ANTI-CLASTOGENIC EFFECT OF ROSIGLITAZONE AGAINST THE NICOTINAMIDE-STREPTOZOTOCIN INDUCED NUCLEAR DAMAGES IN WISTAR RATS

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Summary

Increased oxidative stress due to the chronic hyperglycemia plays a basic role in the mutagenic changes that contribute in the secondary complications including cancer. Rosiglitazone (RSG), a PPAR-γ ligand was tested against the nicotinamide (230 mg/kg, ip) and streptozotocin (65 mg/kg, ip) induced nuclear damages in the experimental type-2 diabetes mellitus (T2DM). The anti-clastogenic effect of RSG (1, 10 and 100 mg/kg, p.o daily for 4 weeks) was evaluated in diabetic male Wistar rats using bone marrow micronucleus test system. α-tocopherol (20 mg/kg, p.o) was used as an internal antioxidant standard agent against the diabetes induced nuclear damage and oxidative stress. The in vitro antioxidant activity of RSG and α-tocopherol was evaluated by DPPH and superoxide anion scavenging methods. The results indicate that administration of RSG produced dose-dependent anti-mutagenic activity. RSG at 10 and 100 mg/kg reduced ($P<0.01$) the frequency of micronuclei in both polychromatic erythrocytes (PCEs) and normochromatic erythrocytes (NCEs) in the diabetic group. However, RSG at higher dose (100 mg/kg) produced further suppression of P/N. α-tocopherol significantly ($p<0.001$) prevented the mutagenic changes in the diabetic rats. The in vitro antioxidant study indicated that the EC_{50} value for RSG and α-tocopherol was 45.5 µg/ml and 7.75 µg/ml respectively in DPPH method, while in superoxide anion scavenging method it was found to be 65.5 µg/ml for RSG and 15.5 µg/ml for α-tocopherol. The observations suggest that RSG posses antimutagenic property against the diabetes induced nuclear damages and this effect could be related to the antioxidant potential of RSG.

Key words: Rosiglitazone, nicotinamide, streptozotocin, type-2 diabetes mellitus, bone marrow micronucleus test, antioxidant**.**

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Introduction

The peroxisome proliferator activated receptor – γ (PPAR- γ) is a member of the nuclear receptor super family. The agonists of PPAR- γ (Thiazolidinediones; TZDs) are used as oral anti-hyperglycemic drugs. Troglitazone was the first agent to be approved but was later withdrawn from the market due to severe hepatotoxicity [1]. Rosiglitazone and pioglitazone are the other agonists currently available for the clinical use and are reported to possess no/less hepatotoxicity. Both these agents are known to implicate in the adipocyte differentiation, insulin sensitivity and inflammatory process [2]. The studies conducted on TZDs reported that the compounds are effective against lipodystrophy, polycystic ovary syndrome, cancer [2-5]. Besides, the drugs are also known to possess antioxidant potential against several free radicals [6].

Diabetes mellitus type-2 (T2DM) is associated with a variety of metabolic abnormalities, principle among them is hyperglycemia. A considerable amount of evidence suggests that oxidative stress may play an important role in the pathogenesis and complications of diabetes. The accumulation of the products of the oxidative stress, especially the reactive oxygen species can cause the damage to biological macromolecules like proteins, lipids and DNA [7]. Mutations as a result of DNA damage can cause cancer, heart ailments, aging and neurological diseases [7, 8].

Genotoxicity testing assumes importance since the consequences of the mutation can be observed both in the present generation as well in future progeny. Among the battery of tests available to evaluate the DNA damage, rodent bone marrow micronucleus is considered as an important assay since the method identifies the clastogenic and antimutagenic potential of the test compound [8].

Our earlier study on TZDs revealed that the acute and chronic administration of Rosiglitazone (RSG – 1, 10 and 100 mg/kg) to normal animals did not produce significant damage to the nuclear components of erythrocytes although, the higher dose at 100 mg/kg in chronic duration (4 weeks) diminished the erythropoeisis [9]. Further, the literature review suggested that TZDs could possess the cancer chemo preventive potential in diabetic patients [5, 10] and extensive studies still need to be done in this direction. Therefore, to study the anti-clastogenic effect of TZDs in NIDDM, we planned to evaluate the role of RSG against the nuclear damages induced by nicotinamide and streptozotocin in Wistar rats.

Materials and methods

Chemicals

A gift sample of Rosiglitazone (RSG) was obtained from Biocon (India) 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 gm were maintained under standard laboratory conditions such as temperature 22-25^{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.

Induction of Type-2 diabetes

Experimental NIDDM was developed in adult rats by administering streptozotocin (STZ) and nicotinamide (NA). 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 ≈ 180 ± 8 mg/dl are only selected for the study [11].

Dosage, treatment and sampling

The animals were divided mainly in to three groups ie., control, diabetic and treatment. The treatment group received three doses of RSG (1, 10 and 100 mg/kg) [12, 13] orally per day for 4 weeks after the induction of diabetes. RSG at 100 mg/kg was selected since our earlier study indicated a suppressive effect on P/N ratio after the chronic administration of RSG to the normal animals [9]. The control and diabetic animals were administered saline (0.5 ml/kg) daily through out the treatment period. In this study, α-tocopherol (20 mg/kg, po) [14] was used as standard antioxidant agent. Before the administration, both α-tocopherol and rosiglitazone were suspended in 1% carboxy methyl cellulose (CMC). To study the influence of α-tocopherol alone on the normal condition, a parallel group was maintained where the animals received only the α-tocopherol (20 mg/kg) for 4 weeks.

Bone marrow micronucleus test

The modified method of Schimid was followed to perform the bone marrow MN test (Vijaylaxmi and Venu 1999). The animals after respective treatment were sacrificed by cervical dislocation under light anesthesia. 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 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 as PCEs and normochromatic erythrocytes as NCEs) (Photo-1). About 2000 PCEs and corresponding NCEs were scanned for the presence of MN [15].

In vitro antioxidant activity

a. DPPH method

A stock solution of 1, 1 diphenyl-2-picryl hydrazyl (DPPH) was prepared such that 75 µl of it in 3 ml of methanol gave an initial absorbance of 0.9 at 515 nm. This stock solution was used to measure the anti-radical activity. Decrease in the absorbance in the presence of test compound at different concentration was noted after 15 min. and the percentage inhibition was calculated by comparing the results of the test compound with the control [16].

b. Superoxide anion method

Superoxide radicals (O_2^{-}) were generated from the photo-reduction of riboflavin and were detected by NBT reduction method. The reaction mixture contained 6 mM EDTA with 3 µg NaCN, riboflavin (2 mM), NBT (50 mM), KH_2PO_4-Na_2HPO_4 buffer (67 mM, pH 7.8) and various concentrations of the test compound in a final volume of 3 ml. The tubes were illuminated under incandescent lamp for 15 min. The optical density (OD) at 530 nm was measured before and after illumination. The inhibition of superoxide radical was determined by comparing the absorbance of control with test [17].

Statistics

The statistical analyses of the results were done by One-way Anova followed by multiple comparison by Bonferroni test [18].

Results

A. Effect of Rosiglitazone on bone marrow micronucleus.

The results indicate that the chronic treatment of RSG had shown dose-dependent inhibition in the nuclear damage induced by diabetes type-2 (Table-1).

Experimental T2DM after the administration of NA and STZ had shown a significant ($P<0.001$) increase in the number of micronucleated erythrocytes compared to the control. The P/N ratio (polychromatic vs normochromatic erythrocytes) was also found to be diminished ($P<0.001$) in diabetic condition. RSG was tested in three doses viz., 1, 10 and 100 mg/kg against the mutagenic changes produced by the T2DM. RSG at 10 mg/kg significantly ($P<0.01$) reduced the micronucleated erythrocytes without affecting the P/N ratio. The percentage MN reduction produced by RSG (10 mg/kg) was found to be 15.6 % in PCEs and 17.74 % in NCEs compared to the diabetic group. Administration of RSG at 100 mg/kg had produced significant ($P<0.001$) reduction in MN PCEs (21.27 %) and MN NCEs (26.61 %)

but further suppressed (P<0.001) P/N ratio compared to the diabetes. The administration of lower dose of RSG (1 mg/kg) did not induce any significant change in the diabetic animals. Further, α-tocopherol used as an standard antioxidant had produced a significant (P<0.001) protection against the nuclear damages in the diabetic animals. The treatment had also enhanced (P<0.001) the P/N ratio compared to the NIDDM. However, the administration of α-tocopherol (20 mg/kg) to the normal animals did not show any clastogenicity (Table-1).

Table-1: Effect of Rosiglitazone on bone marrow micronucleus in NA-STZ induced type-2 diabetes

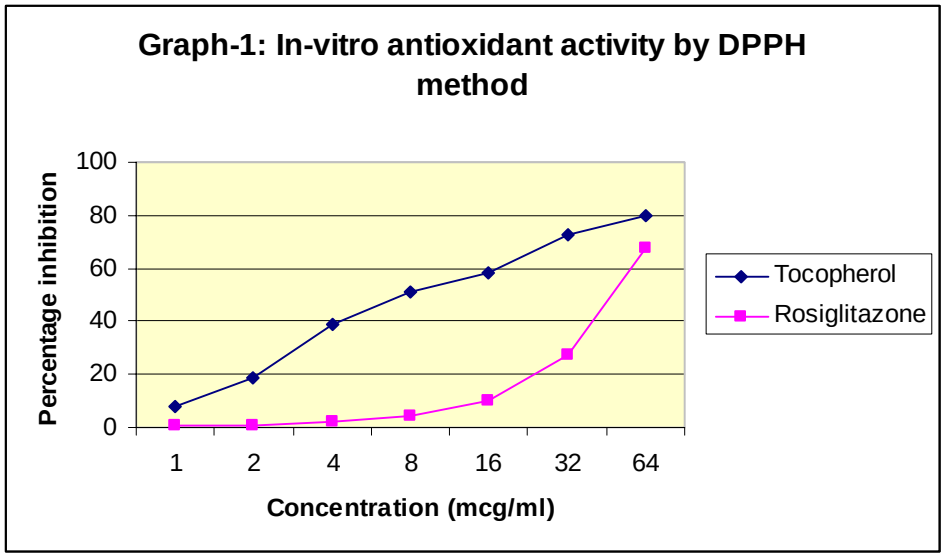
Treatment and Dose (mg/kg)	Micronucleus test		
	% MN in PCEs	% MN in NCEs	P/N ratio
Control (Saline- 1 ml / 500 gm)	0.39 ± 0.01	0.41 ± 0.02	1.08 ± 0.01
α-Tocopherol (20 mg/kg)	0.42 ± 0.04	0.40 ± 0.08	0.99 ± 0.14
NA (230 mg/kg) + **STZ** (65 mg/kg)	1.41 ± 0.08[c]	1.24 ± 0.13[c]	0.80 ± 0.02[c]
NA-STZ + α-Tocopherol (20 mg/kg)	0.82 ± 0.07***	0.82 ± 0.15***	0.94 ± 0.01***
NA-STZ + RSG (1 mg/kg)	1.34 ± 0.06	1.20 ± 0.01	0.80 ± 0.03
NA-STZ + RSG (10 mg/kg)	1.19 ± 0.07***	1.02 ± 0.05**	0.79 ± 0.01
NA-STZ + RSG (100 mg/kg)	1.11 ± 0.06***	0.91 ± 0.03***	0.71 ± 0.01***

Values are expressed as Mean ± SD, NA – Nicotinamide, STZ – Streptozotocin, RSG - Rosiglitazone
Statistics: One way Anova followed by Bonferroni test.
[c]p<0.001 *compared with the Control*
**p<0.01, ***p<0.001 *compared with the Diabetic group*

B. In vitro antioxidant activity

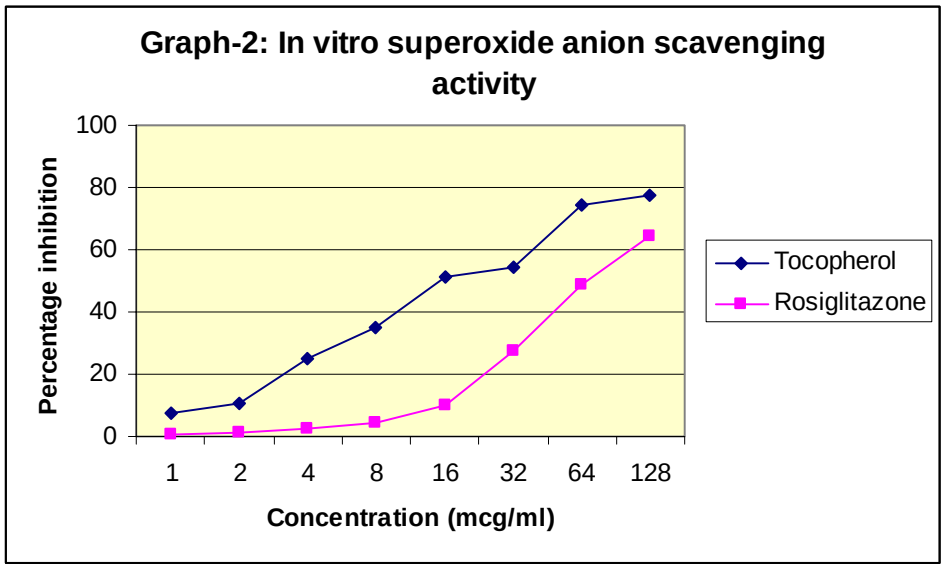
1. **DPPH method**

The antiradical activity of α-tocopherol and RSG measured by the DPPH method indicated that both the agents prevented the formation of free radicals. At lower doses, the RSG produced minimum inhibitory effect as compared to the α-tocopherol. The EC_{50} value for α-tocopherol and RSG was found to be 7.75 µg/ml and 45.5 µg/ml respectively (Graph-1).



2. **Superoxide anion method**

The superoxide anion scavenging activity indicated that RSG reduced the formation of $O_2^{\cdot-}$ and the scavenging activity was found to be less potent than α-tocopherol. The EC_{50} value for RSG and α-tocopherol was found to be 66.5 µg/ml and 15.5 µg/ml respectively (Graph-2).



Discussion

Diabetes mellitus (DM) is one of the common metabolic diseases, affect about 3-4 % of the world's population [6]. The data collected from the earlier studies suggest that there is a close link between hyperglycemia, oxidative stress and diabetic complications. High blood glucose level determines overproduction of reative oxygen species (ROS) by the mitochondria electron transport chain. High reactivity of ROS determines chemical changes in virtually all cellular components leading to DNA and protein modification and lipid peroxidation [7, 19].
In vivo rodent micronucleaus (MN) assay has been widely used to detect genotoxicity. The MN test is devised for evaluating the ability of test agents to induce structural and/or numerical chromosomal damage [7]. Both the kinds of damages are associated with the appearance and/or progression of tumors and with adverse reproductive and development outcomes [8]. The frequency of MN is evaluated in two types of erythrocytes viz., polychromatic erythrocytes (PCE, young erythrocytes still containing RNA) and normochromatic erythrocytes (NCE, mature erythrocytes). Further, agent treated animals and vehicle control animals provide the cytotoxic index as the damage tend to decrease the P/N ratio [15, 19]. In this study, an experimental type-2 DM was induced by the administration of nicotinamide (NA) and STZ. According to Masiello et al, the administration of NA prior to STZ partially protects the β-cells against the cytotoxic damages of STZ in adult rats and the changes that follows is reported to be closer to T2DM specially with regard to the insulin response towards glucose [10]. The observations indicated that the administration of NA-STZ to the Wistar rats had increased the frequency of MN in the erythrocytic population and had reduced the P/N ratio (Table-1). The earlier findings suggest that the changes observed in the frequency of MN and P/N ratio in the diabetic condition is mainly due to the oxidative stress mediated cytogenetic damages [7, 19]. The possible mechanisms involved are: increased polyol pathway flux and advanced glycation endpoint formation (AGE), the activation of protein kinase C (PKC) isoforms and the increased hexosamine pathway flux could all be the effects of hyperglycemia-induced overproduction of superoxide by the mitochondrial electron-transport chain [19, 20]. In addition, the diabetic condition is also reported to impair the response of the antioxidant genes to hyperglycemia, leading to reduced expression of antioxidant enzymes [19]. The present study indicated that administration of Rosiglitazone (RSG) has produced dose-dependent inhibitory effect on the frequency of micronuclei formation in the NA-STZ induced T2DM. However, the chronic administration of RSG (100 mg/kg) to the diabetic animals had shown further suppression of P/N ratio compared to the diabetic group (Table-1). The ability of RSG to prevent the diabetes mediated nuclear damage in the RBCs indicates that the treatment might reduce the mutation related complication in the diabetic patients. However, these observations contradict the earlier findings, where the authors have reported that RSG has the potency to increase the nuclear damage in the lymphocytes [21]. The variation in the action of RSG towards the two formed elements of blood needs to be further studied to find the actual mode of action.

The diminished P/N ratio in normal and diabetic animals indicates that RSG has the capacity to interfere in the cell cycle. This property can be attributed to the anticancer activity of RSG, since PPAR-γ ligands are known to posses the anti-tumor potential [21]. As per the earlier reports, RSG might show the inhibitory effect on the cell proliferation due to the activation of TSC2 (Tuberin) with subsequent suppression of m TOR (molecular target of

rapamycin) signaling [22, 23]. Further, RSG is also found to cause disruption in the cellular integrity by interfering in the transmembrane potential [23]. Similar mechanisms of RSG in this study might have contributed in the reduceed P/N ratio.

Previous studies suggest that antioxidant limits the nuclear damage by preventing the free radical action and exhibit their action by multiple mechanisms such as quenching of free radicals, inhibition of cell growth, induction of apoptosis, enhancement of immune function, modulation of carcinogen metabolizing enzymes etc [7, 19]. The present study indicated that RSG inhibited the free radical formation by DPPH and superoxide anion method. The EC_{50} values for RSG were found to be 45.5 µg/ml and 66.5 µg/ml by DPPH and superoxide anion scavenging methods respectively. The ability of RSG to inhibit the free radical formation indicate that RSG could be useful in reducing the ROS mediated oxidative stress and its complications. Since, α-tocopherol a known antioxidant had reduced the NA-STZ mediated nuclear and cytolytic damages in this study, it can be suggested that antioxidants could play a beneficial role in the ROS induced DNA damages. In addition, the earlier reports indicated that RSG possess antioxidant activity due to its ability to interfere in the MAPK-NF kB pathways [24]. Based on this, we assume that RSG reduced the T2DM mediated cytonuclear damages due to its antioxidant potential.

In conclusion, it can be suggested that the experimental T2DM had induced nuclear damages primarily due to the ROS mediated oxidative stress and RSG could have minimized the damages due to the antioxidant activity.

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