Abstract

Background: Oxidative stress due to improper control of blood glucose in chronic diabetes plays a major role in the development of secondary complications including cancer and birth defect. The aim of this study is to evaluate the protective effect of combination of pioglitazone with metformin or glimepiride against the experimental type-2 diabetes induced nuclear damage and reproductive toxicity in rats.

Methods: The combinations of Pioglitazone (Pio-1 mg/kg) with metformin (Met-50 mg/kg) or glimepiride (Gmp-0.2 mg/kg) given orally daily for 4 weeks were tested against nicotinamide (NA-230 mg/kg, ip)-streptozotocin (STZ-65 mg/kg, ip)-induced micronuclei (MN) formation and sperm abnormalities in male Wistar rats. The antioxidant status was evaluated by measuring the levels of serum lipid peroxidation (LPO), catalase (CAT) and superoxide dismutase (SOD).

Results: The administration of Pio+Met significantly (P<0.01) reduced the number of micronucleated erythrocytes, increased the polychromatic: normochromatic erythrocytes (P/N ratio), reduced (P<0.001) sperm morphology defects and increased (P<0.05) the caudal sperm count compared to the untreated diabetic condition. Furthermore, the Pio+Met combination enhanced the antioxidant status in diabetic animals. However, Pio+Gmp did not attenuate the nuclear and sperm defects or oxidative stress.

Conclusions: The observations suggest that Pio+Met combination reduced nuclear damage and sperm abnormalities by enhancing the antioxidant status in the diabetic animals.

Keywords: antidiabetics, combination therapy, micronuclei, sperm abnormalities, health sciences

Introduction

Type 2 diabetes mellitus (T2DM) is a chronic and progressive disease characterised by impaired insulin secretion and insulin resistance in the liver, adipose tissue and skeletal muscle. These combined abnormalities contribute to abnormal glucose metabolism. The degree and duration of hyperglycaemia is the main reason for the complications of T2DM (1). Chronic hyperglycaemia is the major cause of the generation of reactive oxygen species (ROS), which damage the components of host cells, including DNA. Mutation in the somatic cells often results in secondary problems such as aging, heart ailments, neurological defects and carcinogenesis (2). Damage to the germinal cells can result in male infertility, pre-term pregnancy loss and a variety of pathologies in the offspring, including childhood cancer (3). Genotoxicity testing assumes importance since the consequences of DNA damage can cause defects in both the present generation and the offspring. Bone marrow micronucleus (MN) and sperm abnormality assays in rodents are important tools to assess the mutagenic and anti-mutagenic property of test compounds (3,4).

Data from the United Kingdom Prospective Diabetes Study (UKPDS) have established that tight glucose control reduces the risk of oxidative stress-related disorders in patients with T2DM. The UKPDS data also suggested that approximately 10% of patients do not maintain optimum glycaemic levels even when treated intensively with sulphonylureas (SUs), biguanides or insulin as monotherapy (5). Prior studies propose that addition of a second agent from a different class may improve the glycaemic control in patients who do not maintain glycated haemoglobin (HbA1c) concentration of <7% (6).
However, combination of oral-hypoglycaemics may lead to unwanted side effects (7). The combination of SU with biguanide is the most preferred but this combination carries the risk of hypoglycaemia (8).

Thiazolidinediones (TZDs) can be used in combination therapy with SUs or biguanide, and such combinations have been clinically established to be effective in reducing the HbA1c concentration by 0.8% to 1.2% compared to the monotherapy of individual drugs (9). The combination of TZDs with SU/biguanides has been found to benefit diabetic patients in reducing hyperglycaemia and insulin resistance, in addition to improving cardiovascular complications such as dyslipidemia and endothelial dysfunction (10). However, monotherapy with pioglitazone (Pio), a member of the TZD class, has been reported to induce DNA damage in the hepatocytes and lymphocytes in rats. The mechanism suggested includes increased generation of ROS, especially from those cells that contribute to Pio metabolism and detoxification (11). On the other hand, metformin (Met) and glimepiride (Gmp) have been reported to decrease oxidative stress-mediated nuclear damage in diabetic rats (12,13). Considering the complications associated with genotoxic antidiabetic agents and the influence of the addition of an anti-mutagenic in the combination, this study was designed to evaluate the role of Met or Gmp in combination with Pio against nicotinamide (NA)-streptozotocin (STZ)-mediated nuclear defects and sperm abnormalities in male Wistar rats.

**Materials and methods**

**Chemicals**

Gift samples of Pio, Met and Gmp were obtained from Biocon Pvt. Ltd, Micro Labs Pvt. Ltd. and Bal Pharma Ltd., Bangalore, respectively. Staining reagents and other chemicals used in this study were of analytical grade and procured from the HiMedia Laboratories Pvt. Ltd., Mumbai.

**Animals**

Eight-week-old healthy, laboratory bred, male Wistar rats weighing 180 ± 10 g were maintained under standard laboratory conditions at a temperature of 20 ± 2 °C, 12 hour light / dark cycle and provided water and pellet food *ad libitum*. The experiments were conducted in a 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).

**Dosage, treatment and sampling**

The animals were divided mainly into the following groups:

- **Group 1**: Control (0.5 mL/kg saline, by mouth (p.o))
- **Group 2**: Untreated diabetic (230 mg/kg NA and 65 mg/kg STZ)
- **Group 3**: Diabetic + Combination-1 (1 mg/kg Pio + 50 mg/kg Met, p.o)
- **Group 4**: Diabetic + Combination-2 (1 mg/kg Pio + 0.2 mg/kg Gmp, p.o)
- **Group 5**: Diabetic + α-tocopherol (20 mg/kg, p.o)
- **Group 6**: Diabetic + insulin (1 IU/kg, s.c)

The doses of Pio (15), Met (16) and Gmp (17) were selected as per previous reports and depending on their individual concentrations found in the antidiabetic combination formulation meant for clinical use (18,19). The drugs were administered once daily for 4 weeks after the induction of diabetes. The control and untreated diabetic animals were administered saline (0.5 mL/kg) daily throughout the treatment period. In this study, α-tocopherol (20) and insulin (21) were used as the standard antioxidant agent and hypoglycaemic agent, respectively. Before the administration, Pio, Gmp and α-tocopherol were suspended in 1% w/v carboxy methyl cellulose (CMC), insulin was reconstituted in water for injection and Met was dissolved in distilled water to obtain the required dose. In all of the groups, a 12 h fasting condition was maintained before the experiment, wherein the animals were provided only water *ad libitum*.

**Bone marrow micronucleus test**

The modified method of Schmid was followed to perform the bone marrow MN test (4). Following the treatment, animals were sacrificed by cervical dislocation under light ether anaesthesia (2 mL/kg, open drop method) (22). Animals were cut open to excise the femur and tibia. Bone marrow MN slides were prepared by using the modified method of Schmid. Marrow suspension from the femur and tibia bones of both sides were prepared in 5% bovine serum albumin (BSA), centrifuged at 1000 rpm for 8 min and then the pellet was resuspended in a required quantity of BSA. A drop of this suspension was placed on a clean glass slide and a smear was prepared and air dried. The slides were fixed in absolute methanol, stained with May-Grunwald-Giemsa and MN were identified as dark bluish coloured, round fragments in two forms of RBCs (polychromatic erythrocytes as PCEs and normochromatic erythrocytes as NCEs) (23) (Figure 1). However,
a few exceptions could be possible, especially with high doses of chromosome breaking agents, in which MN could appear as almond-shaped or half-moon-shaped (23). About 2,000 PCEs and corresponding NCEs were scanned for the presence of MN to calculate P/N (Polychromatic: normochromatic erythrocytes) ratio using 100X oil immersion objective.

**Sperm morphology and sperm count assay**

The procedure described by Wyrobek and Bruce (1975) (24) was followed to study the sperm shape abnormalities in the cauda epididymis of the rats. One thousand sperm per animal were screened to find the different types of abnormalities in one of the cauda epididymis. Six types of abnormalities including amorphous, hookless, banana shape, coiled, double-headed and double-tailed (Figure 2) were evaluated and the total abnormality was represented as % abnormal sperm (25).

The caudal sperm count test was performed according to the method described by D'Souza (26). The spermatozoa count was obtained by counting the number of sperm cells in the four chambers of a Neubauer slide.

**In vivo antioxidant activity**

Blood samples were collected from the retro-orbital plexus under light ether anaesthesia (22). The serum was separated by centrifugation (1000 rpm) and immediately analysed to determine the antioxidant enzyme activity.

**Serum lipid peroxidation (LPO)**

The procedure described by Yagi (27) was followed to estimate the lipid peroxidation. The principle depends on the reaction between thiobarbituric acid with malondialdehyde, a secondary product of lipid peroxidation at pH 4. The development of a reddish-pink colour, which indicates the extent of peroxidation, was estimated at 532 nm. The extent of lipid peroxidation was expressed as nmol/mg protein (27).

**Catalase (CAT)**

The estimation of catalase (EC 1.11.1.6) activity was done by determining the decomposition of H₂O₂ at 610 nm in an assay mixture containing phosphate buffer (0.25 M, pH 7). One international unit of catalase utilised is that amount which catalyses the decomposition of 1 mM H₂O₂ per min at 37°C and is expressed in terms of unit/mg protein (28).

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**Figure 1:** Micronucleated polychromatic erythrocytes [arrow indicates the micronucleus in the polychromatic erythrocytes with normal normochromatic erythrocytes, stained with May-Grunwald-Giemsa, 100X magnification]
Superoxide dismutase (SOD)

The principle for measuring the SOD (EC 1.11.1.1) depends on the detection of superoxide ions generated during auto-oxidation of hydroxylamine. During the oxidation, nitro blue tetrazolium (NBT) is reduced and nitrite is produced in the presence of EDTA, which can be detected colourimetrically at 560 nm. The concentration of SOD is expressed as units/mg protein (29).

Blood glucose estimation

Fasting blood glucose estimation was done 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.

Statistics

The statistical analyses were done by One-way ANOVA followed by a multiple comparisons test with Bonferroni test for the bone marrow MN test and Newman-Keuls for the antioxidant study. However, data on epididymal sperm shape abnormalities and sperm count were analysed employing a non-parametric test, the Mann-Whitney U test. P < 0.05 was considered to indicate a significant difference.

Results

Effect of the combination of Pioglitazone with Metformin or Glimepiride on the frequency of bone marrow micronuclei in NA-STZ-induced diabetic rats

The NA-STZ induced T2DM significantly (P < 0.001), increased the frequency of MN in PCEs and NCEs and reduced the P/N ratio compared to the control animals. Combination of Pio+Met inhibited the percentage of MN in PCEs (P < 0.001) and NCEs (P < 0.01), in addition to enhancing the P/N ratio (P < 0.05) compared to the untreated diabetic rats. The percent inhibition was 14.9% for MN PCEs, 21.8% for MN NCEs and 11.4% for P/N ratio compared to the untreated diabetic group. Administration of α-tocopherol reduced (P < 0.001) the number of MN PCEs (17.2%) and NCEs (22.6%), along with an increase (P < 0.001) in the P/N ratio (11.4%) compared to the untreated diabetic rats. However, Pio+Gmp and insulin treatments did not alter the incidence of micronucleated erythrocytes and P/N ratio in NA-STZ diabetic rats (Table 1).
Effect of the combination of Pioglitazone with Metformin or Glimepiride on the sperm morphology and sperm count in NA-STZ-induced diabetic rats

Administration of NA-STZ significantly increased the occurrence of sperm shape abnormalities (P<0.001) and reduced the sperm count (P<0.001) compared to the control animals. Administration of combination of Pio+Met significantly reduced (P<0.001) sperm shape abnormalities and increased (P<0.05) the sperm count compared to the untreated diabetic group. The percent inhibition was 19.5% for sperm abnormalities and 6.7% for sperm count compared to the untreated diabetics. Similarly, α-tocopherol diminished (P<0.001) the sperm abnormalities (26.6%) and sperm count (14.8%) compared to the untreated diabetics. However, Pio+Gmp and insulin treatments did not alter the reproductive damage induced by the diabetic state (Table 2).

Effect of the combination of Pioglitazone with Metformin or Glimepiride on the serum antioxidant status and glucose level in NA-STZ-induced diabetic rats

The experimental T2DM significantly (P<0.001) increased the oxidative stress and blood glucose level compared to the control animals. Diabetic animals treated with Pio+Met combination showed a decreased level of LPO (P<0.05) and enhanced concentrations of CAT (P<0.001) and SOD (P<0.01) compared with the untreated diabetic condition. The percent change was 14.3% for LPO, 68.9% for CAT and 72.7% for SOD compared to untreated hyperglycaemic group. Administration of α-tocopherol elevated the antioxidant status by decreasing LPO level (P<0.001, 28.3%) and improving CAT (P<0.001, 68.9%) and SOD (P<0.001, 95.5%) concentrations compared to untreated experimental T2DM. However, Pio+Gmp and insulin did not produce significant changes in the oxidative stress. In addition, all of the treatments significantly (P<0.001) reduced the hyperglycaemia, α-tocopherol being the least potent (P<0.05) among them (Table 3).

Discussion

The present study indicated that administration of NA-STZ increased the micronuclei frequency, sperm abnormalities, oxidative stress and glycaemia and reduced P/N ratio. The normal P/N ratio is reported to be 1:1 in bone marrow. A n increase in NCEs signals a cytotoxic effect whereas an increase in PCEs reflects a stimulation of erythrocyte proliferative activity (30).

The elevated blood sugar level was observed to be moderate (180 ± 8 mg/dL) and this can be attributed to the role of NA during the development of T2DM. NA functioning as an antioxidant is reported to partially protect β-cells against the cytotoxic damages of STZ (14). The oxidative stress generated during hyperglycaemia is reported to involve several pathways such as accelerated formation of advanced glycation end products (AGEs), polyol pathway, hexosamine and protein kinase-C (PKC) (31,32).

Table 1: Effect of the combination of Pioglitazone with Metformin or Glimepiride on the frequency of bone marrow micronuclei in NA-STZ-induced diabetic rats

<table>
<thead>
<tr>
<th>Bone marrow micronucleus test</th>
<th>Treatment and Dose (mg/kg)</th>
<th>% MN in PCEs</th>
<th>% MN in NCEs</th>
<th>P/N ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Saline-0.5 ml/kg)</td>
<td>NA (230 mg) + STZ (65 mg)</td>
<td>0.39 ± 0.01</td>
<td>0.41 ± 0.02</td>
<td>1.08 ± 0.03</td>
</tr>
<tr>
<td>NA-STZ + Pio (1 mg) + Met (50 mg)</td>
<td>1.41 ± 0.08</td>
<td>1.24 ± 0.12</td>
<td>0.79 ± 0.02</td>
<td>0.79 ± 0.02</td>
</tr>
<tr>
<td>NA-STZ + Pio (1 mg) + Gmp (0.2 mg)</td>
<td>1.20 ± 0.06</td>
<td>0.97 ± 0.09</td>
<td>0.88 ± 0.07</td>
<td>0.88 ± 0.07</td>
</tr>
<tr>
<td>NA-STZ + α-Tocopherol (20 mg/kg)</td>
<td>1.42 ± 0.08</td>
<td>1.02 ± 0.26</td>
<td>0.80 ± 0.02</td>
<td>0.80 ± 0.02</td>
</tr>
<tr>
<td>NA-STZ + Insulin (1 IU/kg)</td>
<td>1.17 ± 0.14</td>
<td>0.96 ± 0.15</td>
<td>0.88 ± 0.07</td>
<td>0.76 ± 0.04</td>
</tr>
<tr>
<td>NA-STZ + Insulin (1 IU/kg)</td>
<td>1.47 ± 0.02</td>
<td>1.27 ± 0.10</td>
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</tr>
</tbody>
</table>

Values are expressed as Mean ± SD, MN – micronucleus, PCE – polychromat erythrocytes, NCE – normochromatic erythrocytes, NA – Nicotinamide, STZ – Streptozotocin, Pio – Pioglitazone, Met – Metformin, Gmp – Glimepiride, N=8

Statistics: One-way Anova followed by Bonferroni test.

*P<0.001, **P<0.05 compared with the Control.

*P<0.001, "P<0.01, "P<0.05 compared with the untreated Diabetic group.
Administration of Pio+Met reduced the incidence of MN formation in PCEs, NCEs and enhanced the P/N ratio in the diabetic animals (Table 1). The combination also reduced the incidence of sperm shape abnormalities and increased the sperm count in the hyperglycaemic rats (Table 2). The antioxidant profile indicated that the combination of Pio+Met elevated the serum levels of CAT and SOD and reduced the LPO, along with a significant anti-hyperglycaemic action (Table 3). As reported, SOD is an enzyme that catalyses the dismutation of superoxide ion to oxygen and hydrogen peroxide, thus protecting the cell from the superoxide toxicity (29,32). CAT efficiently promotes the conversion of hydrogen peroxide to water and molecular oxygen (28,33). LPO occurs when ROS attack the polyunsaturated fatty acid residues of phospholipids of the cell membrane, which is extremely sensitive to oxidation. Spermatozoa are highly susceptible to damage by excess concentrations of ROS due to a high content of polyunsaturated fatty acid within their plasma membrane (27,33).

Additionally, α-tocopherol treatment reduced the nuclear breaks in erythrocytes and minimised spermatozoa abnormalities in addition to reducing the oxidative stress and hyperglycaemia. As a potent antioxidant, α-tocopherol has been

<table>
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<th>Table 2: Effect of the combination of Pioglitazone with Metformin or Glimepiride on sperm morphology and sperm count in NA-STZ-induced diabetic rats</th>
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<tr>
<td><strong>Sperm abnormality test</strong></td>
</tr>
<tr>
<td><strong>Control (Saline-0.5 ml/kg)</strong></td>
</tr>
<tr>
<td><strong>Total % Abnormality</strong></td>
</tr>
<tr>
<td><strong>Sperm count (10⁶)</strong></td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SE, NA – Nicotinamide, STZ – Streptozotocin, Pio – Pioglitazone, Met – Metformin, Gmp – Glimepiride, N=8
Statistics:  Mann-Whitney U test,
εP<0.05, P<0.01, P<0.001 compared with the Control
εP<0.001, εP<0.05, εP<0.01, compared with the untreated Diabetic group

<table>
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<th>Table 3: Effect of the combination of Pioglitazone with Metformin or Glimepiride on serum antioxidant status and glucose level in NA-STZ-induced diabetic rats</th>
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<tr>
<td><strong>Serum antioxidant status and glucose level</strong></td>
</tr>
<tr>
<td><strong>Control (Saline-0.5 ml/kg)</strong></td>
</tr>
<tr>
<td><strong>Lipid peroxidation (μmol/mg protein)</strong></td>
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<tr>
<td><strong>Catalase (units/mg protein)</strong></td>
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<tr>
<td><strong>SOD (units/mg protein)</strong></td>
</tr>
<tr>
<td><strong>Blood glucose (mg/dl)</strong></td>
</tr>
</tbody>
</table>

Values are expressed as Mean ± SD, NA – Nicotinamide, STZ – Streptozotocin, Pio – Pioglitazone, Met – Metformin, Gmp – Glimepiride, N=8
Statistics: One-way Anova followed by Newman-Keuls
εP<0.01, εP<0.05, εP<0.001 compared with the Control
εP<0.05, εP<0.01, εP<0.001, compared with the untreated Diabetic group
reported to reduce ROS-mediated MN formation, sperm abnormalities and hyperglycaemia (34–36). Considering these observations, it can be suggested that the antioxidant property of a compound could play a significant role in averting the nuclear injury and sperm aberrations caused by diabetes (2,33).

Previous research has indicated that Pio and Met possess antioxidant potential (37,38). TZDs were found to suppress the generation of ROS by affecting the activation of NF-κB and interfering with the MAPK signalling cascade (37). Met is reported to modulate the activity of PKC and NADPH oxidase, which in turn counteract ROS by elevating antioxidant enzymes like catalase, SOD and glutathione peroxidase (GPx) (38). As reported earlier, Pio monotherapy has been shown to increase the nuclear damage in hepatocytes and to a small extent, in lymphocytes. This action can be related to the hepatotoxic property of TZDs, including troglitazone and Pio (11), although the mechanism for lymphocyte damage caused by Pio remains to be elucidated. Our data indicate that inclusion of Met with Pio reduced the nuclear damage, suggesting that this combination is effective in combating the ROS-induced nuclear imperfections in the diabetic state.

On the other hand, Pio+Gmp did not protect from nuclear damage and sperm abnormalities in NA-STZ diabetes and the lack of free radical scavenging action can be considered as the possible rationale. The non-significant reduction in the micronuclei and sperm abnormality frequency after the administration of Pio+Gmp and insulin indicated the importance of antioxidant potential in addition to the antidiabetic effect in minimising the hyperglycaemia-mediated oxidative stress and cellular damages. Gmp is reported to possess antioxidant properties (39), but the present study suggests that the dosage and duration of co-exposure with Pio may not be sufficient to exploit the free radical scavenging potential of this drug.

Conclusion

The combination of Pio with Met prevented NA-STZ-mediated oxidative stress and erythrocyte and sperm abnormalities. With optimal control of hyperglycaemia, the combination could play an important role, especially in young diabetic patients, in avoiding the nuclear complications related to hyperglycaemia and oxidative stress.

Author’s contributions

Data collection and assembly: SIR, KD. All authors contributed equally to the conception and design of the study, data analysis and interpretation, as well as drafting and critical revision of the article.

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