Study the Level of Ceruloplasmin Oxidase Activity, Copper and Other Biochemical Parameters in Second and Third Trimester Pregnant Women

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ABSTRACT

Background: Ceruloplasmin (Cp) is a protein having numerous important functions including oxidase activity and super oxidase activity. The aim of study is to measure Cp activity, Copper and other biochemical parameters in second and third trimester pregnant women and determine optimum conditions for enzyme activity in their sera. Objective: Copper, Cp, vitamin C, vitamin E, S. protein, albumin had been measured in group A [Second trimester pregnant women], group B [Third trimester pregnant women] and measurement Km and V max of Cp. Results: There were a significant difference in Hb, copper, Cp, vitamin (C and E), S. protein, and albumin in group A compared to group B. Conclusion: The current study supposed that increase in copper, Cp and deficiency of antioxidant barrier may cause oxidative stress in third trimester pregnant women, and may be antioxidant treatment should be useful for these patients.

INTRODUCTION

Ceruloplasmin oxidase (Cp) is an enzyme (EC 1.16.3.1) synthesized in the liver having 6 atoms of copper in its structure. Ceruloplasmin transports about 70% of total copper in human plasma while albumin carries about 15%. The rest is accounted for by macroglobulins. Ceruloplasmin displays a copper-dependent oxidase activity, which is related with possible oxidation of Fe^{2+} (ferrous iron) in to Fe^{3+} (ferric iron), so supporting in its transport in the plasma in association with transferring, which can carry iron only in the ferric state. The molecular weight of human ceruloplasmin is described to be 151 KDa. The specific oxidase activity of ceruloplasmin was studied in plasma and serum of pregnant women with and without complications (Israa G, and Zaizafoon N, 2013).

Ceruloplasmin is an acute phase protein and is synthesized in response to the tissue damage and inflammation. It is an essential intravascular antioxidant and defends the tunica intima against free radical damage (Feraye G, and Aysen A, 2010). On the other hand, the physiological role of Cp is not well defined but may include extracellular antioxidant activity by promoting Fe mobilization and thus preventing metal-catalyzed free radical tissue damage. Alternatively, several studies suggest that Cp may also exhibit potent pro-oxidant activity. In spite of the unknown function of such Cp pro-oxidative activity, it is probable that the protein is involved in host protection and repair processes mediated by the immune system, namely during inflammation (Dina P, Rui M, and Deborah P, 2008). It furthermore inhibits the lipid peroxidation and the deoxy ribose degradation stimulated by iron and copper salts. It can defend the lipids and the erythrocyte membranes from copper and iron-induced injury. The other proposals are that the ceruloplasmin can decompose lipid peroxides or scavenge organic oxygen radicals (Cao J, et al, 2000).

Pregnancy is a integral stress condition in which many physiological and metabolic functions are changed to considerable extent (Saladin A, Kenneth S, 2012). Pregnancy is associated with increased request of all the micronutrients like iron, copper, zinc vitamin C, and vitamin B12. The insufficiency of these nutrients could affect pregnancy and outcome of pregnancy. Vitamins and minerals are collectively mentioned to as micronutrients and have essential effect on the health of pregnant women and growing fetus (Black RE, 2001). Normal pregnancy is a physiological state described by an increase in the production of reactive oxygen species. Human placenta exerts a lot of effect upon maternal homeostasis together with the involvement in the oxidative stress processes. One of the descriptions is the abundance of mitochondria within the placenta and the high rate of the oxygen partial pressure in the pregnant women (Fialova L, et al, 2006). Normal pregnancy is accompanying with the increase in oxidative stress and lipid peroxidation. The diet deficiency through late
pregnancy increases the lipid peroxidation, changes in the vitamin status and leads to the anemia in progeny.

The research on diets is a central area of fetal origins of diseases that need the significant care (Feraye G, and Aysen A. 2010). The current study was conducted to evaluate ceruloplasmin oxidase and other biochemical parameters in the pregnant women (2nd and 3rd trimesters) and optimum conditions for enzyme activity in their sera.

### MATERIAL AND METHODS

The present study included 80 women enrolled in the following groups: (A) forty women in second trimester of pregnancy (25-36) years (Mean ±SD: 32.38±2.27), and forty women in third trimester of pregnancy (26-35) years (Mean±SD: 30.55±2.55). Women with obstetrical condition like multiple pregnancy, gestational diabetes and history of cesarean, breech delivery were excluded. Laboratory investigations including hemoglobin, copper, Cp, vitamin E, vitamin C and albumin, total protein and globulin had been measured in pregnant women. Five ml of venous blood samples have been collected into two tubes, one containing EDTA for measurement of blood hemoglobin (Hb), and the second tube was centrifuged at 3000 rpm for 5 min after allowing the blood to clot at room temperature.

The Hb, serum protein, Albumin and copper levels were measured by spectrophotometric methods supplied by Giesse Diagnostic. Ascorbic acid levels were estimated by the method of Tietz (Tietz N.W. 1986). Vitamin E levels were determined according to a modified of Hashim and Schuttringer (Hashim S.A. Schuttringer G.R. 1966). The enzymatic assay of ceruloplasmin oxidase activity was accomplished using the modified Rice method and p-phenylene diamine-2HCL as a substrate (Erel O.1998).

### Kinetic Parameters (Km and V max):

**Effect of the pH:**

The enzymatic reaction was carried out using buffers with different pH [4.6, 4.8, 5, 5.2, 5.4, 5.6, 5.8] for Ceruloplasmin Oxidase. The pH optimum was estimated by scheming the relationship between the enzyme activities versus the pH values.

**Effect of the Temperature:**

Ceruloplasmin oxidase enzymatic reaction was carried out under optimum reaction condition using different temperatures [25, 30, 35, 40, 45, 50]. The optimum temperature was assessed by plotting the correlation between the enzyme activities versus the temperature values.

**Effect Of Substrate Concentration:**

Ceruloplasmin oxidase enzymatic reaction was carried out under optimum reaction condition using different concentrations of p-phenylene diamine-2HCL as a substrate [4.0, 4.5, 5, 5.5, 6.0, 6.5, 7.0 mM]. The relationship between each substrate concentration and the enzyme activity was plotted in order to determine the optimum substrate concentration for each enzyme activity. Then the values of Km and Vmax for ceruloplasmin toward substrate were determined using the Lineweaver-Burk plot [the relationship between 1/V versus 1/[S]].

### Ceruloplasmin Oxidase-polyacrylamide gel electrophoresis:

Polyacrylamide gel 7.5% was prepared by mixing 7.5 ml of distilled water, 33 ml of stock buffer (Tris-glycine 0.15 M) pH 8.9, 22.2 ml of acrylamide solution. The mixture was degassed for 15 minutes, then 3.2 ml of ammonium persulfate solution and 0.1 ml of N,N,N,N-tetramethylenediamine (TEMED) were added to the mixture solution (Polymer Electrophoresis technical manual.1999).

The mixture was gently mixed and loaded in the gel plates. The gel was allowed to polymerize for about 40 minutes, pre electrophoresis was carried out at 50 mA and 15 v/cm for 30 min, then of 10 µl of the samples were applied into the wells in the gel. electrophoresis was continued at 40 mA and 15 v/cm for 3 hours or until the bromophenol blue dye reached the gel margin. Finally the gel was removed and have been stained ceruloplasmin oxidase activity.

All statistical analyses in studies were performed using SPSS version 15.0 for Windows [Statistical Package for Social Science, Inc., Chicago, IL, USA]. Descriptive analysis was used to show the mean and standard deviation of variables. The significance of difference between mean values was estimated by student T-Test. The probability p<0.05 = significant, p>0.05 = non-significant.

### Results:

There is no significant different in age between group A [Second trimester pregnant women], group B[Third trimester pregnant women] as shown in table 1.

**Table 1:** The mean and standard deviation of age, weight and gestation age in group A [Second trimester pregnant women], group B[Third trimester pregnant women].
In group B [Third trimester pregnant women] the mean copper and Cp, have been significantly increased \((p < 0.01 \text{ and } p<0.05 \text{ respectively})\) compared with group B [Third trimester pregnant women]. Hb, vitamin E, vitamin C, S. Protein, and S. albumin were found to be significantly decrease with \(p\) value < 0.05 in group B [Third trimester pregnant women] compared with group B [Third trimester pregnant women] as shown in table 2.

Table 2: The mean and standard deviation of Hb, copper, Cp, vitamin C, vitamin E, S. Protein, albumin and globulin in group A [Second trimester pregnant women], group B [Third trimester pregnant women].

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Group A (n=40)</th>
<th>Group B (n=40)</th>
<th>(p) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hb (g/dl)</td>
<td>11.00 ± 0.53</td>
<td>9.98 ± 0.77</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Copper (µg/dl)</td>
<td>145.76± 12.40</td>
<td>160.31±13.29</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>ceruloplasmine Oxidase (mg/dl)</td>
<td>40.78± 6.32</td>
<td>53.23±7.22</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Vitamin E(µmol/l)</td>
<td>18.35± 2.92</td>
<td>16.99±3.34</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Vitamin C(mg/dl)</td>
<td>1.23±0.06</td>
<td>0.98±0.06</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>T.S. Protein[g/dl]</td>
<td>6.33 ± 0.93</td>
<td>6.00 ± 0.55</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>S. Albumin [g/dl]</td>
<td>4.43±0.69</td>
<td>3.95±0.27</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>S. Globulin [g/dl]</td>
<td>2.02±0.45</td>
<td>2.25±0.51</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Alb./Glu. ratio</td>
<td>2.29 ±0.67</td>
<td>1.97±0.49</td>
<td>&gt;0.05</td>
</tr>
</tbody>
</table>

Figures 1 showed the electrophoresis pattern profile of sera of group A [Second trimester pregnant women], group B [Third trimester pregnant women]. The ceruloplasmin was detected on the gel by exploiting its oxidase activity with PPD-2HCL as a substrate. Results in figure 1 showed that enzyme activities appeared as a single band in both groups.

**Fig. 1:** DNA-PAGE 7.5% profile of crude serum ceruloplasmine Oxidase. The gel was stained for ceruloplasmine oxidase activity. (from left right).

Crude serum group A [Second trimester pregnant women].
Crude serum group B [Third trimester pregnant women].

Optimum pH
The \(\text{pH} (4.6, 4.8 , 5.2 , 5.4, 5.6, 5.8)\) effect have been studied on Cp activity as shown in figure 2. The result showed that highest enzyme activity in was at \(\text{pH} 5.4\) in group both group.
Fig. 2: The effect of pH of the enzymatic activity Cp in group A[Second trimester pregnant women], group B[Third trimester pregnant women].

**Temperature:**
In both groups Cp activity increases according to the incubation temperature until it reaches maximum at 35°C as shown in figure 3.

![Temperature](image)

Fig. 3: The effect of temperature of the enzymatic activity Cp in group A[Second trimester pregnant women], group B[Third trimester pregnant women].

- **Effect of substrate concentration:**
  Determination of Cp activity with different substrate concentration [4.0, 4.5, 5.5, 6.0, 6.5, 7.0 mM], p-phenylene diamine-2HCL, and studying this concentration on rate of Cp reaction in both studied groups. A key factor affecting the rate of a reaction catalyzed by an enzyme is the concentration of substrate, [S]. However, studying the effects of substrate concentration is complicated by the fact that [S] changes during the course of an in vitro reaction as substrate is converted to product(Nelson D. 2005). Figures 4 and 5 showed Cp obey to Michaelis–Menten kinetics. Its explain the Lineweaver–Burk plot of Cp from both groups. These plots were used for determination of Km and Vmax values of the enzymes and the results are shown in table(3)

![Substrate Concentration](image)

**Fig. 4:** The effect of substrate concentration of the enzymatic activity of Cp in group A[Second trimester pregnant women], group B[Third trimester pregnant women].
Fig. 5: Determination of Km and Vmax for Cp of in group A [Second trimester pregnant women], group B [Third trimester pregnant women] using Lineweaver-Burk plot.

Table 3: The Km and Vmax for group A [Second Trimester pregnant women], group B [Third trimester pregnant women].

<table>
<thead>
<tr>
<th>Groups</th>
<th>Km value [mM]</th>
<th>V max value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>5.12</td>
<td>45.45</td>
</tr>
<tr>
<td>Group B</td>
<td>3.95</td>
<td>58.62</td>
</tr>
</tbody>
</table>

Discussions:
This result is in agreement with a study reported an increase in copper, ceruloplasmin from the first trimester pregnant also it states that elevated levels of maternal serum copper would be the result of the mobilization of copper stocked in the liver and other tissues (Mehde A et al. 2013), the copper needs would then be increased in order to restore the reserves. moreover high estrogen levels tend to cause increased both the copper absorption (Johnson S. 2001), and hepatic ceruloplasmin synthesis (Ulutas PA. et al. 2009). A ceruloplasmin level avarice in pregnant women due to estrogen secretion (Jonson M. 1990). The present result are in Agreement with early reports which showed that serum copper level increased with period of gestation and its level in the 3rd trimester of pregnancy were significantly higher than during the 2nd trimester (Al-sarrag R, and Altai W. 2007).

The result in current study is in agreement with the study by Rao, which showed a statistically significant decrease in levels of vitamin C in normally pregnant women (Rao GM. Et al 2005) According to study by Roes in uncomplicated pregnancy, concentration of vitamin C decreased during pregnancy. Hence in pregnancy there seems a balance between antioxidant and oxidant concentration despite modest oxidative stress (Roes EM et al. 2006).

Reduced vitamin C as a water-soluble antioxidant was described to role as the first line antioxidant defense in contrast to free radicals present mainly in plasma. It may be possible that ascorbic acid was consumed as a defense against oxidative stress. When the capacity of ascorbic acid is exceeded, free radicals can diffuse the cell membrane initiating lipid peroxidation (Niki E. et al. 1991),Mohanty S. et al. 2006).

Vitamin E is most essential chain breaking antioxidants and they defend polyunsaturated fatty acids from per oxidative injury by giving hydrogen to the lipid peroxyl radical. Since of the lipophilic property of the tocopherol molecule is the major free radical chain terminator in the lipophil environment, Vitamin C as a reducing and antioxidant agent, directly reacts with superoxides, hydroxyl radicals, and various lipid hydroperoxides. In addition it can as well reinstate the antioxidant properties of oxidized vitamin E (Ishihara M. 1978).

The concentration of proteins in pregnancy through pregnancy is within physiological values (Forenbacher S.1993). The slight increase in protein concentrations in the second trimester of pregnancy, compared to the third trimester of pregnancy, may be due to hormonal changes in the organism .The secretion of other hormones (glucocorticoids, thyroxin) increases throughout pregnancy as well, as a importance of increased sex hormone secretion, which in turn increases metabolic events in the organism. Glucocorticoids increase the mobilization of additional hepatic proteins and transport of amino acids to liver cells. Mobilized amino acids in liver cells will assistance synthesize glucose through gluoneogenesis, which is the original source of energy for the embryo (Nett M., Holton W.1973).

In general the pH variant has a marked effect on the rate of the enzymatic reaction, pH can affect the activity by altering the charge of functional residues that complicated in substrate binding or in the catalysis process itself, Enzymes also experience change in the conformation when the pH changes (Murray R. et al. 2009). The decrease in Cp activity at acidic pH due to effect of pH environment of reaction in ionic groups which found in active site or changing in ionic state for substrate or complex enzyme-substrate when the
concentration of substrate over than Michaelis constants (K_m), if the substrate concentration is little, it will depend on enzyme (Segal I 1976).

The present study conclude that Cp (one of the important extracellular antioxidant) concentration increase in the sera of pregnant women in Third trimester, even thought this increase seems to be insufficient to overcome the oxidative stress and may be antioxidant treatment should be useful for these patients.

Enzymes are complex protein molecules, their catalytic activities result from a precise and highly ordered tertiary structure. The tertiary structure of an enzyme is maintained primarily by a large number of weak non covalent bonds, which means that its structure is easily effected if high temperature are used (Rahman Y.E 1966). It was obtained from these curves that the reaction rate is enhanced due to the increase of reaction activation energy as a result of molecules collision until the temperature reaches the optimum value (35-40°C).

As shown in the figure, raising the temperature above the optimum degree, causes a decline in the velocity. Indicating a disruption of the compressed three dimensional structures that is essential for the catalytic activity. These results were nearly resembles to the other studies in normal human serum (Rahman Y.E 1966).

The Michaelis constant indicates the relative suitability of alternate substrates of particular enzyme. That is, the substrate with the lowest K_m value has the highest apparent affinity for the enzyme (Segal I 1976).

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REFERENCES


