Study of Serum Levels of Copper, Ceruloplasmin in Pregnant Women with preeclampsia.
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Abstract:
Pregnancy is a period of increased metabolic demands with changes in Women's physiology and the requirements of a growing foetus. Preeclampsia is a pregnancy-specific condition that increases maternal and infant morbidity and mortality. The present research was undertaken to measure serum levels of copper in normal non-pregnant women, normal pregnant and women with preeclampsia and also to study the level of oxidative stress by measuring the antioxidant, ceruloplasmin in these women. The study shows that the serum copper and ceruloplasmin levels are significantly raised in normal pregnant women and are further more raised in women with preeclampsia.

Keywords: Pregnancy, Pre-eclampsia, Copper and Ceruloplasmin.

Introduction:
Preeclampsia is clinically defined as hypertension and proteinuria with onset following the 20th week of pregnancy. It is a pregnancy-specific syndrome of reduced organ perfusion secondary to vasospasm and endothelial activation.

It was noted that increased blood pressure and urinary protein antedated seizure of eclampsia, from this came the term preeclampsia, as suggested by Roberts [11]. Preeclampsia is a pregnancy-specific condition that increases maternal and infant morbidity and mortality. It is diagnosed by new-onset increased blood pressure and proteinuria during gestation, which for many years were the sole targets for study. More recently, increased attention to the multisystemic nature of the syndrome with involvement of almost all organs, activation of coagulation and increased sensitivity to pressor agents has expanded understanding of the disorder. The epidemiology of preeclampsia, being more common in poor women, long ago suggested that nutrients might be involved in the disorder [4].

Despite its prevalence and severity, the pathophysiology of this multisystem disorder is still poorly understood and its aetiology has not yet been fully elucidated. Deficiencies of trace elements such as zinc, copper, selenium and magnesium have been implicated in preeclampsia [7]. Placental and systemic oxidative stress are components of preeclampsia and contribute to a generalized maternal systemic inflammatory activation [3]. Placental ischemic reperfusion injury has been implicated in excessive production of reactive oxygen species (ROS), causing release of placental factors that mediate inflammatory responses. In 1928 Krebs reported that the concentration of copper in the serum was significantly raised in the terminal stages of pregnancy.

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How to cite this article:
Since that time this observation has been confirmed by a number of workers, who have shown that at full term the serum copper level is approximately twice the value found in non-pregnant subjects. Thompson and Watson showed that in preeclampsia there was statistically significant increase in the serum copper level over the normal elevation which occurs in a healthy pregnancy [10]. Physiological functions related to copper are based on the biochemical functions of one or more copper metalloenzymes [5]. During pregnancy, plasma copper concentrations significantly increase, returning to normal non-pregnant values after delivery [6]. The increase in preeclampsia may be an exaggerated response of normal pregnancies [7].

**Materials and Methods:**

The study was conducted in Department of Biochemistry in association with Obstetrics and Gynaecology Department of Princess Esra Hospital, Deccan College of Medical Sciences, Hyderabad, India. The subjects involved in the study comprised of three groups as follows:

- **Group I (Control)** - 30 Healthy non-pregnant women
- **Group II (Normal pregnant)** - 30 Normotensive pregnant women
- **Group III (Preeclamptic)** - 30 Pregnant women with preeclampsia

All the subjects included were aged between 20-35 years. The control group comprised of the healthy volunteers of same age group. Pregnant women were in their third trimester. Preeclampsia was diagnosed depending on the clinical features, raised blood pressure (140/90), oedema and proteinuria (by urinary dipstick ≥ 1+) as specified in minimum criteria for diagnosis of preeclampsia by NHBEP, 2000 report. All the women belonged to low to medium socioeconomic status.

**Exclusion criteria** included multiple pregnancies, lactating mothers, smoking and alcoholic individuals. Women with any acute and chronic illnesses (including diabetes, hypertension) or taking medications that could potentially affect levels of trace elements were also excluded.

Informed consent was obtained from each woman after the study was explained to them. The study received approval of dissertation committee of Deccan College of Medical Sciences, Hyderabad, India.

**Blood Sample Collection:** Blood was drawn from the cubital vein using a sterile needle and syringe into plain tubes. The samples were allowed to clot undisturbed and serum was separated by centrifugation for 10 minutes at 3000 rpm into plain tubes and stored at -20°C until time of analysis.

**Estimation Of Serum Copper:**

**Method:** Serum copper was analyzed by Colorimetric method on semiaut ana lyzer, Microlab 300.

**Principle:** Copper, released from ceruloplasmin, in an acidic medium, reacts with Di-Br-Paes to form a coloured complex. Intensity of the complex formed is directly proportional to the amount of copper present in the sample.

<table>
<thead>
<tr>
<th>Copper + Di-Br-Paes</th>
<th>Acidic Medium</th>
<th>Coloured complex</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contents:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L1 : Buffer reagent</td>
<td>12.5 ml</td>
<td></td>
</tr>
<tr>
<td>L2 : Colour reagent</td>
<td>12.5 ml</td>
<td></td>
</tr>
<tr>
<td>S : Copper standard (200µg/dl)</td>
<td>2 ml</td>
<td></td>
</tr>
</tbody>
</table>

**Storage/Stability:** Contents are stable at 2 - 8°C till the expiry mentioned on the labels.

**Reagent preparation:** Reagents are ready to use. Protect from bright light.

The cold buffer (L1) when retrieved from 2-8°C may have a particulate suspension. The suspension clears up once the buffer attains a temperature over 25°C.

**Working reagent:** For larger assay series a working reagent may be prepared by mixing equal volumes of L1 (Buffer reagent) and L2 (Colour reagent). The working reagent is stable at 2-8°C for at least 3 weeks. Keep tightly closed.

**Sample Material:** Serum, free from hemolysis. Copper is reported to be stable in the sample for 6 days when stored at 2-8°C.

**Procedure:**
- **Wavelength / filter:** 580 nm / yellow
- **Temperature:** Room temperature
- **Light path:** 1 cm.

Pipette into a clean dry test tubes labeled as blank (B), standard (S), and test (T):

<table>
<thead>
<tr>
<th>Addition Sequence</th>
<th>B (ml)</th>
<th>S (ml)</th>
<th>T (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer reagent L1</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Colour reagent L2</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Distilled water</td>
<td>0.05</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Copper Standard S</td>
<td>-</td>
<td>0.05</td>
<td>-</td>
</tr>
<tr>
<td>Sample</td>
<td>-</td>
<td>-</td>
<td>0.05</td>
</tr>
</tbody>
</table>
Mix well and incubate at room temperature 25°C for 10 minutes. Measure the absorbance of the standard (Abs. S), and test sample (Abs. T) against the blank, within 30 minutes.

Calculations: Copper in µg/dl = Abs. T / Abs. S x 200.

Linearity: This procedure is linear up to 500 µg/dl. If the value exceeds this limit, dilute the serum with normal saline (NaCl 0.9%) and repeat the assay. Calculate the value using the proper dilution factor.

Normal Reference Values Serum
Males: 80 - 140 µg/dl
Females: 80 - 155 µg/dl
Newborns: 12 - 67 µg/dl
Children up to 10 years: 30 - 155 µg/dl

Notes:
- Chelating agents such as EDTA, Oxalate and Citrate present even in traces, prevent the formation of the colour complex, hence necessary care should be taken during the assay.
- Highly lipaemic samples could interfere and should be cleared by centrifugation or filtration before use.
- The assay can be run at 600 nm however the absorbance would be approximately 30% lower as compared to 570 nm.

Estimation of Serum Ceruloplasmin:
Method: Determination of copper oxidase activity as described by Ravin.
Principle: The ceruloplasmin present in the serum oxidizes the dye paraphenylene diamine and resulting violet colour (Bandrowski base) is measured at 540 nm on photoelectric colorimeter.

Reagents
1. Para-phenylenediamine hydrochloride was purified by dissolving it in a minimum volume of hot distilled water. It was decolourised with charcoal, filtered while still hot and allowed to crystallize. The dried crystals were kept over calcium chloride. They can be kept in sealed vials for several weeks.
2. Acetate buffer, 0.4 M, pH 5.5, was prepared by adding sufficient amount of 1M acetic acid to 200 ml of 1M sodium acetate to bring to pH 5.5. It was stored at 4°C in the refrigerator.

3. Sodium azide 0.02%: 100 mg of sodium azide was dissolved in 500 ml of distilled water. This can be kept indefinitely at room temperature.
4. Buffer substrate solution: 0.1% para-phenylenediamine in acetate buffer was prepared by weighing 1 mg PPD and mixing in 1 ml of acetate buffer. This solution was always prepared fresh.

Procedure: Two test tubes, one marked as test and the other as control were taken, 1 ml of buffer substrate was added to each and they were immersed in a water bath at 37°C for 3 minutes. After removing them 0.1 ml of serum was added to the test tube marked test. The test tubes were shaken well and immersed in water bath for 15 minutes at 37°C. After removal 5 ml of sodium azide was added to both the test tubes (this completely inhibits oxidation of the substrate). After shaking the test tubes properly, 0.1 ml of serum was added to the test tube marked as control. The test tubes were again shaken and cooled for 30 minutes, in a refrigerator at 4°C.

Readings were taken in 3 cm cuvettes at 540 nm in spectrophotometer.

Calculations: mg/dl of ceruloplasmin = (Reading of test - reading of control) x 87.5

Normal Range: 35 - 65 mg/dl

Observation & Results:
A Comparative study of serum levels of copper and ceruloplasmin was done in 30 non-pregnant women as controls, 30 normal pregnant women and 30 women with preeclampsia. Serum copper, ceruloplasmin levels were found to be elevated in preeclamptic patients compared to non-pregnant women and normal pregnant women.

Table: 1 Comparison of Mean Serum Copper in Groups: [ANOVA]

<table>
<thead>
<tr>
<th></th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>122.83</td>
<td>268</td>
<td>302.63</td>
</tr>
<tr>
<td>Standard Deviation (SD)</td>
<td>6.32</td>
<td>28.94</td>
<td>22.88</td>
</tr>
<tr>
<td>Standard Error (SE)</td>
<td>1.15</td>
<td>5.28</td>
<td>4.17</td>
</tr>
<tr>
<td>F-value</td>
<td>584.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-value</td>
<td>P &lt; 0.0001</td>
<td>Significant</td>
<td></td>
</tr>
</tbody>
</table>
The mean serum copper in group I was 122.83 ±6.3 and in group II & group III mean & SD serum copper level were 268.0±28.94 & 302.63±22.88 respectively. The mean serum copper level in groups were statistically significant [p<0.001].

Table 2: Comparison Mean Serum Copper of Two Groups [Turkeys Post Hoc test]

<table>
<thead>
<tr>
<th>Groups</th>
<th>P-value</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I versus II</td>
<td>P&lt;0.001 Significant</td>
<td></td>
</tr>
<tr>
<td>Group II versus III</td>
<td>P&lt;0.001 Significant</td>
<td></td>
</tr>
<tr>
<td>Group I versus III</td>
<td>P&lt;0.001 Significant</td>
<td></td>
</tr>
</tbody>
</table>

When the two groups were compared, the group I & group II of mean serum copper were statistically significant [p <0.001]. Mean serum copper level of group II & group III were statistically significant [p <0.001]. Also Mean serum copper level of Group I & group III were also found statistically significant [p <0.001].

Table 3 Comparison of Mean Serum Ceruloplasmin in Groups: [ANOVA]

<table>
<thead>
<tr>
<th>Mean</th>
<th>Group I</th>
<th>Group II</th>
<th>Group III</th>
</tr>
</thead>
<tbody>
<tr>
<td>60.16</td>
<td>114.95</td>
<td>139.7</td>
<td></td>
</tr>
<tr>
<td>4.82</td>
<td>9.04</td>
<td>13.76</td>
<td></td>
</tr>
<tr>
<td>0.88034</td>
<td>1.5620</td>
<td>2.5125</td>
<td></td>
</tr>
<tr>
<td>F-value</td>
<td>P &lt; 0.0001 Significant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-value</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The mean serum ceruloplasmin in group I was 60.16±4.82 and in group II & group III mean & SD serum ceruloplasmin level were 114.95±9.04 & 139.7±13.76 respectively. The mean serum ceruloplasmin level in groups were statistically significant [p<0.0001].

Table 4: Comparison Mean Serum Ceruloplasmin of Two Groups [Turkeys Post Hoc test]

<table>
<thead>
<tr>
<th>Groups</th>
<th>P-value</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I versus II</td>
<td>P&lt;0.001 Significant</td>
<td></td>
</tr>
<tr>
<td>Group II versus III</td>
<td>P&lt;0.001 Significant</td>
<td></td>
</tr>
<tr>
<td>Group I versus III</td>
<td>P&lt;0.001 Significant</td>
<td></td>
</tr>
</tbody>
</table>

When the two groups were compared, the group I & group II of mean serum ceruloplasmin were statistically significant [p <0.001]. Mean serum ceruloplasmin level of group II & group III were statistically significant [p <0.001]. Also Mean serum ceruloplasmin level of Group I & group III were also found statistically significant [p <0.001].

Discussion:
In the present study there was significant difference in serum copper levels in group I (non-pregnant women) and group II (normal pregnant women), the levels being higher in group II. The serum levels of copper were also significantly higher in group III (pregnant women with preeclampsia) when compared to group I (normal pregnant women). Further there has been significant difference in serum levels of copper in group II (normal pregnant women) and group III (pregnant women with preeclampsia), the levels being higher in group III. The P value being <0.001 in between all the three comparisons as shown in Table 1 & 2. In pregnancy, copper levels in maternal serum rise, more or less in parallel with increase in serum ceruloplasmin. At the same time, total body copper levels increase, but not in the tissues normally associated with copper homeostasis. The placental transport system changes during the latter stages of the development resulting in the transport of higher copper values towards the end of gestation than that of earlier pregnancy. In the study of Jelka Vukelic, Aleksandra Kapamadzija et al [5] healthy pregnant women with normal course of pregnancy showed a constant trend of the increase of mean serum copper values corresponding to mean serum copper values in healthy non pregnant women which are similar to present study results.

In the present study there has been significant difference in serum ceruloplasmin levels in group I and group II (P value <0.001), the levels being higher in group II. The serum levels of ceruloplasmin were also significantly higher in group III when compared to
Further there was significant difference in serum levels of group II and group III (p value < 0.001), levels being higher in group III as shown in Table 3 & 4.

Compatible with present study results, Fattah et al. [2] showed that ceruloplasmin levels were significantly elevated in the maternal blood of preeclamptic patients as compared with normal pregnant women.

**Conclusion:**
Serum Copper levels were significantly raised which may be due to the increased synthesis of ceruloplasmin. Significant raised levels of ferroxidase antioxidant, ceruloplasmin suggest that lipid peroxidation may be an important factor in pathogenesis of preeclampsia and that plasma antioxidants are altered in preeclampsia. These findings have implications for better understanding of preeclampsia and suggest that oxidative stress has role in pathogenesis of preeclampsia.

The present study suggests that Nutrients can affect oxidative stress by decreasing free radicals and increasing antioxidants. It is recommended that an organized study should be conducted to assess the supplementation of antioxidant nutrients and vitamins to pregnant women as prophylaxis.

**References:**