Inhibitory effect of Glimepiride on nicotinamide-streptozotocin induced nuclear damages and sperm abnormality in diabetic Wistar rats

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The generation of reactive oxygen species in diabetes is considered to be the major cause for the mutation related defects such as cancer, infertility etc. Glimepiride (Gmp) is a third generation antidiabetic sulphonylurea known to possess the antioxidant effect in streptozotocin (STZ) induced diabetes. In this study, the anti-mutagenic activity of Gmp (0.175, 17.5 and 175 mg/kg, po daily for 4 weeks) was evaluated against the nicotinamide (NA-230 mg/kg) and STZ (65 mg/kg) induced somatic and germinal cells defect using bone marrow micronucleus (MN) test and sperm abnormality test respectively in male Wistar rats. Administration of Gmp at 175 mg prevented the NA-STZ induced increased frequency of MN in polychromatic and normochromatic erythrocytes. The treatment with Gmp also decreased the sperm shape abnormality and enhanced the sperm count besides improving the antioxidant status in the diabetic rats. However, the other doses of Gmp (0.175 and 17.5 mg) did not produce significant change in the MN frequency and sperm abnormality although Gmp at 17.5 mg showed significant antidiabetic effect in the hyperglycemic animals. The results indicated that Gmp inhibited the NA-STZ mediated changes in the MN frequency and sperm abnormality and enhanced the antioxidant defense. The observations suggest that the antioxidant property of Gmp could have contributed for its ability to decrease the NA-STZ mediated defects in somatic and germinal cells.

Keywords: Antioxidant, Glimepiride, Micronucleus, Sperm abnormality

Increasing evidence in both experimental and clinical studies suggests that there is a close link between hyperglycemia, oxidative stress and diabetic complications. High blood sugar level determines overproduction of reactive oxygen species (ROS) by the mitochondria electron transport chain. ROS virtually damages all cellular components, leading to DNA and protein modification and lipid peroxidation¹. In addition, diabetic patients are reported to have reduced antioxidant defences, such as superoxide dismutase and decreased levels of antioxidants. In consequence, the diabetic patients suffer from an increased risk of oxidative stress-related diseases not only in the present generation but can also transmit the nuclear defects to their progeny².

Experimental type-2 diabetes (T2DM) in rats can be induced by several methods. According to Masiello et al³, administration of streptozotocin (STZ) and nicotinamide (NA) produced moderate hyperglycemia which has clinical similarities especially with respect to the insulin response to the glucose. The partial protection of β-cells by the NA against the cytotoxic action of the STZ is reported to play the major role in the development of non-insulin dependent diabetes mellitus (NIDDM) condition in rats³.

Mutational studies assume importance since the alterations in the DNA can lead to several inheritable diseases. Among the battery of tests available, micronucleus test, sperm abnormality, chromosomal aberration assays are commonly employed to evaluate the drug/disease induced mutations⁴. Sulphonylurea class of antidiabetic drugs are widely used in the therapy of NIDDM. Glimepiride belongs to the third generation sulphonylureas and chemically it is a carboxamido phenyl pyrroline sulphonylurea. The primary mechanism of action of glimepiride in lowering the blood glucose appears to be dependent on stimulating the release of insulin from functioning pancreatic beta cells⁵. The extra pancreatic glucose reducing effects include inhibition of gluconeogenesis, ketogenesis, stimulation of peripheral glucose transport, glycogen synthase activity and glycerol-3-P-acyltransferase activity⁶. In the antidiabetic therapy, glimepiride has the
advantage that it does not cause severe hypoglycemic complication due to sudden release of insulin as like the other sulphonylureas. The studies conducted on the BB rats reported that administration of glimepiride prevents the induction of type-1 diabetes due to the immunosuppressive effect. Glimepiride also reported to benefit the patients suffering from coronary artery diseases by stimulating the production of nitric oxide. Besides, it has been reported that glimepiride possess antioxidant effect against the oxidative stress induced by STZ-diabetes. The mutagenic studies conducted by battery of in vitro and in vivo methods concluded that glimepiride do not have mutagenic potential. Since quenching the free radicals generated in the oxidative stress is one of the possible mechanisms to prevent the mutagenic defects in diabetes and there is a need for antidiabetic regimen that also reduce the ROS induced health complications, this study has been planned to evaluate the anti-mutagenic effect of glimepiride in nicotinamide-streptozotocin induced oxidative stress in type-2 diabetes in Wistar rats.

Materials and Methods

Chemicals — A gift sample of glimepiride (Gmp) was obtained from Bal Pharma Ltd, Bangalore. The stains and other reagents/chemicals used in this study were of analytical grade and procured from the regular suppliers.

Animals — Eight week-old healthy, laboratory bred, male Wistar rats weighing 180 ± 10 g were maintained under standard laboratory conditions (20°C±2°C, 12:12 h light / dark cycle) and provided water and pellet food ad libitum. The experiments were conducted in CPCSEA (Committee for the purpose of control and supervision of experiments on animals, Chennai, India) approved animal house after obtaining the prior approval from the Institutional Animal Ethics Committee (AACP/IAEC/P-31/2005).

Induction of type-2 diabetes — Experimental T2DM was developed in adult rats by administering streptozotocin (STZ) and nicotinamide (NA). The animals received i.p administration of NA -230 mg/kg (SD Fine-Chem Ltd, Mumbai, India) dissolved in saline 15 min before an administration of STZ– 65 mg/kg, ip (Sigma Aldrich, USA) dissolved in 0.1 M citrated buffer (pH 4.5) immediately before use. Blood glucose was estimated after 2 days and the animals with glucose level ≈ 180 ± 8 mg/dl were selected for the study.

Dosage, treatment and sampling — The animals were divided mainly in to three groups ie., control, diabetic and treatment, consisting of 8 animals in each group. The criteria for selecting the dose of Glimepiride (Gmp) was to establish the relationship between antioxidant, antihyperglycemic and antimutagenic activities. To achieve this, 0.175, 17.5 and 175 mg/kg doses, which were in the range between the clinical dose (0.18 mg/kg) and reported concentration for rats (200 mg/kg) were selected. The doses of Gmp were administered orally per day for 4 weeks after the induction of diabetes. The control and diabetic animals were administered saline (0.5 ml/kg) daily throughout the treatment period. In this study, α-tocopherol (20 mg/kg, po, 4 weeks) and insulin (1 IU/kg) were used as standard antioxidant and hypoglycemic agents, respectively. Before the administration, α-tocopherol and Gmp were suspended in 1% w/v carboxy methyl cellulose (CMC) whereas insulin was reconstituted in water for injection to obtain the required dose.

Bone marrow micronucleus test — The modified method of Schimid was followed to perform the bone marrow MN test. The animals after respective treatment were sacrificed by cervical dislocation under light anaesthesia. Animals were cut open to excise femur and tibia. Bone marrow MN slides were prepared by using the modified method of Schimid. Marrow suspension from femur and tibia were prepared in 5% bovine serum albumin (BSA), was centrifuged at 1000 rpm for 8 min and the pellet was resuspended in a required quantity of BSA. A drop of this suspension was taken on a clean glass slide and smear was prepared on glass slide and air dried. The slides were fixed in absolute methanol, stained with May-Grunwald-Giemsa and MN were identified in two forms of RBCs [ie, polychromatic erythrocytes (PCEs) and normochromatic erythrocytes (NCEs)]. About 2000 PCEs and corresponding NCEs were scanned for the presence of MN and also to estimate the P/N ratio (polychromatic: normochromatic ratio) using 100X oil immersion objective.

Sperm morphology and sperm count assay — The procedure described by Wyrobek and Bruce was followed to study the sperm shape abnormality in cauda epididymis of the rats. Sperms (1000 per animals) were screened to find the different types of abnormality in one of the cauda epididymis. Five types of abnormalities viz. amorphous, hookless,
banana shape, fused and double headed were evaluated and the total abnormality was represented as % abnormal sperms. The caudal sperm count test was performed according to D’Souza. The spermatozoa count was obtained by counting the number of sperm cells in the four WBC chambers using a neubauer’s slide.

In vivo antioxidant activity — Blood samples were collected from the retro-orbital plexus under light ether anaesthesia. The serum was separated by centrifugation (1000 rpm) and immediately analyzed to determine the antioxidant enzyme activity.

Serum lipid peroxidation (LPO) — The procedure described by Yagi was followed to estimate the lipid peroxidation.

Enzyme estimation — Activities of serum catalase (CAT; EC 1.11.1.6), superoxide dismutase (SOD; EC 1.11.1.1) and glutathione peroxidase (GPx; EC 1.11.1.9) were assayed.

Blood glucose estimation — Fasting blood glucose estimation was done by using the glucometer (Ascensia ENTRUST, Bayer healthcare Ltd, Mumbai). A drop of blood collected from the tail vein was gently applied over the test zone of the glucometer and the blood glucose level was recorded immediately as mg/dl.

Statistical analysis — The statistical analyses of the result was done by One-way Anova followed by multiple comparison by Bonferroni test for bone marrow MN test and Mann-Whitney U test for the sperm abnormality and Newman-Keuls for antioxidant study respectively. P<0.05 was considered to indicate the significance.

Results

Effect of glimepiride on the frequency of bone marrow micronucleus in NA-STZ induced diabetic rats—Experimental type-2 diabetes after the administration of NA-STZ produced significant increase in the percentage of micronucleated cells in both polychromatic and normochromatic erythrocytes besides reducing the P/N ratio compared to the normal animals. The lower doses of Gmp (0.175 and 17.5 mg) did not alter the nuclear damage caused by the NA-STZ diabetes. However, Gmp at 175 mg reduced the percentage polychromatic and normochromatic micronucleated erythrocytes without altering the diminished P/N ratio compared to the diabetic condition. The percentage inhibition in the frequency was observed to be 7.8% for PCEs and 13.7% for NCEs compared to diabetes. Administration of α-tocopherol (20 mg) to the NA-STZ diabetic animals significantly reduced the number of micronucleated cells in both PCEs and NCEs when compared to diabetic group.

<table>
<thead>
<tr>
<th>Treatment and Dose</th>
<th>Bone marrow micronucleus test</th>
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<tr>
<td></td>
<td>Control (saline-0.5ml / kg)</td>
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<tr>
<td>% MN in PCEs</td>
<td>0.39 ± 0.01</td>
</tr>
<tr>
<td>% MN in NCEs</td>
<td>0.41 ± 0.02</td>
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<tr>
<td>P/N ratio</td>
<td>1.08 ± 0.03</td>
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NA = Nicotinamide, STZ = Streptozotocin, One way Anova followed by Bonferroni test. P values: *<0.001 compared with the control **<0.01, ***<0.001 compared with diabetic group.
NCEs and enhanced the P/N ratio as well compared to the NIDDM group. However, the standard hypoglycemic agent insulin (1 IU) did not prevent the nuclear damage induced by the T2DM (Table 1).

**Effect of glimepiride on the sperm morphology and sperm count in NA-STZ induced diabetic rats** — The NA-STZ induced diabetic condition significantly ($P<0.001$) increased the sperm shape abnormality (Fig. 2) and reduced the sperm count and weight of the testis in comparison to the normal animals. Gmp at 0.175 and 17.5 mg did not show significant effect on the tested parameters, however, when Gmp was evaluated at 175 mg, a significant ($P<0.05$) decrease in the sperm shape abnormality and increase in the sperm count was observed but the treatment did not enhance the weight of testis compared to the diabetes. The percentage reduction was found to be 11% for sperm shape abnormality and 6.8% for sperm count in the diabetic condition. Administration of α-tocopherol reduced the sperm abnormality and enhanced the sperm count in the diabetic animals. Further, administration of insulin (1 IU) did not prevent the NA-STZ mediated sperm abnormality and weight of testes (Table 2).

**Effect of glimepiride on the serum blood glucose and antioxidant status in NA-STZ induced diabetic rats** — NA-STZ induced diabetic condition significantly ($P<0.001$) increased the LPO and reduced the levels of antioxidant enzymes such as CAT, SOD and GPx compared to the control animals. Gmp at 175 mg enhanced the serum levels of CAT, SOD and GPx but did not alter the LPO activity compared to the diabetic animals. However, the lower dose of Gmp (0.175 and 17.5 mg) did not produce the significant change in the antioxidant status. Further, α-tocopherol treated diabetic animals showed enhanced the levels of CAT, SOD, GPx besides diminished LPO.

**Table 2**—Effect of glimepiride on the sperm morphology and sperm count in NA-STZ induced diabetic rats

<table>
<thead>
<tr>
<th>Treatment and Dose (mg/kg)</th>
<th>Sperm abnormality test</th>
<th>Weight of testis (g)</th>
<th>Total abnormality(%)</th>
<th>Sperm count ($10^6$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (saline-0.5 ml/kg) + NA (230 mg) STZ (65 mg) + glimepiride (0.175 mg/kg) NA-STZ + glimepiride (17.5 mg/kg)</td>
<td>Control</td>
<td>1.23 ± 0.02</td>
<td>1.04 ± 0.07</td>
<td>33.18 ± 1.36</td>
</tr>
<tr>
<td>NA-STZ + glimepiride (175 mg/kg)</td>
<td>NA-STZ</td>
<td>1.19 ± 0.11</td>
<td>1.58 ± 0.17</td>
<td>27.77 ± 1.31</td>
</tr>
<tr>
<td>NA-STZ + glimepiride (175 mg/kg) + α-tocopherol (20 mg/kg)</td>
<td>NA-STZ</td>
<td>1.20 ± 0.43</td>
<td>1.56 ± 0.09</td>
<td>27.82 ± 2.83</td>
</tr>
<tr>
<td>NA-STZ + insulin (1 IU/kg)</td>
<td>NA-STZ</td>
<td>1.23 ± 0.11</td>
<td>1.46 ± 0.14</td>
<td>29.68 ± 1.07</td>
</tr>
<tr>
<td>NA-STZ + insulin (1 IU/kg)</td>
<td>NA-STZ</td>
<td>1.19 ± 0.07</td>
<td>1.21 ± 0.34</td>
<td>27.69 ± 2.23</td>
</tr>
</tbody>
</table>

NA – Nicotinamide, STZ – Streptozotocin, One way Anova followed by Mann-Whitney U test. $P$ values: * $<0.001$ compared with the control
** $<0.01$, *** $<0.001$ compared with diabetic group.

**Fig 2**— Different types of sperm shape abnormalities [A: normal; B: Hookless; C: banana; D: amorphous; E: fused and F: double head].
Administration of insulin did not alter oxidative stress in the experimental NIDDM.

In addition, administration of Gmp (17.5 and 175 mg) and insulin exhibited potent glucose lowering effect on the NA-STZ induced hyperglycemia, while the effect after the α-tocopherol treatment was observed to be mild in comparison to the diabetic group (Table 3).

**Discussion**

The results of the present study indicated that the NA-STZ diabetes increased the population of micronucleated erythrocytes and reduced the P/N ratio (Table 1). The diabetic condition also increased the sperm shape abnormality besides reducing the weight of testis and caudal sperm count (Table 2). These effects appear to be mediated through the oxidative stress generated due to the hyperglycemia (Table 3). As STZ mediated increase micronuclei frequency and sperm abnormality is already reported, the mechanism suggested include the activation of several cellular damaging pathways by the ROS such as accelerated formation of advanced glycation end products (AGE), polyol pathway, hexosamine pathway and protein kinase (PKC).

Diabetes mediated oxidative stress is known to damage the nuclear component of the host cells and is considered to be a vital cause for the mutation related somatic and germinal cell disorders. The somatic cell defects has the tendency to cause various types of neurological defects, heart ailments, carcinogenesis aging etc while the germinal cell damage results in infertility. In the case of male infertility, the lower levels of oxidative damage to the spermatozoa may retain the capacity for fertilization while carrying significant levels of oxidative damage in their DNA. Epidemiological evidence suggests that subsequent aberrant repair of such damage in the zygote may result in the mutations associated with preterm pregnancy and a variety of pathologies in the offspring, including childhood cancer.

Administration of glimepiride and α-tocopherol to the NA-STZ diabetic rats had reduced the population of micronucleated erythrocytes and sperm shape abnormality besides enhancing the sperm count (Tables 1 and 2). These drugs also enhanced the serum levels of antioxidant enzymes and reduced the LPO and hyperglycemia (Table 3). These observations indicate that increasing the level of CAT, SOD and GPx and reducing the LPO could minimize the cytogenetic damages in somatic and germinal cells. SOD is an enzyme that catalyses the dismutation of superoxide ion in to oxygen and hydrogen peroxide, thus protecting the cell from the superoxide toxicity. CAT present in many plants, animals and aerobic bacteria, efficiently promotes the conversion of hydrogen peroxide to water and molecular oxygen.

The function of GPx is to remove the H$_2$O$_2$ generated by metabolic action or oxidative stress. LPO occurs when ROS attacks the poly unsaturated fatty acid residues of phospholipids of cell membrane which is extremely sensitive to the oxidation. Host cells like spermatozoa are highly susceptible to the damage by excess concentrations of ROS due to high content of

<table>
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<tr>
<th>Serum antioxidant status and glucose level</th>
<th>Control (Saline-0.5ml/kg)</th>
<th>NA (230 mg) + STZ (65 mg)</th>
<th>NA-STZ + glimepiride (0.175 mg/kg)</th>
<th>NA-STZ + glimepiride (17.5 mg/kg)</th>
<th>NA-STZ + glimepiride (175 mg/kg)</th>
<th>NA-STZ + α-tocopherol (20 mg/kg)</th>
<th>NA-STZ + insulin (1 IU/kg)</th>
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<tr>
<td>Lipid peroxidation (μmol/mg protein)</td>
<td>2.39 ± 0.20</td>
<td>3.35 ± 0.22</td>
<td>3.37 ± 0.32</td>
<td>3.19 ± 0.11</td>
<td>3.04 ± 0.29</td>
<td>2.40 ± 0.37</td>
<td>3.36 ± 0.11</td>
</tr>
<tr>
<td>Catalase (units/mg protein)</td>
<td>6.39 ± 0.34</td>
<td>3.12 ± 0.38</td>
<td>3.09 ± 0.19</td>
<td>3.36 ± 0.04</td>
<td>3.74 ± 0.10†</td>
<td>5.27 ± 0.06‡</td>
<td>3.11 ± 0.05</td>
</tr>
<tr>
<td>SOD (units/mg protein)</td>
<td>0.46 ± 0.05</td>
<td>0.22 ± 0.06</td>
<td>0.24 ± 0.09</td>
<td>0.27 ± 0.06</td>
<td>0.31 ± 0.02‡</td>
<td>0.43 ± 0.05</td>
<td>0.23 ± 0.09</td>
</tr>
<tr>
<td>Glutathione peroxidase (GPx) (μg of glutathione consumed/mg protein)</td>
<td>1.52 ± 0.04</td>
<td>1.19 ± 0.17</td>
<td>1.14 ± 0.28</td>
<td>1.16 ± 0.92</td>
<td>1.22 ± 0.23**</td>
<td>1.41 ± 0.10**</td>
<td>1.17 ± 0.86**</td>
</tr>
<tr>
<td>Blood glucose (mg/dl)</td>
<td>92.3 ± 3.44</td>
<td>174.3 ± 6.32</td>
<td>170.17 ± 5.96</td>
<td>143.5 ± 7.08**</td>
<td>116.37 ± 7.15***</td>
<td>157.4 ± 6.47</td>
<td>143.8±5.93**</td>
</tr>
</tbody>
</table>

NA – Nicotinamide, STZ – Streptozotocin, One way Anova followed by Newman-Keuls test. P values: * <0.05, ** <0.01, *** <0.001 compared with the control; *<0.05, **<0.01, *** <0.001 compared with diabetic group.
polyunsaturated fatty acid within their plasma membrane. Increased LPO and altered membrane can affect the sperm function through impaired metabolism, motility, acrosome reaction as well as oxidative damage to sperm DNA\textsuperscript{31,32}.

Antioxidants including \(\alpha\)-tocopherol limit the nuclear damage by preventing the free radical action\textsuperscript{33}. An increase in the level of CAT, SOD, GPx and decrease in LPO followed by \(\alpha\)-tocopherol treatment supports its potential to maintain the redox state and to cope with the oxidative stress. Like \(\alpha\)-tocopherol, the test drug glimepiride (175 mg/kg) exhibited a significant anti-mutagenic activity along with an antioxidant effect. However, \(\alpha\)-tocopherol and glimepiride did not increase the weight of testis in the diabetic animals. These results indicate that both the drugs although possess anti-mutagenic and antioxidant activity but did not reduced the NA-STZ mediated changes on the weight of the testes. The inability of these two agents to reverse the diminished weight of testis in the diabetic condition need to be further evaluated.

The relationship between the antioxidant property and antimutagenic effect can be ascertained from the observations of Gmp, \(\alpha\)-tocopherol and insulin treatment to the diabetic animals. Although, Gmp (17.5 mg) and insulin (1 IU) reduced the elevated blood sugar level, the drugs did not exhibited the antioxidant activity. Probably, due to the lack of this property, Gmp (17.5 mg) and insulin did not reduce the nuclear damage in the diabetic animals. The lower dose of Gmp (0.175 mg) neither produced antimutagenic effect nor antioxidant and antidiabetic activity. On the other hand, \(\alpha\)-tocopherol being a known antioxidant is also reported to decrease the hyperglycemia and oxidative mediated nuclear damage in the diabetic condition\textsuperscript{16,33}. Similar type of response was observed when Gmp was tested at 175 mg in the diabetic rats. These data indicate that compounds possessing glucose lowering property along with an antioxidant effect play a beneficial role in preventing the ROS mediated DNA damages. The antioxidant activity of glimepiride is already reported. Krauss \textit{et al.}\textsuperscript{9} reported that administration of glimepiride increased the plasma levels of SOD, GPx besides reducing the levels of \(\text{H}_2\text{O}_2\) and malondialdehyde. They suggested that glimepiride by increasing the level of antioxidant enzymes decreased the ROS mediated damage in the host cells\textsuperscript{9}.

In conclusion, the present observations suggest that Gmp (175 mg/kg) possess antioxidant activity against the NA-STZ induced oxidative stress. The ability of Gmp to protect against the nuclear damages in both somatic and germinal cells in hyperglycemic condition could help in minimizing the ROS-mediated health complications in the diabetic patients.

References