THE PATHOPHYSIOLOGICAL EFFECTS OF SERUM COPPER IN TYPE 2 DIABETES MELLITUS WITH AND WITHOUT DIABETIC NEPHROPATHY

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ABSTRACT

This study was undertaken to investigate the effects of copper on the development of lipid peroxidation in patients with type 2 diabetes mellitus with and without diabetic nephropathy. Fifty-five patients with type 2 diabetes mellitus were recruited in this study and were divided into 2 sub-groups based on the presence of microalbuminuria, the first group (microalbuminuric group n=31) had a microalbuminuria between 30-299 ug/mg. The second group (normoalbuminuric group, n=29) had an albumin level less than 30 ug/mg. The two diabetic groups were compared to the control group (n=37). The results of our study showed significant elevation in the levels of serum lipid profiles and lipid peroxidation markers in the microalbuminuric group compared to the normoalbuminuric group at P<0.001. This was associated with significant rise of serum copper in the diabetic group with nephropathy compared to the diabetic group with no nephropathy and the control group, P<0.001. The current study illustrates that the presence of microalbuminuria in type 2 DM can be regarded as an index of increased cardiovascular vulnerability and a signal for vigorous efforts at correction of known modifiable risk factors in which serum Cu one of them.

KEYWORDS: Serum copper, lipid peroxidation, microalbuminuria, type 2 diabetes mellitus
INTRODUCTION

Copper (Cu) is an essential nutrient that is required by the body for various metabolic activities. Approximately, 80 to 150 milligrams of Cu exists in the adult human body.\(^1\) The distribution of Cu is affected by sex, age, and the level of Cu in the diet. Brain and liver have the highest tissue levels (about one-third of the total body burden), with lesser concentrations found in the heart, spleen, kidneys, and blood. The iris and choroids of the eye have very high Cu levels.\(^2,3\) Cu can be available in various types of food, mainly, the dried beans and nuts, while milk and dairy products are poor sources.\(^3\) The World Health Organization (WHO) recommendation of daily intake of Cu is 30 mg/kg body weight per day (or 2.1 mg/day) for an adult male and 80 mg/kg body weight per day for infants.\(^4\)

Cu possesses insulin-like activity promoting lipogenesis. Lipids are major targets during Oxidative stress. Reactive oxygen species (ROS) can directly damage polyunsaturated fatty acids in membranes and initiate lipid peroxidation. A primary effect of lipid peroxidation is a decrease in membrane fluidity, which alters membrane properties and can significantly disrupt membrane-bound proteins. This effect acts as an amplifier, more radicals are formed, and polyunsaturated fatty acids are degraded to a variety of products. Some of them, such as aldehydes, are very reactive and can damage molecules such as proteins.\(^5\)

Aldehydes (unlike ROS), have longer half-life and can diffuse from the site of their origin and reach and attack targets which are distant from the initial free-radical event, acting as “second toxic messengers” of the complex chain reactions initiated. Among the many different aldehydes which can form during lipid peroxidation, among the most intensively studied are malondialdehyde (MDA).\(^6\) MDA can provide an index of the overall lipid peroxidation resulting due to an imbalance between the released ROS and the total antioxidants capacity.

Cu deficiency can result in glucose intolerance, decreased insulin response and increased glucose levels in the blood. A Cu deficit can enhances glycation, the deleterious binding of sugars to protein.\(^7\) In addition, Cu deficiency is associated with hypercholesterolemia and may fasten the process of atherosclerosis.\(^8\) Administration of additional Cu result in a further increase in serum Cu, reducing serum cholesterol significantly, and reducing the incidence of atherosclerosis. Furthermore, studies showed that excess dietary cholesterol causes cardiovascular disease by lowering the absorption of Cu, an effect that is preventable by increasing the Cu level in the diet.\(^8\)
MATERIALS AND METHODS

Study protocol and Participants

This study was approved by the Scientific and Ethics Committee of the College of Medicine, Al-Nahrain University. Informed consent was obtained from all participants. Ninety-two participants were recruited for this study (55 participants with type 2 diabetes mellitus and 37 normal control subjects). Type 2 diabetes mellitus was diagnosed as per the WHO definition. Type 2 diabetic patients ($n = 55$) were divided according to the urine protein (albumin) excretion measured in ug per mg creatinine (Table 1) into:

1. Patients with albumin-creatinine ratio that is equal to 30-299 ug/mg were considered to have microalbuminuria ($n = 31$).
2. Patients with albumin excretion less than 30 ug per mg creatinine were considered normoalbuminuric ($n = 24$).

All patients were recruited from the outpatient diabetes clinic at Al-Kadhymia Teaching Hospital. The exclusion criteria included: Patients with any recent medical illness, impaired thyroid or renal function, diagnosis of renal disease, treatment with oestrogen, glucocorticoids, or other drugs except oral hypoglycaemic and/or beta blocker antihypertensive drugs. All patients included in the study were non-smokers; none were taking antioxidant supplements or drugs with known antioxidant activity. The mean duration of diabetes was (7.96 ± 3.45 years).

The control group consisted of 37 healthy, age and gender-matched subjects (48.92 ± 8.9 years). The control group consisted of participants with no known medical history and with no family history of diabetes or nephropathy.

Blood Samples

Ten millilitres of venous blood samples were collected from each subject in the study after 8-12 hours fasting. 2 millilitres were collected into an EDTA containing tubes for glycated haemoglobin (HbA1c) assay. The remaining 8 millilitres were centrifuged at 3000 rpm for 10 minutes after about 30 minutes from the time of blood collection. Sera were separated for measurement of serum creatinine, serum lipids, serum malondialdehyde (MDA) level. Serum MDA level was assayed at the same day of blood collection, the sera were stored at -80°C. All assays were obtained by running duplicates for the test, control and the standard.
Urine samples
Random morning urine Specimens were obtained from each subject in the study, to quantify albuminuria, creatinine and albumin to creatinine ratio. No urine preservatives were used; the samples were stored in appropriate containers and were kept at the refrigerator until the time of measurements.

Parameters of the study
A micro method was employed for the determination of urinary protein based upon the co precipitation of protein and ponceau S dye by trichloracetic acid (TCA), dissolution of the precipitate in dilute alkali and spectrophotometric determination of the dye in alkaline solution. [10] Serum and urine creatinine was estimated by the BioMerieux assay kit based on the method of Bartels et al. [11]

Serum lipids were measure using BioMerieux assay kits for total cholesterol (TC), triglycerides (TG), high density lipoprotein cholesterol (HDL-C), and low density lipoprotein cholesterol (LDL-C).

Malondialdehyde (MDA), an end product of fatty acid peroxidation, can react with thiobarbituric acid reacting substance (TBA) to form a colour complex that has maximum absorbance at 532 nm. It serves as a convenient index of lipid peroxidation to measure total lipid peroxide and oxidized HDL-C.

Cu was measured by Flame Atomic Absorption Spectrophotometer. A stock standard concentration of Cu (50 mol / L) was prepared and subsequent dilutions were made to obtain a calibration curve. Serum samples were diluted (1:10) by deionized water and measured directly against an aqueous standard made from certified standard solution. Cu hallow cathode lamps were used at wavelength of 324.75 nm. These solutions were aspirated directly into air-acetylene flame.

Statistical analysis
Data are expressed as mean ± standard deviation of mean. Statistical significance was determined by ANOVA test followed by unpaired Student’s t-test and Pearson’s correlation (r) to test correlation of regression. P values equal or lower than 0.05 were considered statistically significant.
RESULTS
All groups were closely age-matched, and the two diabetes groups were well matched for duration of disease (Table 1)

Table (1) Demographic and clinical data of the participants included in the study. Values are expressed as mean ± standard deviation. Significant difference was considered when P value equal or less than 0.05.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>(Group 1) Diabetic Microalbuminuric</th>
<th>(Group 2) Diabetic Normoalbuminuric</th>
<th>(Group 3) Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>31</td>
<td>24</td>
<td>37</td>
</tr>
<tr>
<td>Male/female</td>
<td>11/20</td>
<td>13/11</td>
<td>15/22</td>
</tr>
<tr>
<td>Urine Albumin/creatinine ratio (ug/mg)</td>
<td>85.4±30.6 *</td>
<td>15.3±4.8*</td>
<td>11.4±2.5</td>
</tr>
<tr>
<td>Age/ years</td>
<td>49.5±7.6</td>
<td>52.2±8.2</td>
<td>48.9±8.9</td>
</tr>
<tr>
<td>Duration/ Years</td>
<td>8.0 (3.512) NS</td>
<td>7.83 (3.535) NS</td>
<td></td>
</tr>
<tr>
<td>HbA1c %</td>
<td>8.1±1.1*</td>
<td>7.68±0.9*</td>
<td>4.87±1</td>
</tr>
<tr>
<td>FBG/ mmol/L</td>
<td>8.9±2.4*</td>
<td>6.3±1.1</td>
<td>5.1±0.3</td>
</tr>
<tr>
<td>T.C mmol/L</td>
<td>5.6±1*</td>
<td>5.29±0.78</td>
<td>4.4±0.58</td>
</tr>
<tr>
<td>TG mmol/L</td>
<td>1.9±0.2*</td>
<td>1.6±0.48*</td>
<td>1.28±0.4</td>
</tr>
<tr>
<td>HDL-C mmol/L</td>
<td>1.09±0.2*</td>
<td>1.1±0.1*</td>
<td>1.4±0.2</td>
</tr>
<tr>
<td>LDL-C mmol/L</td>
<td>3.5±1.1*</td>
<td>3.46±0.8*</td>
<td>2.3±0.5</td>
</tr>
</tbody>
</table>

* Significant difference between the diabetes groups and the control group (P value equal or less than 0.05).

Data for serum MDA and Cu were included in (Table 2) below:

Table (2) Serum MDA, copper, atherogenic index and LDL size for participants of this study: Values were expressed as mean ± standard deviation. Significant difference was considered when P value equal or less than 0.05.

<table>
<thead>
<tr>
<th>Groups</th>
<th>MDA Umol/L</th>
<th>S. Cu Umol/L</th>
<th>AI (LDLc/HDLc)</th>
<th>LDL-C Size (TG/HDL-C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 Microalbuminurics (n =31)</td>
<td>0.96*† +/- (0.1)</td>
<td>27.7**†† +/- (6.2)</td>
<td>3.39** +/- (1.3)</td>
<td>1.79**† +/- (0.42)</td>
</tr>
<tr>
<td>Group 2 Normoalbuminurics (n =24)</td>
<td>0.833* +/- (0.2)</td>
<td>20.3 +/- (2.9)</td>
<td>3.178** +/- (1.1)</td>
<td>1.49** +/- (0.5)</td>
</tr>
</tbody>
</table>
Serum Cu was significantly elevated in the microalbuminuric patients compared the
normoalbuminurics, and control subjects, (P = 0.001). Serum Cu was also significantly
increased in the normoalbuminuric patients compared with control subjects
(P < 0.001), (Figure 1).

| Controls (n = 37) | 0.580 +/- (0.1) | 18.97 +/- (4.4) | 1.625 +/- (0.560) | 0.9 +/- (0.3) |

*p<0.05 versus controls  †P<0.05 group (1) versus group (2)
**P<0.001 versus controls   ††P<0.001 group (1) versus group (2)

Figure 1. Serum copper levels in the diabetic and control groups of this study. Values
were expressed as mean ± standard deviation.

Figure 2. Correlation between serum copper and serum total MDA in
microalbuminuric type 2 diabetic patients in this study (r = 0.433, p = 0.015).

There was a significant positive correlation between serum Cu and total MDA level
microalbuminuric diabetics (r = 0.433, p = 0.015), (Figure 2). Such a relation was not
significant when comparing these parameters in the normoalbuminurics and the controls group \( (r = 0.312, \ p = 0.138) \), and \( (r = 0.235, \ p = 0.161) \) respectively.

**DISCUSSION**

The accelerated atherosclerosis process associated with type 2 DM has been previously investigated \(^{[12,\ 13]}\) and is a serious problem associated with the epidemics of type 2 DM. \(^{[14]}\) The mechanism of the link between microalbuminuria and cardiovascular mortality is still not clear. However, increased urinary albumin loss has been postulated to be a marker of a generalized increase in vascular permeability, predisposing infiltration of the arterial wall by atherogenic lipoprotein particles. \(^{[15,\ 16]}\)

Serum MDA was elevated in type 2 DM patients with micro-vascular complications compared to type 2 DM patients with no clinical complications and matched healthy subjects.\(^{[17]}\) The increasing levels of blood free fatty acid depending on increased lipolysis results in increase in MDA production. \(^{[17]}\) In the excessive production of free radicals, it leads to microvascular lesions.

Our study showed that the atherogenic index (AI) is higher in the type 2 DM compared to the controls group (table 2) due either to decreased HDL-C or increased LDL-C or both with no significant difference between the micro- and normoalbuminuric T2DM. Low HDL-C, high LDL-C, cholesterol and triglyceride levels are established cardiovascular risk factors in non-diabetic\(^{[18]}\) and type 2 DM individuals. \(^{[19]}\) However, qualitative abnormalities of lipoproteins, such as glycation and oxidation, are also important and these are not detected by conventional measures. The typical dyslipoproteinaemia of type 2 diabetes is characterised by elevated VLDL, small (dense) LDL particles, and decreased HDL. \(^{[20]}\)

Serum Cu showed a significant elevation in diabetics groups as compared to the controls and being significantly higher in the microalbuminuric patients than the normoalbuminuric (table 2) and (figure 1). This hypercupraemia was also shown by \(^{[21]}\), especially with disease such as retinopathy and hypertension. The significant reduction in serum Cu may occur as a result of increasing in urinary loss of Cu and ceruloplasmin in nephrotic patients. There was a significant correlation between serum Cu and total cholesterol / HDL-C ratio confirming the existence of a role for Cu in the development of hyperlipidaemia so serum Cu level is affected by renal excretion and kidney disease which is one of the major complications of diabetes that gives a conflicting data from many researches.
In addition, it is worth to mention that high serum Cu concentrations are significantly associated with an increased mortality from all cardiovascular diseases and from coronary heart disease in particular.\textsuperscript{[22]} These findings, along with the relationship between hypercupraemia and pathologies such as hypertensive states, atherosclerosis, myocardial infarction and stroke\textsuperscript{[23]} point to the increased blood levels of Cu ions as a risk factor for cardiovascular mortality and underscore the pathophysiological significance of the present findings.

Eaton and Qian introduced the hypothesis that glycated proteins bind transition metals such as Cu and iron, and that such `glycochelates' accumulate within the vasculature in diabetes and catalytically inactivate endothelial derived relaxation factor (EDRF).\textsuperscript{[24]}

As both Cu deficiency and excess may lead to disease. In the presence of available cellular reducing agents, Cu in low molecular weight forms may play a catalytic role in the initiation of free radical reactions. The resulting oxyradicals have the potential to damage cellular lipids, nucleic acids, proteins and carbohydrates, resulting in a wide-ranging impairment in cellular function and integrity.

The present work, therefore, furthers our understanding regarding the role of subtle alterations in Cu ions homeostasis on vascular functions, of which microalbuminuria is regarded as one of its first manifestations, and suggests that increased blood concentrations of Cu\textsuperscript{2+} may be a potential contributor for diabetic microvascular complications.

**CONCLUSION**

The presence of microalbuminuria in type 2 DM can be regarded as an index of increased cardiovascular vulnerability and a signal for vigorous efforts at correction of known modifiable risk factors in which serum Cu one of them.

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