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Influence of combination of Rosiglitazone wtih Metformin/Glimepiride on Erythropoeitic and germinal cell damages in male diabetic rats

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Abstract
Monotherapy of oral hypoglycemics in chronic diabetes rarely maintain the optimum glycemic control and is considered the major cause for late onset complications. In this study combination of rosiglitazone (Rosi-0.2 mg/kg) with metformin (Met-50 mg/kg) or glimepiride (Gmp – 0.2 mg/kg) orally once daily for 4 weeks was tested against type 2 diabetes mellitus (T2DM) induced by nicotinamide (NA-230 mg/kg, i.p) and streptozotocin (STZ-65 mg/kg, i.p) in male Wistar rats. Results indicated that experimental T2DM significantly (P<0.001) increased micronucleated erythrocytes, sperm shape abnormality and decreased the P/N (polychromatic: normochromatic erythrocytes) ratio, sperm count and weight of testis along with an increase in the oxidative stress and blood glucose level compared to normal animals. Administration of Rosi+Met significantly (P<0.05) reduced the population of micronucleated erythrocytes, sperm shape abnormality, hyperglycemia besides improving the sperm count and antioxidant defense system in the diabetic rats. However, Rosi+Gmp activity was restricted to only the antidiabetic effect. The observations suggest that Rosi+Met reduced the somatic and germinal cell damages in the diabetic animals owing to the antioxidant property.

Keywords: *Combination therapy, Rosiglitazone, Metformin, Glimepiride, Anti-mutagenic, Antioxidant.*

Introduction

Type 2 diabetes mellitus (T2DM) is characterized by defects both in insulin secretion and insulin action. These abnormalities all together contribute to abnormal glucose metabolism. The degree and duration of hyperglycemia is the main reason for the chronic complications due to T2DM.[1] High blood sugar level determines overproduction of reactive oxygen species (ROS) that virtually damages all cellular components including DNA and thus contributing in oxidative stress related diseases both in the present generation as well in the progeny.[2]

Clinical data suggests that glycemic control with monotherapy cannot be maintained in approximately 10% of patients requiring the addition of another antidiabetic drug. Therefore T2DM patients are often treated with a combination of antidiabetics with different and complimentary mechanism of actions to achieve and maintain the optimal glycemic control.[3] Many a times antidiabetics in combination cause unwanted side effects.[4] Sulphonylureas (SUs) and biguanides are the most preferred combination but their treatment reported to carry a risk of hypoglycemia requiring either alteration of the doses or discontinuation of the combination.[5]

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Thiazolidininediones (TZDs) can be used in combination with SUs or biguanides and these combinations have been found to decrease the HbA1c ~1%, providing long-term glycemic control. In addition, the combination of TZDs with SU or biguanides is reported to reduce hyperinsulinemia, insulin resistance and improves factors that have been implicated in the pathogenesis of cardiovascular complications such as dyslipidemia, inflammation, endothelial dysfunction.[6] However, rosiglitazone (Rosi) a member of TZDs has been reported to interfere in the erythropoeitic cell-cycle both in normal and diabetic rats.[7,8] Further, Rosi treated rats showed higher incidences of nuclear damage in liver cells and leucocytes in normal condition.[9] Though, individually Rosi, metformin (Met) and glimepiride (Gmp) have suppressed the oxidative stress mediated nuclear damages in nicotinamide (NA)-streptozotocin (STZ) induced T2DM,[8,10,11] their role in combination need to be studied. Hence the present study will evaluate the role of Rosi in combination with Met/Glim against the micronuclei frequency and sperm abnormalities in the NA-STZ T2DM in Wistar rats.

Materials and Methods

Chemicals

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

Animals

Eight week-old healthy, laboratory bred, male Wistar rats weighing 180 ± 10 gm were maintained under standard laboratory conditions at a temperature 20±2^{o} C, 12 hour 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 STZ and NA.[12] The animals received intraperitoneal 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 of 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 eight animals in each group. The doses of Rosi (0.2 mg/kg),[13] Met (50 mg/kg)[14] and Gmp (0.2 mg/kg)[15] were selected as per the previous reports and depending on their individual concentration found in the antidiabetic combination formulation meant for clinical use.[16,17] The drugs 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),[18] and insulin (1 IU/kg, sc, 4 weeks),[19] were used as standard antioxidant and hypoglycemic agents, respectively. Before the administration, Rosi, Gmp and α-tocopherol were suspended in 1% w/v carboxy methyl cellulose (CMC) whereas insulin was reconstituted in water for injection and Met was dissolved in distilled water to obtain the required dose. Irrespective of treatment, a 12 hr fasting state was maintained in all the groups where the animals were provided with only water *ad libitum*.

Bone marrow micronucleus test

The modified method of Schmid was followed to perform the bone marrow MN test.[20] The animals after respective treatment were sacrificed by cervical dislocation under light ether (2 ml/kg, open drop method) anesthesia.[21] Animals were cut open to excise femur and tibia. Bone marrow MN slides were prepared by using the modified method of Schmid. Marrow suspension from femur and tibia bones (both the sides) 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 placed on a clean glass slide and 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 color round fragments in two forms of RBCs (ie, polychromatic erythrocytes as PCEs and normochromatic erythrocytes as NCEs).[22] About 2000 PCEs and corresponding NCEs were scanned for the presence of MN and 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,[23] was followed to study the sperm shape abnormality in cauda epididymis of the rats. One thousand sperms per animal were screened to find the different types of abnormality in one of the cauda epididymis. Six types of abnormalities such as hookless, banana shape, amorphous, coiled/curved, double headed and double tailed were evaluated and the total abnormality was represented as % abnormal sperms.[24]

The caudal sperm count test was performed according to the method described by D'Souza.[25] 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 glucose estimation

Blood samples were collected from the retro-orbital plexus under light ether anesthesia.[21] The serum was separated by centrifugation (1000 rpm) and immediately analyzed to determine the antioxidant enzyme activity.

Fasting blood glucose estimation was done using a 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 evaluation was done by One-way ANOVA followed by multiple comparisons by Bonferroni test for bone marrow MN test[28] and Mann- Whitney U test for the sperm abnormality[29] and Newman-Keuls for antioxidant study[30], respectively. $P<0.05$ was considered to indicate the significant difference.

Results

A. Effect of combination of Rosiglitazone with Metformin/Glimepiride on the frequency of bone marrow micronucleus in NA-STZ induced diabetic rats.

The experimental T2DM increased ($p<0.001$) the incidences of MN in both PCEs and NCEs and reduced the P/N ratio compared to the normal animals. Administration of combination of Rosi+Met significantly reduced the percentage MN in both PCEs ($p<0.01$) and NCEs ($p<0.05$) without altering the P/N ratio compared to the diabetic animals. The percentage inhibition was found to be 17.7% for PCEs and 14.5% for NCEs compared to the diabetic group. However, administration of Rosi+Gmp did not alter the frequency of MN in erythrocytes and P/N ratio in the diabetic state. Further, the α-tocopherol treatment to the diabetic animals significantly ($p<0.001$) decreased the number of micronucleated cells in both PCEs and NCEs and enhanced the P/N ratio as well, compared to the T2DM group. The standard hypoglycemic agent insulin did not prevent the nuclear damage induced by the T2DM (Table 1).

Table 1: Effect of combination of Rosiglitazone with Metformin/Glimepiride on the frequency of bone marrow micronucleus in NA-STZ induced diabetic rats

Bone marrow micronucleus test	Treatment and Dose (mg/kg)					
	Control (Saline- 0.5 ml / kg)	NA (230 mg) + STZ (65 mg)	NA-STZ + Rosi (0.2 mg) + Met (50 mg)	NA-STZ + Rosi (0.2 mg) + Gmp (0.2 mg)	NA-STZ + α-Tocopherol (20 mg/kg)	NA-STZ + Insulin (1 IU/kg)
% MN in PCEs	0.39 ± 0.01	1.41 ± 0.08 [a]	1.16 ± 0.10[a] **	1.39 ± 0.61	1.17 ± 0.14 [a] ***	1.47 ± 0.02 [a]
% MN in NCEs	0.41 ± 0.02	1.24 ± 0.12 [a]	1.06 ± 0.08[a] *	1.17 ± 0.11	0.96 ± 0.15 [a] **	1.27 ± 0.10 [a]
P/N ratio	1.08 ± 0.03	0.79 ± 0.02 [a]	0.82 ± 0.06 [a]	0.81 ± 0.06	0.88 ± 0.07 [a] ***	0.76 ± 0.04 [a]

Values are expressed as Mean ± SD, NA – Nicotinamide, STZ – Streptozotocin, Rosi- Rosiglitazone, Met-Metformin, Gmp-Glimepiride, N=8 Statistics: One way Anova followed by Bonferroni test. [a] $P<0.001$ compared with the Control; *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared with the Diabetic group

Table 2: Effect of combination of Rosiglitazone with MetforminGlimepiride on the Sperm morphology and sperm count in NA-STZ induced diabetic rats

Sperm abnormality test	Treatment and Dose (mg/kg)					
	Control (Saline- 0.5 ml / kg)	NA (230 mg) + STZ (65 mg)	NA-STZ + Rosi (0.2 mg) + Met (50 mg)	NA-STZ + Rosi (0.2 mg) + Gmp (0.2 mg)	NA-STZ + α-Tocopherol (20 mg/kg)	NA-STZ + Insulin (1 IU/kg)
Weight of Testis (gm)	1.23 ± 0.02	1.18 ± 0.04 [a]	1.20 ± 0.09	1.20 ± 0.17	1.19 ± 0.07	1.24 ± 0.27
Total % Abnormality	1.04 ± 0.07	1.64 ± 0.07 [b]	1.49 ± 0.09 [a] *	1.60 ± 0.08	1.21 ± 0.34 **	1.59 ± 0.08 [b]
Sperm count (10^6)	33.18 ± 1.36	27.77 ± 1.31 [b]	29.88 ± 1.90 [b] *	28.01 ± 2.32	31.87 ± 2.76 **	27.69 ±2.23 [b]

Values are expressed as Mean ± SD, NA – Nicotinamide, STZ – Streptozotocin, Rosi- Rosiglitazone, Met-Metformin, Gmp-Glimepiride, N=8 Statistics: One way Anova followed by Mann-Whitney U test [a]$P<0.05$, [b] $P<0.001$ compared with the control; *$P<0.05$, **$P<0.01$ compared with the Diabetic group

Table 3: Effect of combination of Rosiglitazone with Metformin/Glimepiride on the serum antioxidant status and glucose level in NA-STZ induced diabetic rats.

Serum antioxidant status and glucose level	Treatment and dose (mg/kg)					
	Control (Saline- 0.5ml / kg)	NA (230 mg) + STZ (65 mg)	NA-STZ + Rosi (0.2 mg) + Met (50 mg)	NA-STZ + Rosi (0.2 mg) + Gmp (0.2 mg)	NA-STZ + α-Tocopherol (20 mg/kg)	NA-STZ + Insulin (1 IU/kg)
Catalase (units/mg protein)	6.39 ± 0.34	3.12 ± 0.38 [b]	3.55 ± 0.27 [b] *	3.12 ± 0.29	5.27 ±0.66 [a] ***	3.11 ± 0.05 [b]
SOD (units/mg protein)	0.46 ± 0.05	0.22 ± 0.06 [b]	0.31 ± 0.04 [b] *	0.22 ± 0.01	0.43 ± 0.05 ***	0.23 ± 0.09 [b]
Blood glucose (mg/dl)	92.3 ± 3.44	174.3±6.32 [b]	131.64±5.01[b]***	162.14±4.14 **	157.4 ± 6.47 [b] *	143.8 ± 5.93 [b] ***

Values are expressed as Mean ± SD, NA – Nicotinamide, STZ – Streptozotocin, Rosi- Rosiglitazone, Met-Metformin, Gmp-Glimepiride, N=8. One way Anova followed by Newman-Keuls. [a] $P<0.01$, [b] $P<0.001$ compared with the Control *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared with the diabetic group

B. Effect of combination of rosiglitazone with metformin/glimepiride on sperm morphology and sperm count in NA-STZ induced diabetic rats.

NA-STZ induced T2DM in Wistar rats significantly increased (p<0.001) the sperm shape abnormalities and reduced the weight of testis (p<0.05) and sperm count (p<0.001) compared to the normal animals. The combination therapy of Rosi+Met suppressed (p<0.05) the sperm morphology defects and enhanced (p<0.05) the sperm count but does not alter the diminished weight of the testis compared to the diabetic animals. The percentage inhibition in the sperm shape abnormality and sperm count was found to be 9.14% and 7.6% respectively compared to the diabetes. Administration of α-tocopherol minimize (P<0.01) the sperm abnormality and improved the sperm count in the diabetic animals. Furthermore, Rosi+Gmp and insulin treatment to diabetic animals did not prevent the NA-STZ mediated sperm abnormalities and diminished testis weight (Table 2).

C. Effect of combination of rosiglitazone with metformin/glimepiride on the serum antioxidant status and glucose level in NA-STZ induced diabetic rats.

The type-2 diabetic condition after the administration of NA and STZ significantly enhanced (p<0.001) both oxidative stress and blood glucose level compared to the control animals. Rosi+Met treatment reduced (p<0.001) the hyperglycemia besides enhancing (p<0.05) the serum catalase and SOD levels compared to the diabetic state. α-tocopherol therapy elevated (p<0.001) the antioxidant defense and showed a mild (p<0.05) anti-hyperglycemic activity too, in the diabetic animals. On the other hand, Rosi+Gmp and insulin treatments does not alter the oxidative stress, though they produced significant (p<0.01) glucose lowering effect in the diabetic rats (Table 3).

Discussion

The results from this study indicated that NA-STZ mediated hyperglycemia enhanced nuclear damage in erythrocytes, increased the sperm shape abnormality, and reduced the sperm count and weight of testis in diabetic rats. The observations suggest that these changes are related to the increased oxidative stress due to the elevated blood sugar level (Table 3). The mechanisms suggested for STZ-mediated hyperglycemia and related somatic and germinal cell complications are due to the activation of several cellular damaging pathways by free radicals such as accelerated formation of advanced glycation end products (AGE), polyol pathway, hexosamine pathway and protein kinase-C.[31, 32]

Oxidative damages to DNA can occur by many routes including oxidative modifications of the nucleotide bases or sugars or by forming cross links. It is known that such modifications can lead to mutations, cellular aging and death.[2] The somatic cell defects has the tendency to cause various types of neurological defects, heart ailments, carcinogenesis, aging etc, while the germinal cell damage, apart from infertility can also result in a variety of pathogenesis in the off-spring, including childhood cancers.[2, 33]

Combination therapy of Rosi+Met reduced the NA-STZ-mediated hyperglycemia, micronuclei (MN) frequency and sperm abnormalities. The combination also enhanced the serum level of CAT and SOD in the diabetic condition. CAT present in many plants, animals and aerobic bacteria, efficiently promotes the conversion of hydrogen peroxide to water and molecular oxygen.[26,32] 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.[27,32] Together, these two enzymes are reported to limit the complications associated due to increased generation of free radicals.[31,32] These observations indicate that compounds possessing the antioxidant potential could limit the oxidative damages to the somatic and germinal cells.[32] The antioxidant potential of Rosi and Met is already reported in the literature.[34,35] The role of antioxidants in reducing the ROS mediated nuclear complications can be further ascertained from the results of α-tocopherol which are in accordance with earlier report.[36]

In case of Rosi+Gmp, although the combination demonstrated antidiabetic effect but did not produced the antioxidant activity, probably the lack of antioxidant property of the combination is responsible for its inability to protect the nuclear damages in diabetes. Though Gmp is also reported to possess the antioxidant potential, [37] the dosage and the duration of the combination might not be sufficient to overcome the ROS action in T2DM. In addition, insulin treatment also produce similar action thus suggesting the relationship between the antioxidant activity of the compound (s) and the ability to suppress the MN frequency and spermatozoa defects in diabetes.[18,33,38]

Different treatments including the α-tocopherol did not alter the diminished weight of testis indicating the inability of the drug(s) to augment the microtubule proliferation in diabetic animals.[39] More studies are required to understand the precise mechanism for this action. Rosi in the previous study has been reported to enhance the nuclear damage in hepatocytes and to some extent in leucocytes.[9] However, recent study contradicts these observations as Rosi was found to reduce the frequency of MN formation in erythrocytes.[8] Our findings too supports the anti-mutagenic property of Rosi in combination with Met in diabetic state. The potent antioxidant property of TZDs has been implicated for averting the nuclear damage.[35, 40] Further, both the combinations do not produce hypoglycemia in the diabetic rats and this property could benefit the diabetics who are at risk from hypoglycemia.[3]

Another finding of the study is that addition of Met/Gmp showed non-significant elevation in P/N ratio, suggesting inclusion of an antioxidant-antidiabetic agent benefited in minimizing the role of Rosi on the cell cycle erythrocytes. As reported earlier, Rosi induces cell growth arrest or death in normal and cancer cells.[7, 8] The mechanism suggested include the modulation in the activity of TSC2 (Tuberin) complex with subsequent suppression of mTOR (molecular target of ripamycin) signaling.[41, 42] On contrary, Met being an antioxidant[35] and an inhibitor of mTOR[43] has been reported to enhance the P/N ratio in the diabetic animals.[10]

This information suggests that Rosi might have suppressed the erythropoiesis through multiple mechanisms apart from inhibiting the mTOR. Further, reports illustrate that compounds having free radical scavenging activity encourage the proliferation of erythrocytes.[18] Since both Met and Gmp are known antioxidants, their inclusion with Rosi could have played a vital role in the enhancement of P/N ratio. Hence, it can be suggested that addition of Met to Rosi has limited some of the nuclear complications associated with the Rosi- monotherapy, besides protecting the host cell from the ROS interceded erythrocyte and germinal cell damages in the diabetic condition.

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