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

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(Received on 16/4/2009) (Accepted for publication 12/1/2010)

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
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 once known as juvenile-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 eighth cause in 2004 and the fourth cause in 2005 for death is diabetes mellitus 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 tests 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 infarction 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 type 1 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 its 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 follow 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 paradigm 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 range=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 bound & 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)

<table>
<thead>
<tr>
<th>Precipitin reaction</th>
<th>Mean concentration of CRP (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>++++ +++</td>
<td>3.3</td>
</tr>
<tr>
<td>++++</td>
<td>2.7</td>
</tr>
<tr>
<td>+++</td>
<td>2.3</td>
</tr>
<tr>
<td>+++</td>
<td>1.2</td>
</tr>
<tr>
<td>++ ±</td>
<td>1.01</td>
</tr>
<tr>
<td>++</td>
<td>0.5</td>
</tr>
<tr>
<td>+ ±</td>
<td>0.4</td>
</tr>
<tr>
<td>+</td>
<td>0.2</td>
</tr>
<tr>
<td>±</td>
<td>0.1</td>
</tr>
<tr>
<td>Trace</td>
<td>0.06</td>
</tr>
</tbody>
</table>
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>
<thead>
<tr>
<th>Groups</th>
<th>Sample No.</th>
<th>Age year</th>
<th>Sex</th>
<th>CRP mg/dl mean±SD</th>
<th>CRP SE</th>
<th>BS mg/dl mean±SD</th>
<th>BS SE</th>
<th>CP(IU) mean±SD</th>
<th>CP SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>21</td>
<td>30→45</td>
<td>Male=14</td>
<td>0.06±0.000</td>
<td>0.000</td>
<td>109.64±17.229</td>
<td>4.605</td>
<td>11.058±2.397</td>
<td>0.640</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Female=17</td>
<td>0.06±0.000</td>
<td>0.000</td>
<td>112.00±12.714</td>
<td>4.805</td>
<td>13.876±8.16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Total mean</td>
<td></td>
<td></td>
<td>0.06</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patients with DM2</td>
<td>30</td>
<td>30→50</td>
<td>Male=17</td>
<td>0.309±0.469</td>
<td>0.135</td>
<td>253.47±71.69</td>
<td>20.357</td>
<td>37.35±17.86</td>
<td>5.283</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Female=13</td>
<td>0.201±0.345</td>
<td>0.096</td>
<td>262.8±56.79</td>
<td>15.752</td>
<td>56.62±12.35</td>
<td>3.425</td>
</tr>
<tr>
<td></td>
<td>Total mean</td>
<td></td>
<td></td>
<td>0.255</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Subject and Parameters</th>
<th>CRP</th>
<th>BS</th>
<th>CP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control patients DM2 (Male)</td>
<td>0.051(N.S.)</td>
<td>0.000(S)</td>
<td>0.000(S)</td>
</tr>
<tr>
<td>Control patients DM2 (Female)</td>
<td>0.000(S)</td>
<td>0.35(N.S.)</td>
<td>0.003(S)</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th>Patients DM2</th>
<th>CRP in male</th>
<th></th>
<th>CRP in female</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Tests</td>
<td>r-value</td>
<td>Significant value</td>
<td>r-value</td>
<td>Significant value</td>
</tr>
<tr>
<td>CP</td>
<td>-0.215</td>
<td>0.407</td>
<td>0.036</td>
<td>0.908</td>
</tr>
<tr>
<td>BS</td>
<td>0.96</td>
<td>0.715</td>
<td>-0.116</td>
<td>0.706</td>
</tr>
</tbody>
</table>

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](image)

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 spectrophotometric method is not suggested as routine 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. CRP concentrations less than 0.05 mg/dl are considered normal; between 0.06 & 10 mg/dl as moderate increases; and more than 10 mg/dl as marked increases. The majority of patients with very high levels have bacterial infection, whereas 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 \(^{(5)}\).

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 DM2 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 (TNF\(\alpha\)) has also been implicated in the pathogenesis of obesity- associated insulin resistance. Plasma TNF\(\alpha\) 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 TNF\(\alpha\), 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 novel 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 agreement with Ramazan \textit{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 \textit{et al.} \(^{(30)}\) reported no significant differences between diabetes and normal adults in the serum levels of CP oxidase activity.

Another study conclude that ceruloplasminemia was an autosomal recessive disorder caused by specific mutations in the CP gene. A ceruloplasminemia 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 abnormalities associated with a ceruloplasminemia are apparent. Therefore, a ceruloplasminemia should be considered in patients with insulin – dependent D.M. who develop progressive neurological abnormalities of unknown aetiology along with microcytic hypochromic anaemia and retinal degeneration \(^{(31)}\). Takuma \textit{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 \textit{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.

References
(6) Betteridge D.J., Gibson J.M., Sager P.T.: Comparison of effectiveness of rosuvastation verses atorvastatin on the achievement of combined CRP (<2mg/dl) and low-denisty lipoprotein cholesterol (<70mg/dl) targets in patients with type 2 diabetes mellitus. Am.J. Cardiol., 2007; 100(8), 1245.


