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Release of iron from haemoglobin—A possible source of free radicals in diabetes mellitus

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Increased blood glucose in diabetes mellitus stimulates nonenzymatic glycosylation of several proteins, including haemoglobin. Although iron is tightly bound to haemoglobin, it is liberated under specific circumstances yielding free reactive iron. Studies with purified haemoglobin from normal individuals and diabetic patients revealed that concentration of free iron was significantly higher in the latter cases and increased progressively with extent of the disease. *In vitro* glycosylation of haemoglobin also led to increase in release of iron from protein. This increase in free iron, acting as a Fenton reagent, might produce free radicals, which, in turn might be causing oxidative stress in diabetes.

Diabetes mellitus is a syndrome characterised by chronic hyperglycemia and disturbance of carbohydrate, fat and protein metabolism associated with absolute or relative deficiencies in insulin secretion and/or insulin action. Free radicals and associated stress have been implicated in eliciting pathological changes of diabetes[1]. However, the possible source of free radicals under such clinical disorder is yet to be understood.

In normoglycemic subjects, a small proportion of haemoglobin is attached to carbohydrate moieties, thus, creating glycosylated or glycated haemoglobin[2]. In diabetes mellitus, proportion of glycosylated haemoglobin increases substantially[3]. Amount of glycosylated haemoglobin reflects the extent as well as management of diabetic condition[4].

Ferrous iron with six coordination states is bound in heme pocket of haemoglobin. Under specific circumstances, iron can be liberated from the heme and ligated to another moiety, probably distal histidine in heme pocket[5]. This iron has been termed as mobile reactive iron[6]. Which can catalyse Haber-Weiss reaction producing free radicals, particularly hydroxyl radicals[7,8] and, in turn, may damage proteins, nucleic acids and lipids[9,10]. Cutler[11] has shown that if desferrioxamine, a specific iron chelator injected repeatedly to diabetic patients, the status of glycosylated haemoglobin and free radical-associated damage (lipid peroxidation and tissue damage) is significantly reduced. This study suggests that iron may have some role in free radical generation and onset of diabetes complications. In this report, we have compared free reactive iron level between normal and diabetic haemoglobin samples.

Sephadex G-100 and ferrozine (monosodium salt) were purchased from Sigma Chemical Co, USA. Other chemicals were of analytical grade and obtained locally. Human blood samples from healthy volunteers (non-smokers and non drug addicts) and patients with diabetes mellitus (from NRS Medical College Hospital, Calcutta) were collected in heparinised conditions.

Haemoglobin was isolated and purified from red blood cells using Sephadex G-100 column chromatography[12]. Concentrations of haemoglobin stock solutions in NaCl (0.15 *M*) were determined at 415 nm (ε=125 $mM^{-1}cm^{-1}$, monomer basis using Hitachi U2000 Spectrophotometer). Solutions of purified haemoglobin were stored in sealed vials at 4°C and were used within two days of preparation. Glycosylated haemoglobin level was determined from whole blood using small ion exchange (Biorex-70) columns purchased from Reddy's laboratory, Hyderabad.

Free iron in whole blood as well as in purified haemoglobin solution was measured according to the method of Panter[13]. Whole blood or haemoglobin solution (250 µl) was taken in Eppendorf tube to which 250 µl of ice cold TCA (20%) was added. The tube was centrifuged at 5000 rpm for 10 min to pellet the precipitated protein. Supernatant (250 µl) was transferred to a test tube

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Table 1—Estimated values o f free iron released from whole blood and haemoglobin in normal and diabetic individuals [Values are mean ± SD. Number of cases has been shown in parenthesis]

Group	Glycosylated haemoglobin (%)	Free iron content	
		Haemoglobin (μg/g of haemoglobin)	Whole blood (μg/g of haemoglobin)
Control	4-7	115±13(12)	144.7±23.2(8)
Diabetic patients	8-10	198±20(5)	204.8±17.58(5)
	11-13	231±14(5)	246±15.2(5)
	16-21	341±38(5)	

Iron release from diabetic patients was significant at 1% as compared to control (*t* test)

containing distilled water (250 μl), iron buffer (2.5 ml) reagent (1.5% hydroxylamine hydrochloride in acetate buffer, *p*H 4.5) and 50 μl of iron colour reagent (0.85% ferrozine in iron buffer reagent) were added to each tube. The colour was developed for 30 min at 37°C and read at 560 nm. Results were expressed as microgram of iron per gram of haemoglobin. A standard curve was made using standard solution of iron in iron buffer reagent.

Iron in whole blood and also in purified haemoglobin solutions from normal individuals and diabetic patients has been determined and shown in Table 1. Individual having 4-7% glycosylated haemoglobin represented control group. Compared to control group, iron release in whole blood and also in purified haemoglobin was found to be significantly higher in diabetes.

In vitro glycosylation of haemoglobin was done according to the method of Cohen and Wu[14]. In this method, isolated haemoglobin (5 mg) was incubated in phosphate buffer saline (*p*H 7.4) containing different amount of glucose (final volume 1 ml). Haemoglobin and glucose solutions were filtered under sterile conditions before incubation. The samples were incubated in stoppered glass vials for 5 days at 25°C. After incubation, the amount of free glucose left in the samples was estimated by glucose oxidase method[15], from which the amount of glucose consumed was determined. Extent of glycosylation in haemoglobin was estimated from the amount of glucose consumed. Initially, the isolated haemoglobin was glycosylated (5%). Incubation with glucose caused further glycosylation. Extent of glycosylation was found to increase with the amount of glucose incubated (Table 2). After incubation, iron was estimated from these haemoglobin solutions. Amount of iron release from haemoglobin was positively correlated with the extent of *in vitro* glycosylation.

From these findings (Tables 1, 2), it is evident that glycosylation of haemoglobin led to iron release and was significant in diabetes mellitus which exhibited increase in the level of glycosylation of protein. One pathophysiological state that can result from increased

Table 2—Amount of free iron release from haemoglobin after *in vitro* glycosylation.
[Values are mean of 3 sets of experiments]

Conc.of glucose (mg/100 ml)	Glucose mg/100 ml)	Total* glycosylation (%)	Free iron (μg/g of haemoglobin)
0	0	5.0	120
90	14.7	7.89	170
180	39.1	12.80	215
270	46.4	14.20	230

*Total glycosylation = Initial glycosylation (5%) + *in vitro* glycosylation.

concentration of free iron is bacterial infection[16]. Such pathophysiological change is often encountered in diabetes and may be correlated with increase in iron release from haemoglobin. High concentration of free iron may be related to its ability to catalyse free radical reactions. Iron, acting as a Fenton reagent, produces free radicals[6,17,18], which can cause considerable damage to cellular constituents[6,9,10,19]. In the case of diabetes mellitus, generation of free radicals and associated oxidative stress has been reported[1,20-22]. However, direct evidence for their accumulation is lacking. Desferrioxamine has been reported to lower the level of fasting glucose, glycosylated haemoglobin as well as free radical-associated pathology in diabetes patients[11]. Present study suggested that increase in iron release from haemoglobin contributed *via* free-radical generation to the development of pathophysiological state in diabetes mellitus. However, further studies are necessary to understand the mechanism of increase in iron release from glycosylated haemoglobin and its possible consequences.

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