Advanced glycosylated end products-mediated activation of polymorphonuclear neutrophils in diabetes mellitus and associated oxidative stress

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Two important consequences of hyperglycemia in diabetes are development of oxidative stress and formation of advanced glycation end products (AGE) which are known to be associated with diabetic complications. Relationship between AGE formation and development of oxidative stress (OS) is yet to be established. In the present study, the involvement of AGE in PMN-mediated ROS generation and the associated OS were investigated in type 2 diabetic mellitus (DM) patients. We assessed OS parameters (serum MDA, FRAP and GSH), PMN oxidative functions (respiratory burst and superoxide production) and total serum AGE in 90 subjects divided equally in three groups — control group, Group I consisting of type 2 diabetic patients without microvascular complications and Group II consisting of type 2 diabetic patients with microvascular complications. PMNs isolated from both groups (I and II) exhibited higher level of respiratory burst (RB) and produced increased amount of superoxide anion as compared to the controls. The increase was more pronounced in diabetes with complications, as compared to those without. Serum malondialdehyde (MDA) level was elevated, whereas glutathione (GSH) and ferric reducing ability of plasma (FRAP) levels were significantly reduced in diabetes as compared to the controls, suggesting the presence of oxidative stress in DM. A positive correlation between PMN oxidative function and OS parameters suggested the involvement of PMN in the development of OS in DM. Serum AGE level was also elevated in diabetic groups as compared to the controls. Further, the positive correlation between serum AGE level and PMN oxidative function suggested the involvement of AGE in increased RB and generation of reactive oxygen species (ROS) by resting diabetic PMN. The results of the study indicate that AGE-PMN interaction possibly upregulates NADPH oxidase, leading to enhanced ROS generation and thus contributes to the pathogenesis in diabetes.

Keywords: Advanced glycosylation end products, Polymorphonuclear neutrophil, Reactive oxygen species, Oxidative stress, Diabetes mellitus

Diabetes is associated with various microvascular and macrovascular complications and oxidative stress (OS) is one of the main causes of diabetic complications1-4. The sources of free radicals leading to OS in diabetes mellitus (DM) are not fully known. Reactive oxygen species (ROS) generation by diabetic polymorphonuclear neutrophil (PMN) is thought to be one of the causes of OS5, although the mechanism leading to enhanced ROS generation by diabetic PMN is not understood. Resting PMN’s do not produce ROS normally, although they are equipped with enzyme for respiratory burst (RB), the NADPH oxidase, which is triggered as a result of phagocytic or inflammatory stimulus6. In diabetes, the possible activator of PMN is yet to be identified. Due to hyperglycemia in diabetes, reducing sugars react non-enzymatically with free amino groups of proteins to form a diverse group of protein-bound moieties known as the advanced glycosylation end products (AGE)7.

The presence of AGE on vascular structures is believed to be important in the pathogenesis of diabetes complications8,9. In a recent study, AGE and MDA are shown to be strongly associated with peripheral artery disease in type 2 DM10. AGE-related pathology might be via induction of OS; however, the role of AGE in contributing to OS is not known. It is also not clear whether AGEs provide the necessary trigger to diabetic PMN, although recently, augmentation of neutrophil RB through the action of AGE has been demonstrated11. Therefore, in the present study, we have estimated blood levels of

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Abbreviations: AGE, advanced glycosylated end products; DM, diabetes mellitus; FRAP, ferric reducing ability of plasma; HBSS, Hank’s balanced salt solution; OS, oxidative stress; PMN, polymorphonuclear neutrophils; RB, respiratory burst; ROS, reactive oxygen species; O2•-, superoxide anion.
AGE, OS parameters (MDA, FRAP, glutathione) and oxidative function of PMN (RB and O$_2^{-}$• generation) to evaluate the contributory role of AGE in PMN-mediated ROS generation, leading to OS in diabetic patients by correlation analysis.

Materials and Methods

A total of 90 subjects, 30 normal healthy controls and 60 diabetic patients attending medical OPD and diabetic clinic at the GTB Hospital, Delhi were used in the study. The diabetic patients were further divided into 2 groups: Group I having type 2 DM without microvascular complications and Group II having type 2 DM with microvascular complication. The subjects were matched for age, sex and body mass index (BMI). Detailed clinical history was taken and general and systematic examinations were done. All patients were >30 yr with type 2 diabetes for at least 1 yr duration, diagnosed according to revised American Diabetic Association Criteria$^{12}$. The groups with microvascular complications included patients with diabetic nephropathy, neuropathy or retinopathy or the presence of one or more simultaneously.

Diabetic nephropathy was defined on the basis of history, clinical examination and presence of microalbuminuria (30-300 mg/d) and/or proteinuria (>0.5 g/d). Diabetic neuropathy was defined on the basis of history and clinical examination in which motor, sensory or reflex examinations were deranged from normal. Diabetic retinopathy was defined on the basis of history and clinical examination of fundus in which there was presence of micro aneurysms, dot and blot hemorrhages or retinal changes such as edema and thickening. Exclusion criteria included no evidence of acute infection, autoimmune disorder, liver disease, malignancy or bronchial asthma. Patients on nitrites, immunosuppressants, antioxidants, corticosteroids and antidepressants were also excluded from the study. The Institutional Ethical Committee approved the protocol of the study and all participants gave informed written consent before being tested.

Determination of plasma glucose and oxidative stress parameters

On the day of the study, fasting blood samples were collected. The levels of plasma glucose, serum MDA, erythrocyte glutathione (GSH) levels were estimated immediately after sample collection. Plasma glucose was estimated by glucose oxidase method$^{13}$. Glycosylated hemoglobin (HbA$1_{c}$) was determined by ion exchange chromatography$^{14}$. Serum MDA was measured as thiobarbituric acid-reactive substance colorimetrically$^{15}$. GSH was estimated by the method previously described$^{16}$. The method is based on the development of a yellow colour when 5,5-dithiobis-2 nitrobenzoic acid (DTNB) is added to sulphahydryl compounds. Ferric reducing ability of plasma (FRAP), as an index of total antioxidant capacity was measured following the reduction of ferric tripyridyltriazine (Fe$^{3+}$-TPTZ) complex$^{17}$.

Neutrophil isolation and assessment of their oxidative function

Neutrophils were isolated from heparinised (100 U/ml) venous blood by dextran (5%) sedimentation, followed by gradient centrifugation over Histopaque1119 (Sigma, USA)$^{18}$. Cell viability was monitored by trypan blue exclusion and the purity was verified by cytology from cytocentrifuged preparations coloured by Geimsa staining. The purity and viability were more than 95%. PMN was suspended in Hank’s balanced salt solution (HBSS) with 0.1% human serum albumin (HSA) at a concentration of 2.5 $\times$ 10$^6$ cells/ml.

Neutrophil oxidative functions were estimated by the method described previously$^{19}$. Assay for respiratory burst (RB) was carried out by nitroblue tetrazolium (NBT) reduction in 96 well microtitre plates. The amount of precipitated formazon (reduced NBT) formed when neutrophils were incubated with NBT was measured directly in the cells present in wells with the aid of an ELISA reader fitted with a 550 nm filter. The results were expressed as respiratory burst unit (RBU)/10$^6$ cells/h. 1 RBU = $\Delta$OD$_{550}$ of 0.1.

Assay of superoxide production by PMN

Superoxide generation (O$_2^{-}$•) by PMN was determined by SOD inhibitable cytochrome $c$ reduction method using a 96-microtitre plate$^{19}$. Briefly, freshly prepared PMN monolayers from different experimental groups in the titer plate, 100 $\mu$l of ferricytochrome (160 $\mu$moles ferricytochrome in HBSS) were added and incubated for 30 min at 37°C. In controls, 100 $\mu$l SOD (300 U/ml) was additionally. At the end of incubation, the absorbance of wells was measured at 550 nm after blanking the control and expressed in nM of O$_2^{-}$• based on the extinction coefficient of cytochrome $c$. $\Delta$E$_{550}$ = 21 $\times$ 10$^3$ M$^{-1}$ cm$^{-1}$. 

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Total AGE in serum was determined by affinity chromatography using agarose column containing covalently bound 3-amino phenyl boronic acid polymer, which formed stable ketomine complex with the glycated proteins, so that glycated proteins were retained in the column and non-glycated proteins passed through the column. After washing, glycated proteins were eluted. Protein estimation of the eluant gave a measure of AGE. The results were expressed as % of total protein.

Statistical analysis

Results were expressed as mean ± SD. The significance of difference was determined using analysis of variance (ANOVA), followed by Tukey’s test for multiple comparisons. Correlation between different variables was tested using Pearson’s correlation analysis. \( P<0.05 \) was considered statistically significant.

Results

The demographic and biochemical data of recruited subjects are shown in Table 1. Group II patients had higher duration of diagnosed diabetes as compared to group I. These diabetic patients had hyperglycemia and increased waist to hip ratio, as compared to controls. Blood urea level was found elevated in group II, although not statistically significant. Serum creatinine level was found significantly raised in group II as compared to controls, possibly as a result of more number of diabetic nephropathy patients.

Oxidative stress parameters

The results of oxidative stress parameters studied are shown in Table 2. While MDA is an indicator of oxidative stress, both GSH and FRAP indicates the level of antioxidant defence status of the body. Serum MDA levels were significantly increased in both group of diabetes, in particular patients with diabetic complications, as compared to controls. Among diabetic groups, significant increase in serum MDA levels was observed in diabetics with complications, compared to those without. Blood GSH and FRAP levels, however, were found decreased in diabetic groups, as compared to controls. Among the diabetic groups, group II patients had significantly lower level of FRAP. These results indicated the increase in OS, on one hand, and concomitant depletion of antioxidant defences on the other in diabetes.

Oxidative functions of PMN

The respiratory burst (RB) and superoxide (\( \text{O}_2^- \)) generation capacity of PMN were estimated as a

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Table 1—Demographic and clinical profile of the study groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Group I</th>
<th>Group II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (Yr)</td>
<td>45.20 ± 6.47</td>
<td>47.33 ± 8.67</td>
<td>48.30 ± 9.89</td>
</tr>
<tr>
<td>Sex (Male/female)</td>
<td>13/17</td>
<td>13/17</td>
<td>13/17</td>
</tr>
<tr>
<td>BMI (kg/m(^2))</td>
<td>25.69 ± 2.94</td>
<td>25.19 ± 2.49</td>
<td>25.31 ± 3.87</td>
</tr>
<tr>
<td>Duration of diabetes (yr)</td>
<td>NA</td>
<td>3.70 ± 1.022</td>
<td>6.50 ± 2.59(^b)</td>
</tr>
<tr>
<td>Waist to hip ratio</td>
<td>0.83 ± 0.04</td>
<td>0.90 ± 0.07(^e)</td>
<td>0.90 ± 0.09(^b)</td>
</tr>
<tr>
<td>Fasting glucose (mg %)</td>
<td>84.47 ± 7.50</td>
<td>113.10 ± 28.99(^a)</td>
<td>166.80 ± 36.93(^{a,b})</td>
</tr>
<tr>
<td>Post-prandial glucose (mg %)</td>
<td>34.51 ± 8.75</td>
<td>202.40 ± 49.73(^a)</td>
<td>255.73 ± 47.16(^{a,b})</td>
</tr>
<tr>
<td>Blood urea (mg %)</td>
<td>28 ± 7.64</td>
<td>34.51 ± 8.75</td>
<td>42.27 ± 11.97</td>
</tr>
<tr>
<td>Serum creatinine (mg %)</td>
<td>1.02 ± 0.14</td>
<td>1.87 ± 0.55</td>
<td>2.92 ± 0.63(^b)</td>
</tr>
</tbody>
</table>

\(^a\)Significant difference compared with controls (\( P<0.05 \)); \(^b\)Significant difference compared with Group I. (\( P<0.05 \))

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Table 2—Oxidative stress parameters (MDA, GSH and FRAP), glycosylated hemoglobin (HbA1\(_c\)) and glycosylated protein (AGE) levels in serum

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Group I</th>
<th>Group II</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum MDA (nM/ml)</td>
<td>2.27 ± 0.36</td>
<td>3.16 ± 0.53(^a)</td>
<td>3.75 ± 0.54(^{b,c})</td>
</tr>
<tr>
<td>GSH (mg/g Hb)</td>
<td>3.73 ± 0.39</td>
<td>2.42 ± 0.46(^e)</td>
<td>2.24 ± 0.39(^a)</td>
</tr>
<tr>
<td>FRAP (mM/l)</td>
<td>1015.03 ± 96.68</td>
<td>612.84 ± 95.90(^a)</td>
<td>517.80 ± 73.56(^{a,b})</td>
</tr>
<tr>
<td>HbA1(_c) (g %)</td>
<td>5.77 ± 0.516</td>
<td>8.19 ± 0.529(^e)</td>
<td>9.80 ± 1.00(^{d,d})</td>
</tr>
<tr>
<td>Serum AGE (%)</td>
<td>11.71 ± 0.97</td>
<td>17.86 ± 1.21(^c)</td>
<td>21.79 ± 1.23(^d)</td>
</tr>
</tbody>
</table>

\(^a\)Significant difference compared with controls (\( P<0.001 \)); \(^b\)Significant difference compared with Group I. (\( P<0.001 \)); \(^c\)Significant difference compared with controls (\( P<0.05 \)); \(^d\)Significant difference compared with Group I. (\( P<0.05 \))
measure of PMN oxidative function and the results are presented in Figs 1 and 2. RB of resting PMN in normal subjects was found to be very low (1.69 ± 0.72 RBU/10⁶ cells/h), whereas in diabetics, there was 5-6 fold increase in RB and this difference was found statistically significant (P<0.001). \( \text{O}_2^- \) generation in normal subjects was also very low, but a 3-fold increase was observed by resting PMN’s in diabetic patients. No significant difference in \( \text{O}_2^- \) generation by PMN was observed between the two diabetic groups.

**Estimation of glycosylated proteins**

To measure glycosylation of blood proteins due to hyperglycemia in diabetes, we estimated blood HbA1c and total serum glycosylated protein (AGE) and the results are shown in Table 2. In controls, 5.7% of Hb was glycosylated, whereas in diabetes, it ranged between 8-10%. This increase was observed statistically significant. Among diabetic groups, HbA1c level was significantly increased in diabetics with microvascular complications. The 11% glycosylation of serum proteins was observed in controls, as compared to 18% and 21% in diabetics without and with complications, respectively. The results indicated higher degree of glycosylation of plasma proteins, as compared to Hb.

**Correlation studies**

MDA showed a positive correlation with RB and superoxide generation by PMN, indicating PMN oxidative function as a contributory factor towards OS

![Fig. 1 — PMN respiratory burst (RB) of control and diabetic subjects [RB was measured by modified NBT reduction assay using 96 well microtitreplate. Results were expressed as RBU/10⁶cells/h. 1 RBU = \( \Delta\text{OD}_{550} \) of 0.1. Values expressed as mean ± SD (n = 30). \(^a\)Significant difference compared with controls (P<0.001), \(^b\) Significant difference compared with Group I. (P<0.001)]](image1)

![Fig. 2 — Superoxide production by PMN of control and diabetic subjects Superoxide was measured spectrophotometrically by SOD inhibitable reduction of ferricytochrome c by PMN at 550 nm using a microtitreplate reader. Superoxide generation was expressed as nM/10⁶cells/h based on the extinction coefficient of cytochrome c, \( \Delta E_{550}=21\times10^3 \text{ M}^{-1} \text{ cm}^{-1} \). Values were expressed as mean ± SD (n = 30) \(^a\)Significant difference compared with controls (P<0.001)](image2)

**Table 3—Correlation between serum MDA level and serum AGE with PMN’s oxidative functions in different study groups**

[Values expressed as Pearson’s correlation co-efficient (r value), n = 30]

<table>
<thead>
<tr>
<th>Independent variable → MDA</th>
<th>Control</th>
<th>Group I</th>
<th>Group II</th>
</tr>
</thead>
<tbody>
<tr>
<td>RB</td>
<td>0.294</td>
<td>0.404(^a)</td>
<td>0.649(^c)</td>
</tr>
<tr>
<td>( \text{O}_2^- )</td>
<td>0.095</td>
<td>0.626(^c)</td>
<td>0.589(^b)</td>
</tr>
</tbody>
</table>

Correlation was significant at \( P<0.05\), \( P<0.005\), and \( P<0.001\) level. A significant positive correlation was viewed between MDA level and RB/superoxide production by resting PMN in diabetics without microvascular complications. Also significant positive correlation was noted between serum AGE and \( \text{O}_2^- \) production by PMN’s in diabetics with complications (Table 3). A significant positive correlation was demonstrated between fasting blood sugar with AGE and HbA1c (\( r = 0.732 \) and \( r = 0.738 \) respectively), indicating that the degree of glycosylation increases with the hyperglycemia.

The total serum AGE and HbA1c were analyzed separately for correlation with OS parameters and pearson’s correlation coefficient values are presented in Table 4. Serum AGE as well as HbA1c showed a significant positive correlation with MDA levels in both group of diabetics and a concomitant negative
correlation between GSH and FRAP levels. Significant positive correlation was also observed between serum AGE and superoxide production by PMN in diabetics with complications (Table 3) indicating association between AGE-mediated ROS release from PMN and diabetic complications, showing contributory effect of AGE towards OS. Thus, the above results suggested relationship between OS, PMN oxidative function and serum AGE level in diabetes.

Discussion

We have evaluated oxidative stress and antioxidant status in patients of type 2 DM with and without microvascular complications. We found significantly higher MDA levels in both the diabetic groups, which is in agreement with previous studies indicating an increased production of free radicals, resulting in higher MDA levels in DM. Low antioxidant status in DM is also indicated by significantly lower erythrocyte GSH and FRAP levels in DM than controls. Earlier, an 11% reduction in GSH content in DM (with and without complications) was reported. Enhanced lipid peroxide levels, as evidenced by high MDA level, can reduce GSH content because glutathione peroxidase catalyzes the oxidation of GSH at the expense of H2O2. Also, in DM, GSH is largely consumed because of the regeneration of vitamin C, which is extensively oxidized in diabetic patients. The mechanism leading to OS and lowered antioxidant defense mechanism in DM is not understood. One of the contributing factors is the production of free radicals by PMN’s through respiratory burst.

We evaluated PMN oxidative function by measuring RB and O2•− generation capacity. Normally, PMNs are in a resting state, but get activated when they encounter any stimulus (phagocytic or chemical) causing RB and generation of ROS. In the present study, control PMNs showed low degree of RB and O2•− generation, while from diabetic patients exhibit 3-4 fold increase in RB and a significant increase in O2•− generation (P < 0.001). These results suggest that some serum factors in diabetics are responsible for enhanced RB and ROS generation in PMNs. Possibly, PMNs in diabetics are in a state of activation, causing RB due to some unknown factors associated with DM. This unwarranted RB by diabetic PMN may be partly responsible for OS in DM.

This view is supported by the fact that O2•− production by resting PMN also shows a significant positive correlation with MDA levels in both diabetic groups (r = 0.626 and 0.589 in Group I and II respectively). Therefore, RB and ROS generation by diabetic PMN is responsible for OS observed in DM. The mechanism leading to PMN activation and subsequent OS in DM is yet to be understood. Some authors have suggested the involvement of diabetic plasma factor for PMN dysfunction. Due to persistent hyperglycemia in DM, proteins undergo non-enzymatic glycosylation leading to formation of AGE. To study whether these glycosylated proteins in plasma are associated with activation of PMN, we have determined the levels of glycosylated proteins in plasma are associated with activation of PMN, we have determined the levels of glycosylated proteins in serum of diabetic patients. Total glycosylated proteins are found significantly increased in both the groups of diabetes. The glycosylated proteins levels (serum AGE, HbA1c) are also significantly positively correlated with rise in glucose levels. Correlation analysis also indicates association of serum AGE with increase in OS and depletion of antioxidant defense sources. The results suggest a contributory role of AGE in the OS observed in diabetics. Significant positive correlation between serum AGE and PMN oxidative function suggest involvement of serum AGE with ROS
production by PMN. Earlier, AGE-PMN interaction was shown to result in enhanced RB. It is not clearly understood, how AGE promote free radical generation from PMN; one of the mechanisms could be the upregulation of neutrophil NADPH oxidase by AGE. It is suggested that AGEs stimulate neutrophil RB by the activation of cytosolic phospholipase A\(_2\) and generation of arachidonic acid (AA). AGEs are also known to induce AA, which in turn may upregulate NADPH oxidase through its p47\(_{phox}\) subunit, as AA increases the functional efficiency of p47\(_{phox}\).  

In summary, our results show that AGEs play a significant role in the development of oxidative stress by acting as an activator of diabetic PMN’s causing increase in respiratory burst and ROS generation.

References