Polymorphonuclear leukocyte function in type-2 diabetes mellitus patients and its correlation with glycaemic control

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ABSTRACT
Infection is an important cause of morbidity and mortality in diabetic patients. Chronic hyperglycaemia impairs host defense mechanism such as cell mediated immunity, polymorphonuclear leukocyte (PMNL) function, antibody formation etc. PMNL serves as bodies first line of defense against various infections. The present study was undertaken to establish a correlation between impaired PMNL function, blood glucose levels and its improvement with good glycaemic control with glibenclamide and glimepiride, with special reference to parameters such as respiratory burst and O₂⁻ and H₂O₂ production by diabetic neutrophils.

Keywords: Type 2 diabetes mellitus (DM), PMNL function, respiratory burst, O₂⁻ and H₂O₂ production, glycaemic control, glibenclamide and glimepiride.

INTRODUCTION
Once regarded as a single disease entity diabetes is now seen as a heterogeneous group of diseases, characterized by state of chronic hyperglycaemia resulting from a diversity of aetiologies, environment and genetic, acting jointly. The underlying cause of diabetes is the defective production or action of insulin, hormone that controls glucose, fat and amino acid metabolism. Type-2 diabetes mellitus (DM) is a more prevalent form of diabetes and its prevalence is increasing world wide due to change in life style. Characteristically, type-2 diabetes is a long-term disease with variable clinical manifestations and progression. Chronic hyperglycaemia from whatever cause leads to a number of complications such as cardiovascular, renal, neurological, ocular and intercurrent infections.

Infection is an important cause of morbidity and mortality in diabetic patients. About 16.0% of patients present with infection at onset. Infection is responsible for 6.0% of diabetic deaths, is one of the precipitating cause of ketoacidosis contributing to 25.0% deaths associated with it. Infection is severe, prolonged and difficult to treat in diabetic subjects.2,3 Chronic hyperglycaemia impairs host defense mechanism, such as, cell mediated immunity, polymorphonuclear leukocyte (PMNL) function, antibody formation, decreased complement levels, along with various factors such as drug induced hypoglycaemia, ketoacidosis, decreased blood supply, capillary basement thickening, neuropathy, malnutrition and dehydration.4 PMNL serve as bodies first line of defense against invading microorganism and an abnormality of PMNL function may predispose to increased susceptibility to various infections in diabetics. Chronic hyperglycaemia can impair a wide range of functions in neutrophils including chemotaxis, adherence, phagocytosis, intracellular killing, superoxide anion O₂⁻, hydrogen peroxide H₂O₂ and sorbitol production.4 The importance of several oxygen derived free radicals, O₂⁻, H₂O₂ and hypochlorite (OCI) has been well recognized in the oxygen dependent bactericidal mechanisms. These oxygen derived free radicals are produced during burst of oxidative metabolism called the "respiratory burst". A major portion of the increased oxygen uptake is used to form O₂⁻ which is weakly antimicrobial in itself.5 However, it is a major intermediate in the formation of H₂O₂,6 which in turn participates in the well established hydrogen peroxide-myeloperoxidase- hypochlorite (H₂O₂ – MPO - OCI) system having the most powerful oxidative activity.7 Several reports are available regarding decreased O₂⁻ production8-10 and higher levels of O₂⁻ release by stimulated diabetic neutrophil.11,12 Various other researchers have demonstrated that H₂O₂ production by diabetic neutrophil is reduced on stimulation,13-15 in contrast, reports of increased H₂O₂ production by stimulated diabetic neutrophil is also available.16 However, evidence of no significant difference in H₂O₂ production by stimulated diabetic and control neutrophils has been documented.9 Since 1960's oral hypoglycaemic agents have been used in type-2 DM to achieve glycaemic control. Most commonly used agents are oral sulfonylureas (SU) because of their better tolerability and side effect profile. Second generation SU's are most frequently used as they are dose to dose more potent and have lesser side effect as compared to first generation agents.17 Glibenclamide is generally preferred second generation SU because of its low cost, high potency and longer duration of action.18 But its use is limited by its ability to
induce frequent hypoglycemia, which depresses the mononuclear cells and PMNL function. Glimepiride, a new SU on the other hand, has better glycaemic control and lower incidence of hypoglycaemia due to lower insulin secretion even at high glucose concentration because of its greater insulin sensitizing effect in muscle tissue as compared to glibenclamide.

Many workers have shown a correlation between impaired PMNL function, blood glucose levels and its improvement with good glycaemic control. However to the best of our knowledge there has been not many studies delineating the effect of glibenclamide and glimepiride on PMNL function especially with reference to parameters such as respiratory burst, $O_2^-$ and $H_2O_2$ production. Hence the present study was designed to assess and compare the glycaemic control achieved with glibenclamide and glimepiride and its relationship with PMNL function.

MATERIALS AND METHODS
The study was carried out in the departments of Pharmacology and Biochemistry, University College of Medical Sciences (U. C. M. S.) and GTB Hospital, Delhi, India. All the patients were recruited from the diabetic clinic and medical out patient department of GTB Hospital, Delhi, after obtaining their written informed consent. Sixty, age (minimum 35 years and maximum 70 years) and sex matched patients of type 2 DM were divided into two groups (A and B) each consisting of 30 patients. In group A, Glibenclamide (2.5-15 mg) and in group B, Glimepiride (1-4 mg) was administered.

**Inclusion criteria:** All patient above 30 years of age diagnosed as type-2 DM by WHO criteria (WHO TRS 1985):

a) 1. Fasting plasma glucose ≥ 140 mg/dl
2. 2 h post-prandial plasma glucose ≥ 200 mg/dl

b) Patients who did not achieve their glycaemic targets after 4 weeks of diabetic diet.

**Exclusion criteria:** Patients with any history of acute or chronic infection, autoimmune disorder, malignancy, liver disease, bronchial asthma and patients on nitrates, immunosuppressants, corticosteroids and antioxidants were excluded from the study; pregnant women and patient showing raised CRP, ESR and leukocytosis were also excluded. Patient's name, age, sex, history and examination findings were systematically recorded in predesigned proforma. A detailed clinical history was obtained and thorough physical examination performed to rule out any disease other than DM. Patients who were already on oral hypoglycaemic agents, their medication was stopped for the period of two weeks. Newly diagnosed diabetic patients were given trial of diabetic diet for 4 weeks prior to drug treatment. All subjects underwent the following routine and special investigations:

a. **Routine investigations:**
   1. Haematological: Hb, TLC, DLC and ESR
   2. Biochemical: Glucose (fasting and postprandial), Kidney function test, Liver function test, Lipid profile, Serum total protein and albumin.
   3. Microbiological: CRP, urine (routine and microscopic)
   4. Chest skiagram PA view
   5. ECG
   6. Fundus examination
b. **Special investigations**
   1. Assay of respiratory burst by Nitrobluetetrazolium (NBT) reduction
   2. Superoxide (O$_2^-$) anion production
   3. Hydrogen peroxide (H$_2$O$_2$) production
   4. Glycosylated haemoglobin (HbA$_1C$)

The patients were followed up for 6-8 weeks. At the end of the study routine investigations such as: blood sugar fasting, and postprandial (FBS, PPBS), lipid profile and all special investigations were repeated.

**METHODOLOGY OF SPEICAL INVESTIGATIONS**
**Isolation of neutrophil:** Neutrophils were isolated from heparinised venous blood after erythrocyte gravity sedimentation in 6.0% dextran followed by ficoll-paque centrifugation and hypotonic lysis and cell viability was assessed by trypan blue exclusion.
Assay of respiratory burst by NBT reduction: The assay represents a modification of quantitative NBT reduction assay for use in microplates. The amount of reduced NBT is measured directly in the cells present in the wells, with the aid of an ELISA reader at 550 nm filter.

Estimation of superoxide anion (O$_2^-$): The assay is based on reduction of ferricytochrome C by O$_2^-$. The specificity of reduction being controlled by its inhibition by superoxide dismutase (SOD).

Estimation of Hydrogen Peroxide (H$_2$O$_2$) Production: The assay is based on horse-radish peroxidase (HRPO) dependent oxidation of phenol red by H$_2$O$_2$ leading to formation of compound that, at an alkaline pH, exhibits increased absorbance at 600 nm.

Estimation of glycosylated haemoglobin (HbA$_1$C): Using haemolysed preparation of whole blood, total glycated haemoglobin was determined by using affinity resin that has affinity for glucose molecules attached to haemoglobin. The bound glycated haemoglobin was eluted and measured at 415 nm.

STATISTICAL ANALYSIS
The significance was obtained by using repeated measure analysis of variance (F-test) and multiple comparisons. Significance was done by Tukey's test at 5.0% level of significance.

RESULTS
The routine haematological, biochemical and microbiological investigations of the patients of both the treatment groups were within the normal reference range. No significant change was observed in body weight, body mass index (BMI), HDL, VLDL and triglyceride levels in both the groups. A significant reduction (p< 0.001) in both FBS and PPBS levels was observed after treatment with Glibenclamide (214.07± 86.10 to 131.53 ± 45.25) and glimepiride (229.33 ± 88.22 to 151.90 ± 68.75). Similarly, HbA$_1$C was significantly reduced in both the groups after treatment (P< 0.001). However, changes in overall glycaemic control (as measured by FBS, PPBS and HbA$_1$C) was found to be similar between the two groups. Effect of glibenclamide and glimepiride on respiratory burst (RB) as measured by NBT reduction assay before and after treatment in both the groups showed improved post-treatment RB in both the groups (P< 0.001) (Table-1). However, no significant difference was seen between the groups (P= .399).

The changes in resting and stimulated superoxide (O$_2^-$) production by glibenclamide and glimepiride as assayed by Ferricytochrome-C reduction assay exhibited a significant increase in O$_2^-$ production by resting PMNL after treatment in glibenclamide group, whereas in the glimepiride group, no significant change was seen before and after treatment; on the contrary, O$_2^-$ production by stimulated PMNL was found to be increased significantly in both the groups. (P< 0.001) (Table-2, 3).

A similar increasing trend though not statistically significant, was observed in H$_2$O$_2$ production in resting PMNL in both the groups, while in the stimulated PMNL, H$_2$O$_2$ production improved significantly after treatment in both the groups (P< 0.005) (Table-4, 5)

Studies to evaluate any correlation between impaired PMNL functions, blood glucose levels (FBS, PPBS, HbA$_1$C) and its possible improvement with glycaemic control by glibenclamide or glimepiride did not show any significant correlation both before and after treatment with these drugs.

DISCUSSION
Diabetic state renders the host more susceptible to infection and infection is found to be more common in poorly controlled diabetics. The defect in host defense leading to infection is not understood. PMNL function was believed to be at least partly responsible for this as they play an important role in the host defense mediated through phagocytosis, respiratory burst and oxygen dependent bactericidal activity. A large number of studies are available that clearly indicate PMNL dysfunction in diabetic state. Sulphonylureas (SU) are generally preferred oral hypoglycaemic agents because of their high potency and lesser side effects. Second generation compounds are most commonly used, as they are dose to dose more potent and have fewer side effects. Glibenclamide is the most commonly used and highly potent drug. Glimepiride on the other hand is a relatively new SU, which achieves same pharmacodynamic profile as other SUs at lower insulin levels and thus producing less hypoglycaemic episodes as compared to glibenclamide. Many workers have shown a correlation between glycaemic control and improvement of PMNL function. Hence, this study was designed to assess and compare the effect of glibenclamide and glimepiride on blood sugars (FBS and PPBS) and HbA$_1$C, secondly to assess the PMNL function in both the groups; thirdly to see if any correlation exists between the PMNL function and glycaemic control achieved by glibenclamide and glimepiride.
Firstly, we evaluated the efficacy and safety of glibenclamide and glimepiride as glycemic control agents by assessing their role in lowering FBS and PPBS and HbA1c. We observed that both the drugs lowered the FBS significantly after the treatment. But no significant difference in degree of reduction of blood sugar (fasting) was seen between the two groups. PPBS was also reduced significantly after the treatment with both the drugs as compared to the pretreatment values. Significant reduction in HbA1c was seen after treatment with both the drugs. Similar observation was earlier reported showing therapeutic equivalence of these two drugs. No appreciable increase or decrease was seen in LDL, HDL, TG and total cholesterol levels after treatment in the two groups. Comparison between the two groups did not show any significant differences. Similar observations has already been reported earlier.

Next we studied the oxygen dependent bactericidal mechanisms as measured by respiratory burst, superoxide, and hydrogen peroxide production. Respiratory burst as measured by NBT reduction assay is a measure of both the phagocytic capacity and oxidative burst of neutrophils. PMNL respiratory burst capacity was observed to be about one-third in both groups of patients as compared to the normal value in our laboratory at the time of enrollment into the study. Respiratory burst improved significantly in both groups and the degree of increase in respiratory burst was not significantly different in the two groups. (Table-1)

Production of superoxide and hydrogen peroxide by PMNL was estimated to assess the oxygen dependent bactericidal mechanism. PMNL on their encounter with bacteria get activated and as a result their membrane bound NADPH oxidase gets stimulated and results in reactive oxygen intermediate production, resulting in killing of bacteria. In this study, resting superoxide production by PMNL before treatment with the drug was found to be increased. The resting superoxide production increased further significantly in glibenclamide group but no significant difference was seen in glimepiride group (Table-2). O$_2^-$ production by stimulated PMNL was also increased significantly in both the groups (Table-3). The rise in post treatment hydrogen peroxide production by resting PMNL was significantly higher in glimepiride group as compared to patient taking glibenclamide (Table-4). Increase in superoxide production and hydrogen peroxide production by resting PMNL is an indicator of activation of PMNL. Hyperglycaemia possibly cause glycosylation of the PMNL proteins, making them activated although the exact mechanism is not known. This activated condition leads to reactive oxygen species which causes oxidative damage and is believed to be associated with complications of DM. Marginal increase of resting reactive oxygen species production in our study is possible associated with the drug action. Reactive oxygen species production by stimulated PMNL is an indicator of its bactericidal activity. It is well known that there is less production of O$_2^-$ from PMNL in diabetic patients. Treatment with SUs (glibenclamide and glimepiride) for 6-8 weeks improved O$_2^-$ production by stimulated PMNL in both the groups as compared to their pretreatment values. Similarly H$_2$O$_2$ production by stimulated PMNL improved significantly on treatment with glibenclamide and glimepiride. Our observation also corroborates similar findings of improvement of PMNL function with glycaemic control and of bactericidal property of neutrophil. Since phagocytic and bactericidal activity of neutrophil is associated with respiratory burst and reactive oxygen species production, therefore, results of this study provides a mechanism to higher phagocytic and bactericidal activity that is achieved on attaining glycaemic control by use of SUs.

Finally, we aimed at evaluating any correlation between the degree of increase in respiratory burst, O$_2^-$ and H$_2$O$_2$ production by stimulated PMNL (responsible for controlling infection) with that of decrease in the level of FBS and PPBS and HbA1c, obtained as a result of treatment with either glibenclamide or glimepiride. With these pretreatment and post treatment values, attempts were made to see if any correlation exists between them. Though there was a definite increase in respiratory burst mediated by PMNL of patients on treatment with glibenclamide and glimepiride, we could not find any correlation with increase in respiratory burst, O$_2$ and H$_2$O$_2$ production with that of change in blood glucose levels. This is probably due to the fact that patients recruited were having different magnitude of glycaemia control and varied duration of diabetes.

In conclusion, our studies showed that Glibenelamide and glimepiride have a similar efficacy in lowering blood sugar and glycosylated haemoglobin levels. This improves oxygen dependent mechanism of PMNL and thus reduce the number of infectious episodes in type- 2 DM.
Table-1: Effect of glibenclamide and glimepiride on respiratory burst

<table>
<thead>
<tr>
<th>Group</th>
<th>Respiratory burst OD&lt;sub&gt;550&lt;/sub&gt;/10&lt;sup&gt;6&lt;/sup&gt; cell</th>
<th>Change after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pretreatment</td>
<td>Post treatment</td>
</tr>
<tr>
<td>Group A</td>
<td>0.48 ± 0.10</td>
<td>0.79 ± 0.25*</td>
</tr>
<tr>
<td>(Glibenclamide)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group B</td>
<td>0.46 ± 0.11</td>
<td>0.72 ± 0.15 *</td>
</tr>
<tr>
<td>(Glimepiride)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Pretreatment vs Post treatment P < 0.001, ** Change in respiratory burst P = .399 NS

Table-2: Effect of glibenclamide and glimepiride on super oxide (O<sub>2</sub>−) production by resting PMNL

<table>
<thead>
<tr>
<th>Group</th>
<th>O&lt;sub&gt;2&lt;/sub&gt;− production nmol/10&lt;sup&gt;6&lt;/sup&gt; cell/30 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pretreatment</td>
</tr>
<tr>
<td>Group A</td>
<td>2.24 ± 0.98</td>
</tr>
<tr>
<td>(Glibenclamide)</td>
<td></td>
</tr>
<tr>
<td>Group B</td>
<td>3.76 ± 1.33**</td>
</tr>
<tr>
<td>(Glimepiride)</td>
<td></td>
</tr>
</tbody>
</table>

*<sup>a</sup> Pretreatment vs Post treatment P < 0.001, *<sup>b</sup> Pretreatment vs Post treatment P = 0.013 NS, ** Between the two groups P < 0.001

Table-3: Effect of glibenclamide and glimepiride on super oxide production by stimulated PMNL

<table>
<thead>
<tr>
<th>Group</th>
<th>O&lt;sub&gt;2&lt;/sub&gt;− production nmol/10&lt;sup&gt;6&lt;/sup&gt; cell/30 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pretreatment</td>
</tr>
<tr>
<td>Group A</td>
<td>7.75 ± 1.96</td>
</tr>
<tr>
<td>(Glibenclamide)</td>
<td></td>
</tr>
<tr>
<td>Group B</td>
<td>7.11 ± 1.55**</td>
</tr>
<tr>
<td>(Glimepiride)</td>
<td></td>
</tr>
</tbody>
</table>

* Pretreatment vs Post treatment P < 0.001, ** Between the groups P= 0.013 NS
Table – 4. Effect of glibenclamide and glimepiride on hydrogen peroxide production by resting PMNL.

<table>
<thead>
<tr>
<th>Group</th>
<th>Pretreatment</th>
<th>Post treatment</th>
<th>*a Pretreatment vs Post treatment P = 0.160 NS, *b Between the two groups P &lt; 0.045</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A (Glibenclamide)</td>
<td>4.11 ± 1.57</td>
<td>4.23 ± 1.29 *a</td>
<td></td>
</tr>
<tr>
<td>Group B (Glimepiride)</td>
<td>4.39 ± 1.17</td>
<td>4.93 ± 1.08 *a *b</td>
<td></td>
</tr>
</tbody>
</table>

Table-5: Effect of glibenclamide and glimepiride on hydrogen peroxide production by stimulated PMNL

<table>
<thead>
<tr>
<th>Group</th>
<th>Pretreatment</th>
<th>Post treatment</th>
<th>* Pretreatment vs Post treatment P &lt; 0.001</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A (Glibenclamide)</td>
<td>10.96 ± 2.45</td>
<td>11.83 ± 2.04 *</td>
<td></td>
</tr>
<tr>
<td>Group B (Glimepiride)</td>
<td>8.97 ± 1.77</td>
<td>11.21 ± 1.12 *</td>
<td></td>
</tr>
</tbody>
</table>

* Pretreatment vs Post treatment P < 0.001