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Correspondence

Free iron status & insulin resistance in type 2 diabetes mellitus: Analyzing the probable role of a peanut protein

Sir,

South Asian population is known to have an increased predisposition to type 2 diabetes mellitus (T2DM), which turns out to be an important health concern in this Region¹. As per an earlier report about 11.7 per cent people of Kolkata, West Bengal, suffered from T2DM and the prevalence was on a rise². Anaemia is also present in the Region as a significant public health problem, having a prevalence of greater than 40 per cent in South Asia³. However, the actual status of iron remains unclear because of extensive prevalence of haemoglobinopathies, possible genetic mutations contributing to iron overload, indiscriminate over-the-counter use of iron pills and traditional formulations containing undefined concentrations of iron. Higher heme iron intake and increased body iron stores were found to be significantly associated with a greater risk of T2DM^{4,5}. Patients suffering from haemoglobinopathies or undergoing repeated blood transfusions also suffer from secondary iron loading disorder⁶.

Insulin influences the iron uptake and storage in cells by increasing the cell surface transferrin receptors⁷. Whether a patient with diabetes has excess iron due to increased insulin resistance still remains an unanswered question. Insulin resistance has been shown to have an association with chronic kidney diseases^{8,9}. Iron accumulation has been reported in the proximal renal tubules in diabetic nephropathy¹⁰. Iron in its free form^{11,12} *i.e.* in non-transferrin bound form is known to induce oxidation of biomolecules and in the formation of reactive oxygen species. We, therefore, studied the free iron status in patients with T2DM and compared with healthy individuals and to find a suitable biocompatible reagent which can bind the free iron.

A cross-sectional, pilot study was conducted on consecutive patients attending the General Medicine outpatients department of M.R. Bangur Hospital,

Kolkata, India, between August 2012 and February 2014. Fasting blood and urine samples (10 ml each) were collected from 111 patients with T2DM (53.7 ± 12.4 yr, M:F 60 : 51) and 30 healthy controls (75.6 ± 2.5 yr, M:F 8:7). Approval of ethical committee of Institute of Post Graduate Medical Education & Research (IPGMER), Kolkata, was obtained prior to the study.

Those (i) having anaemia, (ii) suffering from any form of haemoglobinopathy, (iii) who were on iron therapy within one year, (iv) having non-diabetic kidney disease, (v) having febrile illness, (vi) having benign prostatic hypertrophy or prostatic cancer, (vii) having urinary tract infection, or (viii) with uncontrolled hypertension, were excluded. The total iron analysis was done by Ferrozine method¹³ and free iron by HPLC¹⁴. Fasting plasma glucose was estimated by glucose oxidase-peroxidase (GOD-POD) method¹⁵, serum insulin by ELISA monobind kit and creatinine analysis was done by a kinetic assay¹⁵ of Jaffe's involving alkaline solution of sodium picrate¹⁶. The plasma creatinine clearance or estimated glomerular filtration rate (eGFR) were estimated as per Cockcroft and Gault formulae in ml/min¹⁷. Insulin resistance was calculated by homeostatic model assessment - insulin resistance (HOMA-IR) formula¹⁸. Urinary microalbumin analysis was done by immunoturbidimetry¹⁹.

Conarachin I was extracted from peanut and purified²⁰, and was used as a complexing agent for free iron. Conarachin I was characterized by molecular weight determination and absorption spectrometry²⁰. A 15 per cent resolving sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel was used for molecular weight determination and confirmation of protein purification from crude peanut protein²⁰. Presence of band at about 18 kD (Figure, lane 1) showed the presence of conarachin I in the

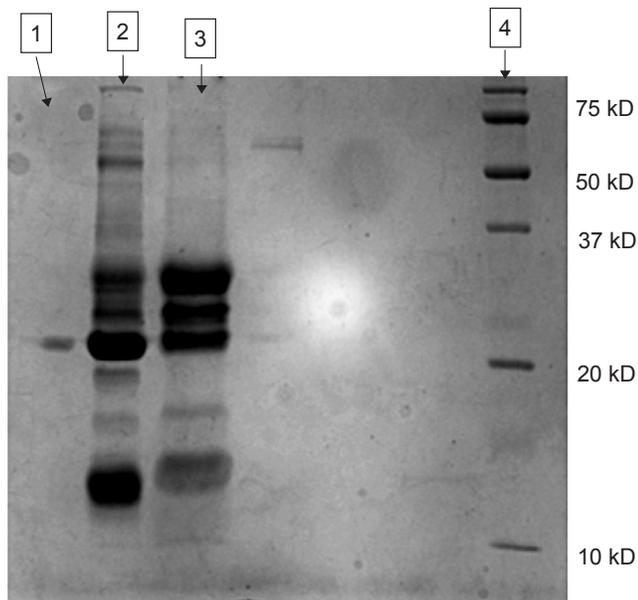


Figure. Purified conarachin I fraction along with its precursors subjected to sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE). Lane 1, pure conarachin I; Lanes 2 and 3, protein part before purification; Lane 4, mol.wt.marker.

purified protein fraction as shown earlier¹⁹. Conarachin I (0.1 ml) was mixed with 0.4 ml of the diluted serum, injected to HPLC column and analyzed to compare the amount of free Fe^{2+} and Fe^{3+} of the same serum samples²⁰.

Qualitative data were grouped and compared by chi square test. Yates correction was done when the cell frequency was below five²¹. Quantitative data were subjected to comparison by their differences in mean by unpaired t test. A HOMA value of 2.4 was taken as comparator as the value exhibited evidence of diabetic kidney disease in an Indian study²². Serum iron value of 150 $\mu\text{g}/\text{dl}$ was taken as upper reference limit for both sexes considering 145 and 160 as upper reference limit (URL) for females and males, respectively as per the kit insert.

Insulin resistance was not found to be significantly associated with total iron. Serum total iron values <150 and >150 $\mu\text{g}/\text{dl}$ were compared with HOMA values <2.4 and >2.4 for both sexes. No association was seen when the means of total free iron were compared with HOMA <2.4 and HOMA >2.4 (15.7 ± 1.64 vs 16.02 ± 2.56 ppm); insulin resistance was not related to $\text{Fe}^{3+}/\text{Fe}^{2+}$ ratio either in males or in females. Obesity, as body mass index (BMI in kg/m^2) >25 ²³ was compared with HOMA, the association was significant at ($P < 0.01$).

Free iron has been known to cause damage by generation of free radicals. As obesity with raised amount of adipose tissue contributes to more serum iron and serum iron is known to contribute to insulin resistance, we decided to multiply the BMI with the ferric-ferrous ratio, and took the product ($\text{BMI} \times \text{Fe}^{3+}/\text{Fe}^{2+}$) as the index value. The median value of the distribution was around 0.37, the mode was 0.3, and HOMA was compared with values above and below 0.3 in females and males.

Iron contributes to insulin resistance (IR) by hindering its action on liver. It also retards the catabolism of insulin leading to hyperinsulinaemia. Insulin contributes to iron overload by generating more transferrin receptors, more ferritin and entry of iron into the fat cells²⁴. So with more number of fat cells, more iron will be present in the body. This will contribute to insulin resistance. A significant difference was obtained in females ($P < 0.001$) but not in males when the index was compared to HOMA of 2.4. Also, concordance of our index values between females and males showed significance ($P < 0.001$) (Table I). Thus, one can assume that the product of free iron ratios and BMI bears a stronger association with IR in females than in males or BMI alone. A mean free iron value of 15.82 ± 1.38 ppm ($n=111$) was found in patients with diabetes, whereas it was 9.28 ± 1.21 ppm in healthy controls ($n=30$) ($P < 0.001$).

The stronger relationship with the index in females may be due to lesser amount of iron stores in them²⁵. Ferric form contributes more to IR possibly as the origin of free iron is supposed to be from transferrin which contains iron in its ferric form. Ferric form may represent the initial active redox state of iron before being reduced to ferrous form. This led us to hypothesize that with more iron in ferric form, there would be more free iron turnover and hence more damage. Though higher amounts of both free and total iron were found amongst patients with microalbuminuria and lower eGFR, the results were not significant in our population which may be due to the presence of lesser amount of nephropathy in patients.

Free iron was considerably reduced in the serum of most patients with diabetes upon addition of conarachin I with a few showing increase whereas serum samples of healthy subjects showed increase in the free iron concentration upon addition of conarachin I. The difference between the two groups was significant ($P < 0.001$) (Table II). Patients with diabetes (mean fasting plasma glucose = 164.77 ± 16.84 mg/dl) had a

Table I. Comparison of sex concordance to index

Patients	HOMA concordant to index	HOMA discordant to index	Total (N)
Females	48	12	60
Males	21	30	51
Total	69	42	111

HOMA >2.4 and index>0.3 or HOMA<2.4 and index <0.3 are taken as positive concordance
HOMA, homeostatic model assessment

Table II. Comparison between effect of conarachin I in serum of patients with and without diabetes

Subject	Decrease in free iron (Fe ³⁺ + Fe ²⁺)	Increase in free iron (Fe ³⁺ + Fe ²⁺)	Total
With diabetes	90	21	111
Without diabetes	0	30	30
Total	90	51	141

P<0.001 (χ^2 test)

higher level of free iron than healthy individuals possibly due to more generation of non-transferrin bound iron (NTBI) by glycation of apotransferrin which does not bind iron avidly²⁶. It was interesting to observe that the higher plasma glucose level in patients with diabetes renders the medium reducing so as the initial Fe³⁺/Fe²⁺ equilibrium in plasma is maintained even after being exposed to aerial oxidation for up to 72 h²⁷. The serum of patients with diabetes having higher mean fasting plasma glucose levels might enhance the complexing ability of conarachin I and reduce the free iron level. In healthy controls with normal glucose levels, addition of the protein conarachin I from outside probably disturbs the equilibrium of transferrin bound iron and releases some bound iron free thus increasing the free iron level. The results indicate that peanut proteins may serve to design therapeutics to reduce excess free iron in patients with diabetes and hence control sugar levels especially in insulin resistant female patients.

In conclusion, free iron was significantly raised in serum of patients with T2DM when compared with healthy subjects. The calculated index of the product of BMI with the ferric-ferrous ratio may be important in assessing insulin resistance, particularly in females or BMI at any level of glycaemia. A peanut protein, conarachin I binds with the free iron in the serum

of patients with diabetes and may contribute to the reduction of insulin resistance. The limitation of the study was that serum ferritin and total iron binding capacity (TIBC) were not measured. As serum ferritin is falsely raised in inflammatory states, thus may contribute as a confounding factor. The use of peanut protein to bind serum free iron is a subject of further investigations in animal models.

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