
- Copper Transport and storage.
- Degradation of Organic Substrates.
- Antioxidant Activity, Defence against Oxidative Stress.
- Oxidation of Nitric Oxide.
- The Metabolism of Nitrosothiols.

Subjects & Methods

Patients with diabetes mellitus with no cardiovascular disease (n=30, F=13, M=17, age range=30-45 years collecting from advisory center in Nursing Home Hospital participated in the study.

Non had overt evidence of infection or connective tissue diseases.Where is the control subjects n=21, F=7, M=14, age rang=30-45 year collecting from blood.

1.C-Reactive protein assay

The CRP latex reagent kit used here is based on an immunological reaction between CRP antisera bound to biologically inert latex particales & CRP in the test specimen. When serum containing greater than 0.8 mg/dl CRP is mixed with the latex reagent, visible agglutination occurs [13]. The latex kit is provided by Atlas medical/ Cambridge. Then we correlated our results of the precipitin test with the quantitative data on CRP, as shown in table 1.

Table (1): Correlation of the result of the precipitin test with the quantitative data on CRP [17]

Precipitin reaction	Mean concentration of CRP (mg/dl)
+++ +++	**3.3**
+++++	**2.7**
++++	**2.3**
+++	**1.2**
++±	**1.01**
++	**0.5**
+±	**0.4**
+	**0.2**
±	**0.1**
Trace	**0.06**

2-Blood Glucose Assay:-

In the trinder reaction the glucose is oxidized to D-gluconate by glucose oxidase with the formation of hydrogen peroxide. In the presence of peroxidase, a mature of phenol and 4-aminoantipyrine is oxidized by hydrogen peroxide, to form a red quinoneimine dye proportional to the concentration of glucose sample. Kit was provided by LINEAR chemical-Spain.

3- Determination of ceruloplasmin oxidase activity:

The enzymatic assay of ceruloplasmin oxidase activity was accomplished using the modified rice method and p-phenylene diamine - 2HCl as a substrate [15].

Results

Table 2 lists the characteristics of the diabetes mellitus who participated in this study with the mean of their, CRP, FBS, CP.

Table 2: Results of CRP,BS & CP expressed as mean ±SD and SC in patients with DM2 and their corresponding controls

Groups	Sample No.	Age year	Sex	CRP mg/dl		BS mg/dl		CP(IU)	
				mean±SD	SE	mean±SD	SE	mean±SD	SE
Control	21	30→45	Male=14	0.06±0.000	0.000	109.64±17.229	4.605	11.058±2.397	0.640
			Female=17	0.06±0.000	0.000	112.00±12.714	4.805	13.876±8.16	
			Total mean	0.06					
Patients with DM2	30	30→50	Male=17	0.309±0.469	0.135	253.47±71.69	20.357	37.35±17.86	5.283
			Female=13	0.201±0.345	0.096	262.8±56.79	15.752	56.62±12.35	3.425
			Total mean	0.255					

The results are compared by using the analysis of variance (ANOVA). The differences with P-value of less than 0.05 are considered statistically significant table (3).

Table (3):Results of analysis of variance (ANOVA) for the two sex of diabetic patients type 2 and the control groups.

Subject	Parameters		
	CRP	BS	CP
Control and patients DM2 (Male)	0.051(N.S.)	0.000(S)	0.000(S)
Control and patients DM2 (Female)	0.000(S)	0.35(N.S.)	0.003(S)

S=significant; N.S.= non-significant

Figure 2 shows compression CRP concentration found in these patients, generally CRP values in patients were more than in control subjects, patients mean = 0.255 mg/dl control mean =0.06 mg/dl.

As shown in the same figure male had more CRP concentration than in female patients with DM Female

This study report that the mean activity of ceruloplasmin oxidase in sera from healthy male & female were m=10.29 IU, F=12.83 IU. Where is mean activity
of CP oxidase in diabetes mellitus patients M=37.06IU, F=56.62IU.

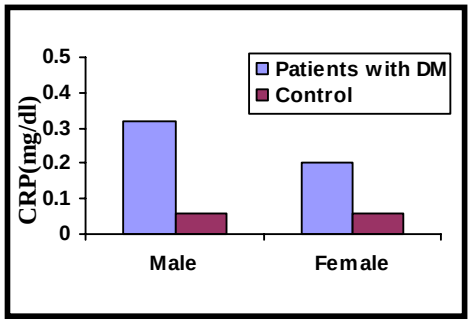

Figure (1): Compression CRP concentration in serum between control & patient with Diabetes Mellitus in both sex

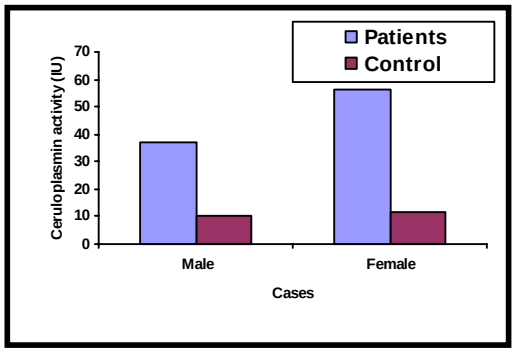

Figure (2): Compression ceruloplasmin oxidase activity in serum between control & patient with Diabetes Mellitus in both sex.

Serum Cp level in diabetic patients were higher than those of controls for each groups, Female had more CP level than in male patients(Figure 2).

The correlation test done by using Excel 2003, according to the program ±(0.1-0.35) consider as weak correlation ±(0.35-0.5) consider correlation of, ±(0.5-1.0) consider as strong correlation.

Table (4) shown the value of correlation coefficient (r) with significant value for the possible correlation between CRP with CP activity and BS individually in each, sex of the patients under this study. As weak negative correlation regarded between CRP & CP for male, while it is a weak positive for female (r=-0.215,0.036) respectively The correlation of CRP with BS is strong positive for male, while it is weak negative for female (r=0.96,-0.116) respectively, all the correlations are in significant relation.

Table (4): The correlation coefficient of CRP with CP & BS in sera of male & female with DM2.

Patients DM2	CRP in male		CRP in female	
Tests	r-value	Significant value	r-value	Significant value
CP	-0.215	0.407	0.036	0.908
BS	0.96	0.715	-0.116	0.706

In general positive correlation factor found between CRP & blood sugar as shown in figure (3-a). Where is ,a negative correlation factor found between CRP & CP as in figure (3-b).

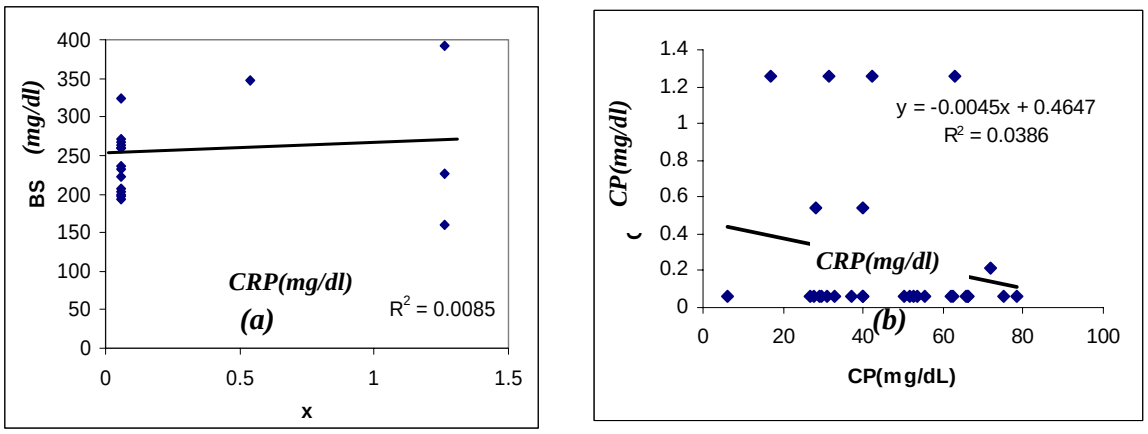

Figure (3): The correlation between
a. CRP & BS
b. CRP & CP

Discussion

As a result of the use of quantitative procedure for the estimation of CRP, it is now possible to give reasonably accurate values for the concentration of this substance in the blood during various disease states. While the spectrophotomertric method is not suggested asaroutine clinical procedure, it appears to have value in the study of certain cases of special interest which more exact data concerning the fluctuations in concentration of CRP desirable.

Moreover, the quantitative data allow a more precise comparison of the sensitivity of the two tests which have clinical applicability, and it is apparent that the test with specific antibody is greatly superior to that with the C-polysaccharide [17].

CRP concentrations less than 0.05 mg/dl are considered normal; between 0.06 & 10 mg/dl as moderate increases; and more than 10mg/dl as marked increases. The majority of patients with very high levels have bacterial infection, wherease more moderate degrees of elevation are seen in most chronic inflammatory states. In general, CRP values rarely exceed 6 or 8 mg/dl in patients with chronic inflammatory states or malignancies. Concentration greater than this should

raise the possibility of superimposed bacterial infection [2].

Type II diabetic patients have significant elevations ($P<0.05$) in C-RP value for female & CP activity for both sex compared to the age matched controls. The same increasing of C-RP value were observed in type II diabetic male patients with differences in the P-value ($M>0.05$) non significant (Table 2 & 3).

These results were in a good agreement with Rory et.al[12];who found that elevated plasma levels of CRP have been reported to be sensitive indicators of infection in adults with diabetic ketoacidosis (DKA). However both CRP hypothesis was that CRP is increased in young regulate CRP, Can be elevated without infection. They hypothesis that CRP is increased in young patients with DKA, even in the absence of an infection, and may serve as a marker for systemic inflammatory response syndrome [18].

Where's, Gibson et al., concluded that patients with DM_2 was effectively had decreasing in CRP levels.[6]

Also the results were agreed with Susanne *et al*;[4] who postulated that CRP signal was observed in the cytoplasm of tubules in 17 out of 20 kidneys from diabetic patient while normal renal tissue showed no CRP expression[4] .

Preventia study (WOSCOPS) group that included 127 new cases of diabetes reported a strong association between CRP and diabetes risk independent of BMI, blood pressure, smoking, and fasting lipids and glucose concentrations[16].

Jagger et al. found that the female /male difference may simply be associated with an increased prevalence of subclinical urinary infection in women. However, compared to diabetic men, diabetic women have a higher relative risk of ischaemic heart disease than their non – diabetic counterparts and this disagreement with our value (female/male=0.649). [19].

Auerucan study find that CRP is strongly linked to metabolic syndrome. This relationship is stronger in women that men. A person with metabolic syndrome and a high level of CRP protein is at an increase risk for getting heart disease[14].

We readily agree that is unclear why inflammation might be more important to the pathogenesis of type 2 diabetes in women compared with men or indeed vice versa. The relation between CRP and DM remains strong in women, even after inclusion of waist-to-hip (WHR), but is absent in men. We acknowledge that our inability to find an association between CRP and diabetes risk in men simply be due to a lack of power.

CRP production by hepatocytes is stimulated by inflammatory cytokines. One such cytokine, tumour necrosis factor (TNFα) has also been implicated in the pathogenosis of obesity- associated insulin resistance. Plasma TNFα levels correlate positively with percentage body fat & body mass index (BMI). Since BMI is known to relate to increasing subject age, it may help to explain some of the associations found here. Also, women have a larger percentage body fat than men for a given BMI, so they may produce relatively more TNFα, thereby explaining their higher CRP values [29].These findings were in disagreement with our results. In the other hand, we noted asignificant increase in Cp oxidase activity in both sex groups with D.M. than normal groups.

And CP is an abundant seventy two-serum glycoprotein which contains >95% of the copper present in human plasma [21].

The region of CP gene located in chromosome 39 which spans N36Kb and is composed of 19 exons. This protein functions as a multi-copper oxidase and is synthesized in hepatocytes with six atoms of copper incorporated prior to secretion[21]. Although copper does not affect the synthesis or secretion of the apoprotein, if copper is unavailable during hepatic biosynthesis an unstable protein lacking oxidase activity is secreted [22].

In Wilson disease a failure to deliver copper into the hepatocyte secretory pathway impairs both biliary copper excretion and copper availability to newly synthesized CP, resulting secondarily in a decreased serum CP concentration as a result of rapid turn over of secreted apoprotein [24]. Consistent with this model, the gene encoding Wilson disease has recently been cloned & showed to be a cation transporting P-type ATpase essential for copper trafficking hepatocytes [25,26,27]).

Some studies defined ceruloplasminemia as a noval human genetic disease and revealed an essential and unique role for CP in iron metabolism and reported a new CP gene mutation in a Kindred with a ceruloplasminemia and expand on the clinical spectrum and implications on diabetes and neurologic disease [28]

Our results are in a good agreament with Ramazan *et al*. [29]Who evaluated patients with DM 2 and clinically healthy subjects, serum CP & CRP levels in diabetic patients were significantly higher than those of control. Also found that CP & C-RP levels in patients with diabetes complications were significantly higher than those of patients without diabetic complications.

R.Awadullab *&et.al*. [30] reported no significant differences between diabetes and normal adults in the serum levels of CP oxidase activity.

Another study conclude that ceruloplasminaemia was an autosomal recessive disorder caused by specific mutations in the CP gene. A ceruloplasminaemia was clinically characterized by D.M. and added it is possible that some diabetic patients with a ceruloplasminemia are mistakenly diagnosed as having Type 1 D.M,as they have reduced insulin secretion and develop diabetes at a younger age, before neurological abnormlities associated with a ceruloplasminaemia are apparent .Therefore, a ceruloplasminaemia should be considered in patients with insulin – dependent D.M. who develop progressive neurological abnormalities of unknown aetiology along with amicrocytic hypochromic anaemia and retinal degeneration [31].

Takuma *et.al.* [32] Suggested that urinary excretion of several plasma proteins with different molecular radii <55Å and different isoelectric point (pI) such as CP increased independently to precede the development of microalbuminuria in D.M.patients, where is Tani *et.al*.indicated that the urinary CP excretion rate (CER) and clearance of CP increased in parallel with the progression of albuminuria. The highest CER was found in macroalbuminuric patients, followed by micro-and nomalalbuminuric patients and the healthy control subjects, the differences between the groups being significant. In view of the fact that the isoelectric point of CP(4.4) is more acidic than that of albumin. The present finding suggested that an enhanced CER was due either to the alteration of charge selectivity in the glomerular basement membrane with unaltered tubular function or to a defect of the non – discriminatory

pores (shunt pathway) with unaltered tubular function.

Also the study found a positive correlation between CRP with blood sugar in male and CP in female with DM2, this refer to when the sugar level increases too much in person with damage in pancreas or error in excretion of insulin hormone or metabolic disorder, the CRP and CP will be increase as a response to any type of inflammation or tissue injury or infection. While the negative correlation referred independent CRP & CP in screening the patients with DM2.

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