Research article

A correlative study of copper, ceruloplasmin, iron, total iron binding capacity and total antioxidant capacity in diabetic nephropathy

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ABSTRACT

Introduction and Aim: Diabetic nephropathy is the common cause of final stage of kidney disease. Studies on trace elements and oxidant-antioxidant status in diabetes mellitus are required for establishing the mechanisms involved in pathogenesis of diabetic complications, and also to establish biomarkers of diabetic nephropathy in addition to the conventional markers. The present study aimed to assess and correlate the blood levels of copper, iron, ceruloplasmin, total iron binding capacity (TIBC) and total antioxidant capacity (TAC) in diabetic nephropathy patients in comparison to diabetic individuals without complications and normal healthy controls.

Materials and Methods: The study subjects were, diabetic patients with nephropathy (group 1), diabetic patients without complications (group 2), and healthy controls (group 3). In the serum samples of all study subjects levels of copper, iron, ceruloplasmin, TIBC and TAC were estimated by standard spectrophotometric methods.

Results: Levels of copper, ceruloplasmin, iron and TIBC in serum were significantly higher and TAC was lower in diabetic patients when compared to controls, and more pronounced changes were seen in diabetic nephropathy patients when compared to diabetic patients with no complications. There was significant positive correlation among glycated hemoglobin, copper, iron and microalbumin in diabetic patients with or without nephropathy. Serum Total antioxidant capacity showed significant negative correlation with HbA1c, microalbuminuria, copper, and iron in diabetic patients with and without nephropathy.

Conclusion: Serum levels of trace elements could serve as diagnostic and prognostic biomarkers of diabetic nephropathy complimentary to microalbuminuria and glycated haemoglobin. Monitoring the trace elements and oxidative stress biomarkers in diabetic patients could be beneficial to prevent oxidative stress and pathogenesis of diabetic complications.

Keywords: Ceruloplasmin; copper; diabetic nephropathy; iron; total antioxidant capacity.

INTRODUCTION

Diabetes mellitus is a major public health condition, affecting millions of people globally, causing long term complications, leading to morbidity and mortality. Diabetes has gradually increased in India over the last four decades, and accounting for a notable portion of the global burden (1).

Nephropathy is one of the earliest microvascular complications of diabetes mellitus, and is the leading cause of kidney failure. The most severe manifestation of diabetic renal disease, the End Stage Renal Disease (ESRD) is the leading cause of reduced life span in people with diabetes. Hence, it is very important to detect diabetic nephropathy at early stages using biomarkers. Routinely used markers include, microalbuminuria and glomerular filtration rate. However, these biomarkers lack sensitivity and specificity and the search is on for newer biomarkers. Novel biomarkers assayed in previous studies include neutrophil-gelatinase associated lipocalin, kidney injury molecule-1, and various pro-inflammatory molecules (2).

It is important to analyze the mechanism of etiopathogenesis of diabetic nephropathy and role of various substances in its complications. Induction of oxidative stress and inflammation has been proposed to be the major mechanism involved in microvascular complications of diabetes including nephropathy (3). Pharmacologic interventions for insulin resistance and diabetes mellitus have been attempted targeting oxidative stress and inflammation (4).

Trace elements play a vital role in human health and disease. Copper and iron are crucial elements in human nutrition and involve in numerous biochemical reactions. Both these trace elements are considered to be pro-oxidants. Copper has been suggested to bind to proteins which undergo glycation in diabetes, and

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ceruloplasmin has been shown to stimulate glycation, release copper to promote oxidative stress in diabetes mellitus. Dysregulation of copper homeostasis has been proposed as a mechanism involved in diabetic nephropathy, and copper chelation therapy has been shown to protect against pathogenesis of diabetic nephropathy. Iron overload has been shown to promote pathogenesis of diabetic nephropathy in animal studies (1,5).

This study aimed to assess blood levels of copper, ceruloplasmin, iron status and total antioxidant capacity in diabetic nephropathy patients in comparison to diabetes patients without complications and healthy individuals.

MATERIALS AND METHODS

Source of data
It was a case control study done at Medical College and Hospital. Patients with diabetic nephropathy (n=30); diabetic individuals without complications (n=30); normal healthy individuals were the subjects of the study (n=30). The diabetic subjects were diagnosed based on WHO (World Health Organization) criteria (6), and Nephropathy was diagnosed based on ADA (American Diabetes Association criteria) criteria (7). Chronic alcoholics, smokers, tobacco chewers, pregnant women, and individuals with non-diabetic systemic illness were excluded.

Collection of samples and analysis
Five ml of venous blood sample was collected under aseptic precautions, serum for the assays of copper, ceruloplasmin, iron, TIBC, and TAC separated after centrifugation at 3000 rpm for 15 minutes. The data of plasma glucose, glycated hemoglobin (HbA1c) and urine albumin were obtained from the medical records.

Serum iron and unsaturated iron binding capacity (UIBC) were assayed by ferrozine method, TIBC was calculated from iron and UIBC (8). Serum copper was assayed by the spectrophotometric method using Di-Br-PAESA (3,5-Dibromo-2-pyridylazo-N-Ethyl-N-3-sulphopropylaniline)(9). Serum ceruloplasmin was analysed using p-phenylene-diamine dihydrochloride as the substrate (10). Serum TAC was analysed by the method of Korocenevic et al., (11) based on reduction in formation of thiobarbituric acid-reactive substances (TBARS). All the above assays except TAC were done in the automated chemistry analyser of Roche Diagnostics, and TAC was done in a double beam spectrophotometer.

Ethical approval
The present research study involving human participants was performed according to ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments. It was approved by Institutional ethics committee (FMMC/FMIEC/325/2011).

Statistical analysis
All statistical analyses were conducted by using Statistical Package for the Social Sciences (SPSS V.17.0). Continuous variables were analyzed by measures such as mean, standard deviation, and statistical significance was tested by independent sample-t-test. Kruskal Wallis test and Mann-Whitney were used to analyse the significance of difference of values among the groups. Correlation among the biochemical parameters was analysed by Karl Pearson’s Correlation Analysis.

RESULTS
The results of the present study were presented in Tables (1-3). Subjects of the study were patients with diabetic nephropathy (Group 1), diabetic patients without any complications (Group 2) and normal healthy individuals (Group 3).

| Parameters                  | Group-1 Diabetic nephropathy (n=30) | Group-2 Diabetic without complications (n=30) | Group-3 Healthy controls (n=30) |
|-----------------------------|-------------------------------------|-----------------------------------------------|---------------------------------|
| Age (Years)                 | 58.66±12.44 (46.22-71.1)           | 47.26±12.32 (34.94-59.58)                     | 40.8±16.06 (24.74-56.86)       |
| Fasting Plasma Glucose (mg/dL) | 167.66±64.35 (103.31-232.01)      | 141.4±45.13 (96.27-186.13)                    | 83.96±7.93 (76.03-91.89)       |
| Post Prandial Plasma glucose (mg/dL) | 247.13±84.99 (162.14-332.12)     | 208.1±74.44 (133.66-282.54)                   | 115.13±7.45 (107.68-122.58)   |
| HbA1c (%)                   | 9.80±2.21 * (7.59-12.01)           | 8.62 ± 2.61 * (6.01-11.23)                     | 5.78 ± 0.62 (5.16-6.4)         |
| Microalbumin (mg/L)         | 208.77 ± 88.59 * (120.18-297.36)   | 14.37 ± 2.94 (11.43-17.31)                    | 6.12 ± 0.87 (5.25-6.99)        |

* Significant difference when compared to Group-3, p<0.001
** Significant difference when compared to diabetic patients without complications, p<0.001

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**Table 2:** Serum levels of copper, ceruloplasmin, iron, TIBC and TAC in diabetic nephropathy (Group-1), Diabetic patients without complications (Group-2), and normal healthy controls (Group-3).

| Parameters                  | Group-1 Diabetic nephropathy (n=30) | Group-2 Diabetic without complications (n=30) | Group-3 Healthy controls (n=30) |
|-----------------------------|-------------------------------------|-----------------------------------------------|---------------------------------|
| HbA₁c (%)                   | 9.80 ± 2.21 * (7.59-12.01)          | 8.62 ± 2.61 * (6.01-11.23)                    | 5.78 ± 0.62 (5.16-6.44)         |
| Microalbumin (mg/L)         | 208.77 ± 88.59 *, ** (120.18-297.36) | 14.37±2.94 (11.43-17.31)                      | 6.12 ± 0.87 (5.25-6.99)         |
| Serum Copper (μg/dl)        | 217.45 ± 128.84 *, ** (88.61-346.29)| 161.75 ± 27.14 * (134.61-188.89)              | 115.37 ± 21.25 (94.12-136.62)   |
| Serum Ceruloplasmin (mg/dl)| 4.25 ± 13.58 *, ** (28.93-56.09)    | 36.52 ± 4.86 (31.66-41.38)                    | 33.07 ± 5.17 (27.9-38.24)       |
| Serum Iron (μg/dl)          | 105.30 ± 16.27 *, ** (89.03-121.57) | 91.73 ± 17.40 (74.33-109.13)                  | 85.10 ± 27.36 (57.74-112.46)    |
| Serum TIBC (μg/dl)          | 344.13 ± 69.09 *, ** (275.04-413.22)| 295.43 ± 34.20 (261.23-329.63)                | 290.80 ± 50.57 (240.23-341.37)  |
| Serum TAC (mmol/L)          | 0.43 ± 0.33 *, ** (0.10-0.76)        | 1.10 ± 0.38 * (0.72-1.48)                     | 3.33 ± 1.59 (1.74-4.92)         |

The values are mean ± S.D. of number of samples indicated.
The range of the values, 95% confidence interval is given in parentheses
* Significant difference when compared to Group-3, p <0.001
** Significant difference when compared to diabetic patients without complications, p<0.001

**Table 3:** Correlation among HbA₁c, microalbumin, copper, ceruloplasmin, iron, TIBC and TAC in diabetic patients with nephropathy (Group-1) and diabetic patients without complications (Group-2).

| Parameters                  | Group 1 Diabetic nephropathy | Group 2 Diabetic without complications |
|-----------------------------|-----------------------------|---------------------------------------|
|                             | Correlation coefficient (r) | Significance                           |
| HbA₁c-Microalbumin           | .866*                       | .869*                                 |
| Copper-HbA₁c                 | .386**                      | .653*                                 |
| Iron-HbA₁c                   | .719*                       | .792*                                 |
| TIBC-HbA₁c                   | .542*                       | .477*                                 |
| Ceruloplasmin-HbA₁c          | .254***                     | .797*                                 |
| TAC-HbA₁c                    | - .0821*                    | - .0852*                              |
| Copper-Microalbumin          | .400**                      | .551*                                 |
| Iron-Microalbumin            | .751*                       | .576*                                 |
| TIBC-Microalbumin            | .568*                       | .279***                               |
| Ceruloplasmin-Microalbumin   | 0.398**                     | .628*                                 |
| TAC-Microalbumin             | - .0896*                    | - .0856*                              |
| Iron-TIBC                    | .342**                      | .718*                                 |
| Iron-Copper                  | .341**                      | .704*                                 |
| Iron-Ceruloplasmin           | .304***                     | .841*                                 |
| Iron-TAC                     | - .0702*                    | - .0524*                              |
| Copper-Ceruloplasmin         | .650*                       | .753*                                 |
| Copper-TIBC                  | .423**                      | .499*                                 |
| Copper-TAC                   | - .0389**                   | - .0504*                              |
| TAC-TIBC                     | - .0646*                    | - .311**                              |

*HS=Highly significant (P<0.001), **S= Significant (P<0.05), ***NS= Not significant

This study observed significantly higher levels of HbA₁c, microalbuminuria, serum iron, TIBC, copper, and ceruloplasmin, and significantly decreased serum TAC in diabetic patients in comparison to controls (Table 2). The changes were more pronounced in diabetic nephropathy patients than Group 2 cases, but there was no significant difference among controls and diabetics without complications with respect to ceruloplasmin, iron and TIBC.

A significant positive correlation of blood HbA₁c with microalbumin, serum levels of iron, TIBC, copper, and negative correlation with TAC in both the groups of diabetics and there was statistical significant in both
the groups, but there was no significant correlation with ceruloplasmin in diabetic nephropathy (Table 3).

This study observed that microalbumin was having significant positive correlation with HbA1c, serum levels of iron, copper, ceruloplasmin and negative correlation with TAC in both the groups of diabetics & there was statistical significant in both the groups, but there was no significant correlation with TIBC, in diabetic patients without complications (Table 3).

Serum copper showed significant positive correlation with TIBC and ceruloplasmin, and negative correlation with TAC but, no significant correlation with serum iron in diabetic nephropathic subjects. However, in diabetic patients without complications serum copper showed significant positive correlation with iron, TIBC, ceruloplasmin and negative correlation with TAC. Serum iron showed significant negative correlation with TAC in all diabetic subjects (group-1 and group- 2). There was significant positive correlation of iron with copper and ceruloplasmin in diabetic without complications but, no significant correlation with ceruloplasmin and copper in diabetic nephropathic subjects (Table-3).

**DISCUSSION**

This study aimed to assess blood levels of copper, ceruloplasmin, iron status and TAC in diabetic nephropathy patients in comparison to diabetic individuals without complications and normal healthy individuals. The study also aimed to correlate these levels with glycated haemoglobin and albuminuria in diabetic patients. There were significant alterations in serum copper, iron, TIBC, ceruloplasmin and TAC in diabetic patients when compared to controls, and diabetic nephropathic patients showed more pronounced changes than the diabetic without complications. Serum iron, copper and ceruloplasmin showed positive correlation with glycated haemoglobin and microalbuminuria while serum TAC showed negative correlation with glycated haemoglobin and micro albuminuria.

Copper is considered as pro-oxidant and increased copper levels might cause or be caused by diabetes mellitus and its complications. In this study, serum copper level was significantly increased in diabetic patients with or without nephropathy, and diabetic nephropathic group showed more pronounced changes. Serum copper showed significant positive correlation with glycemic status & microalbumin in all diabetic patients. The results were in concordance with earlier studies on serum copper in diabetes mellitus (12,13). Free copper participates in Fenton and Heiber-Weiss reactions to generate reactive oxygen species. Increased urinary copper in diabetic nephropathy and it’s correlation with microalbuminuria has been observed in previous studies, and increased deposition of urinary copper to damaged kidney tubules indicates its role in the progression of nephropathy (14). In the present study, serum copper level was positively correlated with microalbuminuria and negatively correlated with total antioxidant capacity among diabetic patients with or without nephropathy (Table 3).

Iron is known to be harmful when deposited excess in tissues, leads to generation of free radicals and induction of oxidative stress. Excess levels of iron in the human blood of renal disease patient, suggests its role in the etiopathogenesis of diabetic nephropathy (15,16). Present study observed significantly higher serum iron and TIBC in diabetic patients without complications, diabetic nephropathic patients in comparison to healthy controls. Serum iron levels showed positive correlation with glycemic status (HbA1c) and microalbuminuria, negative correlation with total antioxidant capacity in diabetic patients with or without microalbuminuria. Various studies have suggested the role of iron in pathogenesis of diabetes mainly through induction of oxidative stress (16,17). Correlation between insulin resistance and iron overload has been reported by previous studies, which indicates its significant role in diabetes and its complications (17,18). Findings of the present study showing correlation of serum iron with microalbuminuria and glycated hemoglobin level and a negative correlation with antioxidant capacity, and more pronounced changes in serum iron status among diabetic patients with nephropathy indicate the mechanisms involved in diabetic nephropathy.

Ceruloplasmin, a copper-containing protein having ferroxidase activity acts as an antioxidant. It also act as a pro-oxidant by donating free copper ions, which induces reactive oxygen species (ROS) formation in case of increased oxidative stress. Various studies have observed increased serum ceruloplasmin in diabetes mellitus (13,19). Lee and co-workers demonstrated serum ceruloplasmin level to be marker for development of diabetic nephropathy in patients with diabetes mellitus (20). The finding of increased serum ceruloplasmin among diabetic subjects and more pronounced increase among subjects with diabetic nephropathy, and its significant positive correlation with microalbuminuria in diabetic nephropathy patients indicates its potential role as a biomarker of diabetic nephropathy.

Serum TAC is an indicator of the non-enzymatic antioxidant defense system (11). This study observed significantly lower serum TAC in diabetic patients with or without nephropathy in comparison to healthy controls, and more pronounced decrease among patients with diabetic nephropathy. Previous studies observed decreased TAC in diabetic patients with or without nephropathy, and more pronounced decrease among diabetic nephropathy patients (21,22). The decrease in TAC correlated with microalbuminuria.

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We observed significant negative correlation of serum TAC with glycated hemoglobin, microalbuminuria, copper and iron levels in diabetic patients with or without nephropathy.

CONCLUSION

This study has observed significant changes in copper, ceruloplasmin, iron status and TAC in diabetic patients with or without nephropathy. The changes in these parameters were more pronounced in diabetic nephropathy patients than diabetic patients without complications. The findings of this study suggest involvement of oxidative stress and trace elements in the pathogenesis of diabetic nephropathy. Serum levels of trace elements could serve as diagnostic markers of diabetic nephropathy complimentary to microalbuminuria and glycated haemoglobin. This study suggests including the antioxidants in the therapeutic regimen of diabetes and its complications, and monitoring the antioxidant status using total antioxidant capacity in serum. Further studies with larger sample size, and correlating the trace elements and oxidative stress markers with progression of diabetic nephropathy are required for establishing the biomarkers of diabetic nephropathy.

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CONFLICT OF INTEREST

None declared

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