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Research Article

Study of impairment in collaboration between ceruloplasmin and transferrin in development of complications in type 2 diabetes mellitus

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ABSTRACT

Background: Hyperglycemia in diabetic patients can increase the levels of free radicals through various metabolic alterations. One of the most reactive pro-oxidants in peroxidation reactions is free, redox-active iron and generates highly reactive hydroxyl radicals that initiate lipid peroxidation. The purpose of the study was to elucidate the role of CP and TRF in type 2 DM and analyse the effect of disturbance in collaboration between these parameters in pathogenesis of type 2 diabetic complications.
Methods: We included 100 Type 2 DM subjects (with and without complications) and 100 healthy controls. The duration of type 2 DM in study subjects ranged from 5 to 16 years. Blood samples in fasting condition were collected for analysis of serum malondialdehyde (MDA), CP and TRF.
Results: In the study serum CP levels were higher in Type 2 diabetic subjects with complications compared to diabetic subjects without complications (P=0.01). However, significantly low transferrin values were obtained in diabetic subjects with complications compared to diabetic subjects without complications (P=0.007). Significantly high MDA levels were observed (P=0.002) in type 2 diabetic subjects with complications compared to type 2 diabetic subjects without complications.
Conclusions: The results of the present study indicate oxidative stress plays a role in precipitating complications in Type 2 DM reflecting in disturbance of CP and TRF collaboration.

Keywords: Type 2 diabetes mellitus, Ceruloplasmin, Transferrin, Malondialdehyde

INTRODUCTION

According to the World Health Organisation 2008 fact sheet diabetes mellitus (DM) is a chronic disease that occurs when the pancreas does not produce enough insulin, or when the body cannot effectively use the insulin it produces. Hyperglycemia or raised blood sugar is a common effect of uncontrolled diabetes and over time leads to serious damage to many of the body's systems, especially the nerves and blood vessels.

DM has emerged as a major health care problem in India. According to the Diabetes Atlas published by the international diabetes federation (IDF), there were an estimated 40 million persons with DM in India in 2007 and this number is predicted to rise to almost 70 million people by 2025 by which time every fifth diabetic subject in the world would be an Indian.[1]

In the pathological events, the increased free radical activity is suggested to play an important role in the lipid peroxidation and protein oxidation of cellular structures causing cell injury.[2] Free radicals are formed disproportionately in DM by glucose degradation, which may play an important role in the development of complications in diabetic patients. The generation of free

radicals may lead to lipid peroxidation and cause severe damage in DM patients. Hyperglycemia in diabetic patients can increase the levels of free radicals through glucose auto-oxidation, non-enzymatic post translational modification of proteins resulting from chemical reaction between glucose and primary amino groups of proteins and also through polyol pathway and protein kinase activation.

In DM iron metabolism is disturbed but little information is available on the effect of DM on the antioxidant capacity of plasma to protect against iron driven lipid peroxidation.[3] One of the most reactive pro-oxidants in peroxidation reactions is, free redox-active iron (Fe^{2+}) since it catalyses the Fenton reaction and generates highly reactive hydroxyl radicals that are able to initiate lipid peroxidation.

In order to prevent these reactions, iron in plasma is sequestered in a safe redox-inactive form by the combined iron oxidising action of ceruloplasmin (CP) and the iron binding capacity of transferrin (TRF). In healthy subjects without iron overload, the iron-binding capacity of TRF is more than sufficient to strongly bind the amount of iron present in serum and it is generally accepted that only a negligible amount of serum iron is found as non-transferrin bound iron (NTBI).[3]

Iron in a biological system attach to biological molecules, where they cause site specific formation of OHº radicals and consequent damage to lipid, protein and DNA.[4] Extensive lipid peroxidation in biological membranes causes loss of fluidity, fall in membrane potential, increased permeability to H^+ and other ions and eventual rupture leading to release of cell and organelle contents. Some end products of peroxide fragmentation are also cytotoxic. Reactive oxygen species degrade polyunsaturated lipids, forming MDA.

This compound is a reactive aldehyde and is one of the many reactive electrophile species that cause toxic stress in cells and form advanced glycation end products (AGEs). The production of this aldehyde is used as a biomarker to measure the level of oxidative stress in an organism.[5]

MDA is a highly toxic by–product formed in part by lipid oxidation derived free radicals. Many studies have shown that its concentration increases considerably in DM. Increased oxidative stress may be present in Type 2 DM due to impaired iron sequestering capability of Transferrin and impaired collaboration of transferrin with ceruloplasmin. These mechanisms may be the factors leading to complications in Type 2 DM.

METHODS

The present study was conducted in the Department of Biochemistry, Jawaharlal Nehru (J.L.N) Medical College and its Associated Group of Hospitals, Ajmer, Rajasthan, India. The subjects included in the study were:

- 100 Healthy controls –Group 1 (50 males and 50 females) selected from volunteers such as doctors, resident doctors, paramedical staff and healthy relatives / attendants of patients.
- 25 males, 25 females) type 2 DM patients without complications-group 2 and
- 50 (25 males, 25 females) type 2 DM patients with complications-group 3 attending the outpatient clinics or admitted in wards of department of medicine and department of cardiology, J.L.N. medical college and associated group of hospitals, Ajmer. The duration of DM in diabetic subjects ranged from 5 to 16 years.

All subjects were age; body mass index and sex matched.

The duration of DM in diabetic subjects ranged from 5 to 16 years.

We excluded the subjects who were habitual smokers, alcoholics, hypertensive and having active inflammatory diseases, nutritional deficiencies, estrogen therapy, malignancy and active immunological diseases. Fasting blood samples were collected in plain vacutainers for estimation of serum ceruloplasmin, transferrin and malondialdehyde. Blood was allowed to clot for 30 minutes at room temperature and then centrifuged at 3000 rotations per minute (rpm) for 10 minutes to obtain clear unhemolysed serum. Serum TRF was determined by Immunoturbidimetric end-point Method, Serum MDA was determined by Colorimetric method of measurement, Serum CP was determined by Para-Phenylenediamine oxidase (p-PPD) manual method.[10]

Statistical analysis

Differences in the parameters between the groups were analyzed by means of the student's t test. Variables were presented as mean±standard deviation (S.D). Partial correlation analysis (first order) was employed to determine the correlation between two variables with the third variable held constant (effect of third variable removed). The accepted level of significance for all statistical analyses used in the study was $P \leq 0.05$ (two tailed P value). In correlation analysis, $P \leq 0.02$ (one tailed P value) was also considered. Correlations between variables were tested using the Pearson rho (r: correlation coefficient) correlation test. Chi-square (χ^2) analysis was used for comparison of groups. The accepted level of significance for all statistical analyses used in the study was $P \leq 0.05$.

Websites used for statistics:

1. www.physics.Qbsju.edu/ stats/ t-test (data entry site for calculating P and t values).

2. Vassar Stats: website for statistical computation (used for correlation and chi square analysis).

RESULTS

In the present study significantly low (P=0.007) TRF values were observed in type 2 diabetic subjects with complications (Mean±SD: 204.0±38.9mg/dl) compared to diabetic subjects without complications (Mean±SD 224.0±34.4mg/dl). Type 2 diabetic subjects with complications (Mean±SD: 46.2±10.5mg/dl) showed significantly high (P=0.01) CP values compared to type 2 diabetic subjects without complications (Mean CP: 41.0±10.4 mg/dl) (Table 1).

Table 1: Mean serum CP, TRF and MDA levels in type 2 DM subjects.

Parameters	Groups Studied		P Value
	Type 2 DM without complications	Type 2 DM with complications	
CP (mg/dl)	41.0±10.4	46.2±10.5	0.013 (S)
TRF (mg/dl)	224.0±34.4	204.0±38.9	0.007 (S)
MDA (nmol/ml)	4.87±1.65	5.90±1.61	0.002 (S)

CP: Ceruloplasmin TRF: Transferrin MDA: Malondialdehyde S: Significant (p<0.05 is significant)

Table 2: Distribution of subjects in various ranges of serum CP, TRF and MDA.

Parameters	Reference Range		Groups		χ^2	P value
			Type 2 DM subject n / 50 (%)	Type 2 DM subjects n / 50 (%)		
CP (mg/dl)	Normal	21.0 – 43.0	29/50 (58%)	15/50 (30%)		
	High	> 43.0	21/50 (42%)	35/50 (70%)	6.86	0.008 (S)
TRF (mg/dl)	Normal	150 – 200	15/50 (30%)	30/50 (60%)		
	High	201 – 340	35/50 (70%)	20/50 (40%)	7.92	0.005 (S)
MDA (nmol/ml)	Normal	1.0 – 5.0	31/50 (62%)	19/50 (38%)	2.35	0.12 (NS)
	Moderately High	5.1 – 7.0	13/50 (26%)	18/50 (36%)	3.97	0.05 (S)
	Very High	> 7.0	6/50 (12%)	13/50 (26%)	0.19	0.66 (NS)

CP: Ceruloplasmin TRF: Transferrin MDA: Malondialdehyde S: Significant (p<0.05 is significant) NS: Non significant

Table 3: Correlation of serum TRF values with CP and MDA values in various groups of subjects.

Groups Studied	CP			MDA		
	r	T	P: One tail Two tail	r	T	P: One tail Two tail
Healthy Control subjects (n=100)	+0.30	3.16	0.001 (S) 0.002 (S)	+0.11	1.06	0.14 (NS) 0.29 (NS)
Type 2 DM subjects without complications (n=50)	-0.26	1.91	0.03 (S) 0.06 (NS)	-0.16	1.15	0.12 (NS) 0.25 (NS)
Type 2 DM subjects with complications (n=50)	-0.56	4.66	< 0.0001 (HS) < 0.0001 (HS)	-0.30	2.15	0.01 (S) 0.03 (S)

CP: Ceruloplasmin TRF: Transferrin MDA: Malondialdehyde S: Significant (p<0.05 is significant).

Mean values of MDA in type 2 diabetic subjects with complications (5.90±1.61nmol/ml) and without complications (4.87±1.65nmol/ml) were significantly higher (P<0.001; P=0.003) than values observed in healthy controls (4.06±1.51 nmol/ml) (Table 1). In the present study 70% of diabetic subjects with complications had raised CP levels (>43.0 mg/dl) compared to 42% diabetics without complications and 24% healthy controls. TRF levels were lower (150-200 mg/dl) in 60% of diabetics with complications compared to 30% diabetics without complications and 26% healthy controls.

Comparison of percentage distribution of diabetic subjects with complications and without complications with CP>43.0mg/dl and TRF 150-200 mg/dl yielded significantly high chi-square values (CP>43.0mg/dl: χ^2=6.86, P=0.008; TRF 150-200mg/dl: χ^2=7.92,

P=0.005) (Table 2). CP and TRF association was impaired in diabetic subjects with complications (r=-0.56; P<0.0001) compared to diabetic subjects without complications (r=-0.26; P=0.06) (Table 3). TRF and CP also correlate with age and duration of diabetes in diabetic subjects with complications. To evaluate further the role of other factors in disturbing CP and TRF association in diabetic subjects with complications partial correlation analysis was used.

In the present study a significantly positive correlation was observed between CP and MDA in type 2 diabetic subjects with complications (r=+0.41; P = 0.003). An inverse correlation was observed between TRF and MDA in diabetic subjects with and without complications. (r=-0.30; P=0.03: r=-0.16; P=0.25). The association of TRF and MDA was significantly negative in diabetics with complications (Table 3).

DISCUSSION

Mean values of malondialdehyde in type 2 DM subjects with complications and without complications were significantly higher (P=0.003; P<0.001) than values observed in healthy controls. Further, significantly high MDA levels were observed (P=0.002) in type 2 diabetic subjects with complications compared to type 2 diabetic subjects without complications. Free radicals are formed disproportionately in diabetes mellitus by glucose degradation, which may play an important role in the development of complications in diabetic patients. The generation of free radicals may lead to lipid peroxidation and cause severe damage in diabetes mellitus patients.

In the present study serum ceruloplasmin levels were significantly high in diabetic subjects both with and without complications compared to healthy controls (P<0.0001). Further, ceruloplasmin levels were higher in type 2 diabetic subjects with complications compared to diabetic subjects without complications (P=0.01).

The raised CP levels found in diabetic subjects in the present study are due to a compensatory mechanism. By keeping Fe in Fe^{3+} state, CP prevents it from undergoing the redox cycles necessary to initiate toxic effects.

However, low transferrin values were obtained in diabetic subjects with complications compared to diabetic subjects without complications (P=0.007). The significantly decreased levels of TRF observed in the diabetic subjects with complications could be due to disturbances in iron (Fe) metabolism.[6,7] TRF plays a central role in the body's metabolism of iron because it transports iron (2 mols of Fe^{3+} per mole of TRF) in the circulation to sites where it is required. Free iron is toxic, but association with TRF diminishes its potential toxicity and also directs iron to where it is required in the body.

TRF regulates iron fluxes between the sites of absorption, storage and utilization. It has also been attributed a very important antioxidant function in plasma. Free ferrous iron (Fe^{2+}) is very reactive and capable of producing free radicals that cause oxidative damage to biomolecules.[8] Typically, no free Fe^{2+} is present because all iron is bound to TRF in a redox inactive ferric (Fe^{3+}) form.

The ways in which modifications or abnormalities of TRF affect its Fe^{3+} binding and antioxidant capacity may be elucidated by the mechanism proposed by singh et. al.[9] In DM protein structure and function are significantly affected by glycation. The aldehyde group of glucose reacts non-enzymatically with amino groups of proteins to form schiff's bases, which undergo amadori rearrangements, forming fructosamines. These modified proteins degrade slowly and irreversibly to advanced glycation end products. It can be suggested that glycation of TRF contributes to OS due to impairment of its antioxidant functions. According to Campenhout et al., (2004) in vitro glycation of TRF decreases its Fe^{3+} binding capacity and makes it less effective in protecting against lipid peroxidation.

A significant positive correlation between CP and TRF values was found in healthy control subjects (r=0.30; P=0.002). This suggests presence of collaboration between these two extracellular antioxidants (CP and TRF). However, this collaboration seems to be impaired in patients with DM, both with and without complications. This is supported by the inverse correlation observed between CP and TRF in type 2 diabetic subjects with and without complications (r=0.56; P<0.0001, r=- 0.26; P=0.06).

Ceruloplasmin converts Fe^{2+} into Fe^{3+} and inhibits Fenton reaction. Transferrin is the iron binding protein and it sequesters iron in a safe redox-inactive form by iron oxidizing action of ceruloplasmin. Thus, the positive significant association observed in the present study between CP and TRF in healthy controls proves the importance of ceruloplasmin and transferrin interaction. This prevents free redox-active iron to generate highly reactive hydroxyl radicals that are able to initiate lipid peroxidation.

This association is lost in diabetic subjects but to a greater extent in diabetics with complications as observed in our study. This suggests that there is collaboration between these two extracellular antioxidants. However, the significant negative correlation between transferrin and ceruloplasmin in diabetic subjects suggests impairment of this collaboration.

According to the study by Campenhout et al., the incidence of complications is increased in diabetic patients. The mechanisms underlying this increased risk may in part be attributed to the imbalance between pro-oxidants (free radicals) and antioxidants, which results in oxidative damage to biomolecules.[3]

CONCLUSION

Collaboration of transferrin and ceruloplasmin is impaired in diabetics. This impairment is much more significant in diabetics with complications as observed in the present study. This could result in iron induced oxidative stress. Thus, the results of the present study indicate that a pro-oxidant / oxidant imbalance is involved in oxidative stress and play a role in precipitating Type 2 diabetic complications.

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