

## Cross sectional comparative study of serum free iron in type 2 diabetes mellitus patients with and without retinopathy and nephropathy complications

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### Abstract

**Introduction:** Non protein Bound iron has the capacity to generate reactive oxygen species is called Free iron causing lipid peroxidation and protein oxidation. Free radicals have been implicated in the pathogenesis of diabetes mellitus. Hence the present study was conducted to compare serum levels of free iron, superoxide dismutase, malondialdehyde & glycated hemoglobin between type 2 diabetes mellitus with microvascular complications (retinopathy and nephropathy) and type 2 diabetes mellitus without complications.

**Methods:** Cross sectional observational study included total 121 subjects. 60 were healthy controls, 31 cases with type 2 diabetes mellitus without complications, 30 cases with microvascular complications (15 nephropathy and 15 retinopathy cases). Estimation of serum levels of free iron, superoxide dismutase, malondialdehyde, random plasma glucose, and HbA<sub>1c</sub>, were estimated by spectrophotometric method.

**Results:** Serum levels of random plasma glucose (<0.001), HbA<sub>1c</sub>(<0.001), serum free iron(<0.001), serum malondialdehyde (<0.001) were significantly high and serum superoxide dismutase activity(<0.001) was significantly low in type 2 diabetes mellitus with microvascular complications (retinopathy and nephropathy) when compared to type 2 diabetes mellitus without complications & healthy controls respectively.

**Conclusion:** The study showed higher levels of serum free iron in type 2 diabetes mellitus patients with microvascular complications (retinopathy and nephropathy) when compared to type 2 diabetes mellitus without any complications. Serum free iron can be used as oxidant marker to assess the progression of complications in type 2 diabetes mellitus.

**Keywords:** Free iron, superoxide dismutase, HbA<sub>1c</sub>, Malondialdehyde, Oxidative stress, Antioxidants, Type 2 diabetes mellitus, Retinopathy, Nephropathy.

### Introduction

Diabetes Mellitus is a group of metabolic diseases characterized by a state of chronic hyperglycaemia, resulting from defects in insulin secretion, insulin action, or both. Diabetes is a long-term disease leading to a number of complications –cardiovascular, renal, ocular, and neurological and others.<sup>1</sup> Microvascular complications of diabetes comprises of retinopathy, nephropathy and neuropathy.<sup>2</sup> Chronic hyperglycaemia causes increased glycation of proteins including hemoglobin resulting in the formation of Advanced Glycated End products (AGE).<sup>3</sup>

There is growing evidence that excess generation of highly reactive free radicals due to chronic hyperglycemia causes oxidative stress, which further exacerbates the development and progression of diabetes and its complications.<sup>4</sup> Malondialdehyde (MDA) marker of lipid peroxidation, are shown to increase with an increase in the concentration of plasma glucose, HbA<sub>1c</sub> and duration of diabetes.<sup>5</sup> Superoxide dismutase (SOD), present in cytosol functions mainly as a first order antioxidant enzyme by neutralizing the effect of superoxide anion which is an important precursor for oxidative stress. Oxidative stress leads to decreased levels of serum SOD (S-SOD) and patients with low levels of S-SOD are prone to microvascular complications.<sup>6</sup>

Free iron is any form of iron bound to proteins in such way that, it retains the catalysing power to generate free radicals.<sup>7</sup> Excess free iron has been implicated in the pathogenesis of diabetes and its complications.<sup>8</sup> Previous studies have observed elevated serum iron levels in type 2 diabetic patients, and decreasing iron stores are proposed to ameliorate insulin resistance by reducing the cascade of events responsible for toxicity associated with advanced glycation end products (AGE) and reactive oxygen species (ROS).<sup>9</sup> There is paucity of studies on serum free iron levels in type 2 diabetes patients with retinopathy and nephropathy microvascular complications. Based on these findings, the present study aimed to compare serum levels of free iron, SOD, MDA and glycated hemoglobin between type 2 diabetes mellitus with microvascular complications (retinopathy and nephropathy) with type 2 diabetes mellitus without any complications and healthy individuals.

### Materials and Methods

This study was carried out for duration of one year, in the Department of Biochemistry, JJM Medical College Hospital, Davangere. Ethical clearance was obtained from the institutional ethics committee. Samples were collected from the JJM Medical college

hospital, Davangere after obtaining informed consent from all the study subjects. Study involved 3 groups:

Group-1: Normal, Healthy subjects, both males and females, aged 20-60 years; n = 60

Group-2: Clinically and Biochemically proven cases of type 2 Diabetes mellitus without any complications; both males and females; n =31

Group-3: Clinically and Biochemically proven cases of type 2 diabetes mellitus with microvascular complications like retinopathy and nephropathy; n=30

Retinopathy patients with any of the fundoscopic evidences of microaneurysms, cotton wool spots, retinal haemorrhages, neovascularisation and nephropathy patients who had non-infective persistent proteinuria with evidence of renal failure were selected for the study.

Any subjects with type 1 diabetes mellitus, gestational diabetes mellitus, cancer, on iron supplementation, on oral contraceptives (progestogen) therapy, history of repeated blood transfusion, hemochromatosis, hemosiderosis, thalassemia, chronic infections, alcoholics, and chronic smokers were excluded from the study.

All the blood samples were collected in a plain tube under strict aseptic conditions. Serum was separated after twenty minutes of collection by centrifuging the tube at 3000g for 10 minutes for estimation of serum SOD, serum free iron & serum MDA. 2ml of blood was collected into EDTA containing tube for estimation of HbA<sub>1c</sub>. And 2ml blood

was collected into sodium fluoride/oxalate containing tube for plasma glucose estimation. Random plasma glucose was estimated by GOD-POD (Glucose Oxidase – Peroxidase) method.<sup>10</sup> HbA<sub>1c</sub> was estimated by cation-exchange resin method.<sup>11</sup> Serum free iron was estimated by using Bathophenanthroline-disulphonate method.<sup>7</sup> Bathophenanthroline-disulphonate (BPS) is a chromogenic chelator which is specific for ferrous iron. The ferrous chelator complex of BPS absorbs strongly at 535nm, detection limit less than 1µM in sample of 50ul. The chelator does not liberate iron from haemoglobin or transferrin. It measures iron as such rather than its catalytic effects. The method estimates ferric as well as ferrous iron, in the same sample and discriminates between ferrous and ferric iron. Serum superoxide dismutase was estimated by Marklund and Marklund method<sup>5</sup> and serum malondialdehyde was analyzed by Satoh method.<sup>12</sup>

**Statistical Analysis:** Results are expressed as mean ±SE. Statistical analysis of biochemical parameters was done by one way ANOVA, Post-hoc Tukey's test and unpaired t-test. p value of <0.05 is considered as statistical significance.

## Results

Results of the study are presented in Tables 1, 2 and 3 and Fig. 1.

**Table 1: Comparison of random plasma glucose, HbA<sub>1c</sub>, serum free iron, serum SOD, serum MDA levels in controls, Type 2 DM without complications and Type2 DM with microvascular complications**

	Group 1-Controls (n=60)	Group 2-Type 2DM without complications(n=31)	Group 3-Type 2DM with microvascular complications(n=30)
	mean±SE (95% CI)	mean±SE (95% CI)	mean±SE (95% CI)
Random plasma glucose(mg/dl)***	102.0±2.20 (106.327-97.672)	158.1±10.521 (178.722-137.477)	207.0±12.614 (231.723-182.276)
HbA <sub>1c</sub> (%) ***	6.23±0.124 (6.472-5.987)	8.97±0.357 (9.671-8.268)	10.29±0.482 (11.235-9.344)
Serum free Iron(mmol/L)***	4.54±0.249 (5.028-4.051)	16.44±1.149 (18.692-14.187)	45.58±3.257 (5.965-39.194)
Serum SOD (units/ml)***	12.85±0.342 (13.52-12.179)	6.77±0.469 (7.690-5.849)	3.88±0.259 (4.388-3.371)
Serum MDA (nmol/ml)***	4.19±0.255 (4.691-3.688)	9.63±0.582 (10.772-8.487)	13.27±0.691 (14.48-12.05)

\*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001.

**Legend 1:** Type2 DM with microvascular patients have higher random plasma glucose, HbA<sub>1c</sub>, serum free iron, serum MDA and lower serum SOD compared to Type 2 DM without complications and control groups.

**Table 2: Comparison between groups -controls vs. type 2 DM without complications; controls vs. Type2 DM with microvascular complications and type 2DM without complications vs. type 2 DM with microvascular complications**

	Controls VS. Type2DM without complications	Controls VS. Type2DM with microvascular complications	Type2DM with microvascular complications vs. Type2DM without complications
	mean difference	mean difference	mean difference
Random plasma glucose(mg/dl)	56.1***	105***	48.9***
HbA <sub>1c</sub> (%)	2.73***	4.06***	1.32**
Serum free iron(mmol/L)	11.9***	41.04***	29.14***
Serum SOD (units/ml)	6.08***	8.97***	2.89***
Serum MDA (nmol/ml)	5.44***	9.08***	3.64***

\*p < 0.05; \*p < 0.01; \*\*\*p < 0.001.

**Legend 2:** Analysis done by Post -hoc Tukey's Test.

Mean difference of random plasma glucose, HbA<sub>1c</sub>, serum free iron, Serum SOD, Serum MDA showed significant differences between controls and type 2DM without complications; controls and type 2 DM with microvascular complications and; Type 2 DM without complications compared to Type 2 DM with microvascular complications.

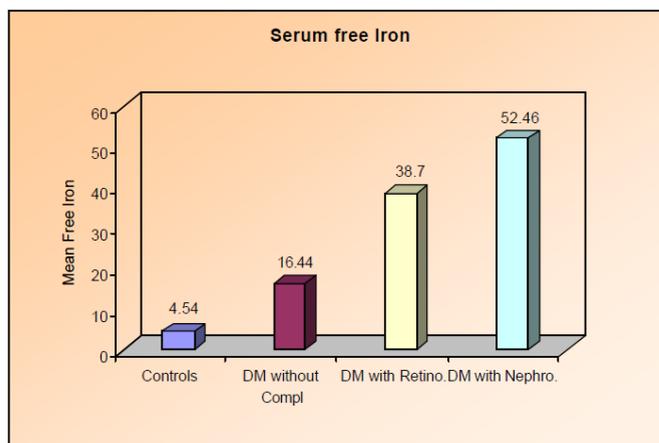
**Table 3: Comparison of random plasma glucose, HbA<sub>1c</sub>, serum free iron, SOD, and MDA in type 2 DM without complications and type 2 DM with nephropathy and type 2 DM with retinopathy**

	Type 2 DM without complications	Type 2 DM with retinopathy	Type 2 DM with nephropathy	Type 2 DM without complications VS Type 2 DM with Retinopathy	Type 2 DM without complications VS Type 2 DM with nephropathy
	(n=31)	(n=15)	(n=15)		
	mean±SE	mean±SE	mean±SE	mean difference	mean difference
Random plasma glucose (mg/dl)	158.1±10.5	184.2±14.41	229.7±19.40	26.1*	71.6**
HbA <sub>1c</sub> (%)	8.97±0.357	9.13±0.475	11.46±0.736	0.16*	2.49**
Serum Free iron(mmol/L)	16.44±1.147	38.70±4.01	52.46±4.59		36.02***
Serum SOD(units/ml)	6.77±0.468	3.37±0.403	4.36±0.286		2.41**
Serum MDA(nmol/ml)	9.63±0.582	11.26±0.754	15.29±0.661		5.66***

\*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001

**Legend 3:** Analysis done by Unpaired 't' test. In type 2 Diabetic nephropathy and in type 2 Diabetic retinopathy patient's serum free iron levels and serum MDA were significantly high when compared to diabetic patients without any complications.

There was no statistically significant difference between type 2 diabetics with retinopathy and type 2 diabetics with nephropathy with respect to serum MDA, serum SOD and serum free iron.



**Fig. 1: Comparison of serum free iron levels between controls, type 2DM without complications, type 2 DM with retinopathy and type 2 DM with nephropathy**

**Legend 1:** Significantly high serum free iron levels seen in type 2 Diabetic patients with microvascular complications when compared to type 2 Diabetic patients without any complications and controls.

## Discussion

Diabetes mellitus in all its heterogeneity has taken the centre stage as one of the ultimate medical challenges.<sup>13</sup> Diabetic vascular complication is a leading cause of end stage renal failure, acquired blindness, neuropathies and accelerated atherosclerosis which are the major cause of morbidity and mortality.<sup>14</sup> Chronic hyperglycaemia is a major initiator of diabetic microvascular complications. It induces various metabolic and hemodynamic derangements, including increased advanced glycation end (AGE) product formation, enhanced production of reactive oxygen species (ROS), activation of protein kinase C (PKC), stimulation of the polyol pathway and the renin angiotensin system (RAS), contributing to the characteristic histopathological changes observed in diabetic vascular complications.<sup>13</sup>

In our study type 2 diabetic patients with retinopathy and nephropathy microvascular complications, showed more significant increase in glycated haemoglobin when compared to diabetic patients without complications. An elevated HbA<sub>1c</sub> level has been shown to be a dominant predictor of the development and progression of microvascular complications in patients with type 2 DM. Patients with type 2 DM with HbA<sub>1c</sub> levels  $\geq 7.5\%$  are shown to have a 2.5 to 5 fold relative risk of developing microvascular complications.<sup>16</sup>

Hyperglycemia in poor glycemic controlled state causes glycation of proteins, especially hemoglobin and transferrin releasing iron in Free State. This makes a vicious cycle of hyperglycemia, glycation of hemoglobin and increase in levels of free iron. Free iron either in ferrous or ferric form, is proposed to react with superoxide or hydrogen peroxide via Fenton or Haber-Weiss reaction to generate highly reactive hydroxyl radical(OH<sup>\*</sup>), oxidizing lipid molecules in the cell membrane, DNA bases and DNA sugars. The

increase in oxidative stress may be associated with risk of developing diabetes mellitus and its associated complications.<sup>8,16,17,18,19</sup>

We observed significantly increased serum free iron levels in type 2 diabetic patients. Patients with retinopathy and nephropathy microvascular complications showed more pronounced increase when compared to those without complications. Findings of our study are in accordance with previous studies.<sup>20,21,22,23,24,25</sup>

Hydroperoxides react with transition metals to form stable aldehydes, such as malondialdehyde (MDA), a marker of lipid peroxidation. The rise in the plasma MDA levels in the later stages of DM reflects increased oxidative stress causing oxidative damage of lipids. Present study observed significant increase in serum MDA in diabetic patients. Serum MDA is shown to increase with progression of diabetes as evident by significantly higher MDA in diabetic patients with complications in comparison to diabetic patients without microvascular complications. These findings are in accordance with the previous studies.<sup>24,26,28</sup>

Oxidative stress causes decreased levels of antioxidants such as SOD, and the proposed reasons are- utilization of SOD for scavenging free radicals, non-enzymatic glycation of SOD causing it to undergo fragmentation, and reduced synthesis of SOD.<sup>5,27</sup> In our study, serum SOD activity was significantly lower in type 2 DM. When compared to type 2 diabetics without complications, diabetics with retinopathy and nephropathy microvascular complications showed significant decreased activity. Our findings are in accordance with previous studies.<sup>8,16,26,27-29</sup>

Limitations of our study are low sample size and the study needs to be extended in type 2 diabetes mellitus with neuropathy cases, to understand complete oxidant-antioxidant balance in microvascular complications.

## Conclusion

Type 2 diabetic patients showed significantly increased levels of random plasma glucose, glycated hemoglobin, serum free iron, serum malondialdehyde compared to healthy controls. The increase was more pronounced in type 2 diabetic retinopathy and nephropathy microvascular complication cases than in type 2 diabetes mellitus without complications. Serum SOD level was significantly decreased in all type 2 DM cases when compared to controls. The SOD levels were more significantly decreased in type 2 DM with microvascular complications including nephropathy and retinopathy cases when compared to type 2 DM without microvascular complications. The present study suggests excess free radical generated, under excess free iron and hyperglycemia can be considered as risk factors for development and progression of diabetic microvascular complications.

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