Research Paper

Protective role of glibenclamide against nicotinamide-streptozotocin induced nuclear damage in diabetic Wistar rats

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ABSTRACT

Objective: To evaluate the protective effect of glibenclamide against the experimental diabetes-induced nuclear damage in Wistar rats. **Materials and Methods:** The anti-mutagenic effect of glibenclamide (0.5, 5 and 50 mg/kg, p.o daily for 4 weeks) was evaluated against the nicotinamide (NA)-streptozotocin (STZ) induced type-2 diabetes mellitus using bone marrow micronucleus and sperm abnormalities tests. The antioxidant status was tested by estimating the serum levels of lipid peroxidation (LPO), catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx). **Results:** The results indicated that glibenclamide at 50 mg/kg decreased the frequency of micronuclei in erythrocytes ($P < 0.05$) and sperm shape abnormality ($P < 0.01$) besides enhancing the antioxidant status ($P < 0.05$) in the diabetic rats. However, glibenclamide treatment did not enhance the polychromatic and normochromatic erythrocytes (P/N) ratio and sperm count in the diabetic condition. **Conclusion:** The observations indicate that the glibenclamide has anti-mutagenic potential which could be related to the antioxidant effect and might also possess anti-proliferative property.

Key words: Antioxidant, glibenclamide, micronucleus test, sperm abnormality

INTRODUCTION

Sulfonylurea antidiabetic agents such as glibenclamide (GL), long used in the treatment of non-insulin dependent diabetes mellitus, are known to promote insulin secretion through inhibition of ATP-sensitive K+ (K_{ATP}) channels in the pancreatic cells.[1] Chemically GL (also known as glyburide) is a derivative of sulfonyl cyclo hexylurea.[2] Apart from the antidiabetic property, GL has also been reported to exhibit anti-nociceptive, anti-tumor and platelet aggregation inhibitor activities.[3-6] In addition, GL is found to cause the release of nitric oxide which is related to its stimulatory effect on endothelial Ca^{2+} levels causing endothelium dependent relaxation activity.[7] Research from the antioxidant studies suggests that GL has the potential to counteract the reactive oxygen species mediated oxidative stress.[8-10]

The uncontrolled hyperglycemia is considered to be the major cause for the oxidative stress induced diabetic complications.[11] The complications range from mild modification in the protein structures to genetic abnormalities. The nuclear defects in diabetic patients not only contribute to disorders like cancer, heart ailments in the present generation but also transmit the mutations to the future generation in form of defective genes in the reproductive cells like spermatozoa.[12,13] Thus, mutation tests like micronucleus and sperm abnormality test assume importance as they identify the somatic and germinal cells defects induced by the drug/disease.[13]

Since compounds possessing antioxidant and anticancer activities are strongly implicated to exhibit the anti-mutagenic potential[13,14] and the mutagenic test for GL was found to be negative,[15] we examined in this study the anti-clastogenic

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effect of GL against the nicotinamide-STZ induced DNA damage using bone marrow micronucleus and sperm abnormalities tests in male Wistar rats.

MATERIALS AND METHODS

Chemicals

A gift sample of glibenclamide (GL) was obtained from Micro Labs Pvt Ltd, Bangalore. The stains and other reagents/ chemicals used in this study were of analytical grade and procured from Himedia Labs (P) Ltd, Mumbai.

Animals

Eight week-old healthy, laboratory in-bred, male Wistar rats weighing 180 ± 10 g were maintained under standard laboratory conditions such as temperature 20 ± 2°C, 12 h light / dark cycle and was provided with water and pellet food (Amruth Biotech Ltd, Maharashtra) *ad libitum*. The experiments were conducted after obtaining the prior approval from the Institutional Animal Ethics Committee.

Induction of Type-2 diabetes

Experimental type-2 diabetes mellitus (T2DM) was developed in adult rats by administering streptozotocin (STZ) and nicotinamide (NA).[16] 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, i.p (Sigma Aldrich, USA) dissolved in 0.1 M citrated buffer (pH 4.5) immediately before use. A drop of blood collected from tail vein was estimated for blood glucose level after 2 days and the animals with glucose level ≈ 180 ± 8 mg/dl are only selected for the study. The moderate hyperglycemia was found to observe in 71.2% of rats treated with NA and STZ.

Dosage, treatment and sampling

The animals were divided mainly into seven groups ie., control, diabetic and treatment, consisting of eight animals in each group. Three doses of GL were selected viz., 0.5, 5 and 50 mg/kg, p.o as per the OECD and standard guidelines for the mutagenicity test,[17,18] the lowest and highest dose being 10 fold lesser and higher than the therapeutic dose (5 mg/kg)[19] respectively. The doses of GL were administered orally per day as gavage 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)[20] and insulin (1 IU/kg, s.c, 4 weeks)[21] were used as standard antioxidant and hypoglycemic agents, respectively. Before the administration, α-tocopherol and GL 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 Schmid was followed to perform the bone marrow MN test.[22] The animals after respective treatment were sacrificed by cervical dislocation under light anesthesia (Diethyl ether, 2 ml/kg, open drop method). 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 micronuclei (MN) were identified in two forms of RBCs (ie, polychromatic erythrocytes as PCEs and normochromatic erythrocytes as NCEs) [Figure 1]. About 2000 PCEs and corresponding NCEs were scanned for the presence of MN using 100X oil immersion objective. The P/N ratio was calculated by dividing total number of PCEs with corresponding NCEs per animal.[18]

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 animals were screened to find the different types of abnormality in one of the cauda epididymis. Six types of abnormalities such as amorphous, hookless, banana shape, fused, double headed and double tailed [Figure 2] were evaluated and finally represented as percentage total abnormality.[24]

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.[25]

In vivo antioxidant activity

Blood samples were collected from the retro-orbital plexus under light ether anesthesia. The serum was separated by centrifugation (1000 rpm, for 2 min) and immediately analyzed to determine the antioxidant enzyme activity.

Serum lipid peroxidation (LPO)

The procedure described by Ohkawa *et al*, 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. A reddish pink color developed was estimated at 532 nm which indicates the extent of peroxidation. The extent of lipid peroxidation was expressed as η mol/ mg protein.[26]

Catalase (CAT)

The estimation of catalase (EC 1.11.1.6) activity was done by determining the decomposition of H_2O_2 at 240 nm in an assay

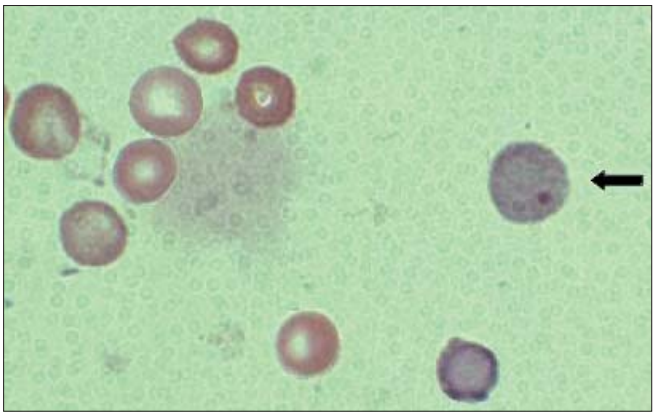

Figure 1: Micronucleated polychromatic erythrocytes [arrow indicates the MN in the PCEs with normal NCEs, stained with May-Grunwald-Giemsa, 100×magnifications]

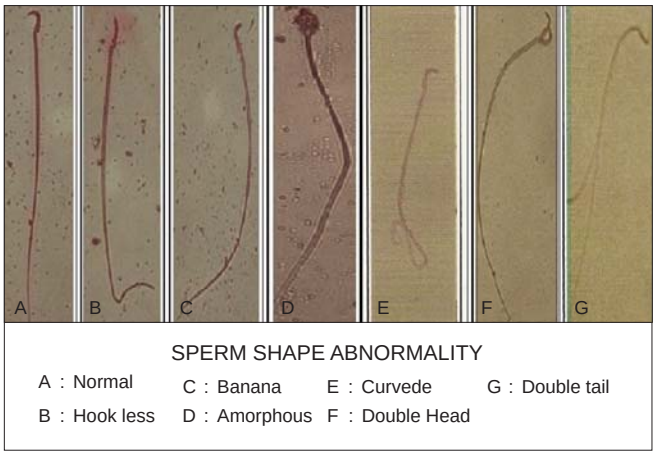

Figure 2: Different types of sperm shape abnormalities [A: normal; B: Hookless; C: banana; D: amorphous; E: fused and F: double head, stained with 1% aqueous eosin Y, 40 X magnification].

mixture containing the phosphate buffer (0.25 M, pH 7). One international unit of catalase utilized is that amount which catalyzes the decomposition of 1 mM H_2O_2 per min at 37°C and expressed in terms of unit / mg protein.[27]

Superoxide dismutase

The principle for measuring the Superoxide dismutase (SOD) (EC 1.11.1.1) depends on the detection of the O_2^- 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 colorimetrically at 560 nm. The concentration of SOD is expressed as units / mg protein.[28]

Glutathione peroxidase

Glutathione peroxidase (GPx) (EC 1.11.1.9) activity was assayed based on the modified method of Paglia and Valentine described by Heath and Tappel.[29] A 100 µl of the serum sample is incubated for 5 min at 37° with stock solution (0.12 mM NADPH and 1 unit of glutathione reductase prepared in the tris buffer) in a final volume of 1.65 ml. About 50 µl of cumene hydroperoxide (1 mg/ml) is added to start the reaction, and the absorbance at 340 nm is monitored for the rate of disappearance of NADPH and the GPx value was represented as µg of glutathione consumed/min/mg protein.

Blood glucose estimation

A drop of blood was collected from the tail vein and applied to the test zone of the glucose strip for immediate measurement of the fasting glycemia (mg / dl) using the Ascensia ENTRUST glucometer (Bayer healthcare Ltd, Mumbai).

Statistics

The statistical analyses were done by one-way ANOVA followed by multiple comparisons by Bonferroni test for bone marrow MN test,[30] while data on epididymal sperm shape abnormalities and sperm count were analyzed by employing a non parametric test, the Mann-Whitney U test.[31] The antioxidant data was analyzed by one way ANOVA. $P < 0.05$ was considered to indicate significant difference.

RESULTS

Effect of GL on the frequency of bone marrow micronuclei and P/N ratio in NA-STZ induced diabetic rats

Type-2 diabetes in Wistar rats after the administration of NA-STZ produced significant ($P < 0.001$) increase in the percentage micronucleated cells in both polychromatic and normochromatic erythrocytes besides reducing the P/N ratio compared to the normal animals. Administration of GL at 50 mg/kg reduced the incidences of MN in PCEs ($P < 0.01$) and NCEs ($P < 0.05$) but did not alter the P/N ratio compared to the NA-STZ animals. The percentage inhibition in the frequency was found to be 14.2% for PCEs and 11.3% for NCEs compared to the diabetes. Although GL at 5 mg/kg decreased ($P < 0.05$) the % MN in PCEs (% inhibition – 8.5%), this dose did not show any other alteration on MN in NCEs and P/N ratio. The lower dose of GL (0.5 mg/kg) did not minimize the nuclear damage induced by the diabetic condition. Further, administration of α-tocopherol (20 mg/kg) produced significant ($P < 0.01$) inhibition in the micronucleated cell populations besides enhancing the P/N ratio compared to the diabetic rats. However, insulin (1 IU/kg) did not induce significant change in the DNA damage mediated by the NA-STZ diabetic state [Table 1].

Effect of GL on the sperm morphology and sperm count in NA-STZ induced diabetic rats

The diabetic condition after the administration of NA-STZ significantly ($P < 0.001$) increased the sperm shape abnormality and reduced the sperm count and weight of the testis in comparison to the normal animals. The treatment of GL at 5 and 50 mg/kg significantly ($P < 0.01$) decreased the sperm

shape abnormality without affecting the diminished weight of testis and sperm count in the diabetic state. However, the lower dose of GL (0.5 mg/kg) did not prevent the sperm abnormalities and defects in the weight of testis induced by the diabetic condition. Administration of α-tocopherol reduced ($P < 0.01$) 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 GL on serum antioxidant status and glucose level in NA-STZ induced diabetic rats

NA-STZ diabetes significantly ($P < 0.001$) increased the LPO and reduced the levels of antioxidants enzymes such as CAT, SOD and GPx compared to the control group. GL at 50 mg/kg showed significant ($P < 0.05$) decrease in the LPO levels and enhanced the levels of antioxidant enzymes compared to the type-2 diabetes. The lower doses of GL (0.5 and 5 mg/kg), however, did not modify the oxidative condition. Further, administration of α-tocopherol to the diabetic animals significantly ($P < 0.001$) enhanced the antioxidant status. However, insulin treatment did not alter the oxidative condition in the experimental NIDDM. In addition, both GL and insulin significantly ($P < 0.001$) reduced the hyperglycemia in the diabetic rats, while the effect after α-tocopherol treatment was observed to be mild ($P < 0.05$) in comparison to the diabetic group [Table 3].

DISCUSSION

In this study, the experimental T2DM after the administration of NA-STZ produced significant defects in MN and sperm abnormality tests, besides enhancing the oxidative stress in diabetic rats. The GL treated diabetic rats showed decreased incidences of MN in erythrocytes, sperm abnormalities and blood glucose along with an enhanced antioxidant status [Tables 1-3].

According to Masiello *et al*, co-administration of NA and STZ produces stable, moderate hyperglycemia suitable for chronic diabetic studies.[16] NA partially protects the β-cells against the STZ-mediated cytotoxic damages. NA was found to preserve the intra cellular pool of NAD either by acting as a precursor of NAD or by inhibiting the activity of poly (ADP-ribose) synthetase which is an NAD consuming enzyme activated by STZ.[16] Earlier studies indicate that STZ can cause nuclear defect and sperm abnormalities, and 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).[32,33]

Among the oral hypoglycemics, metformin (Met) has been reported to exhibit the anti-mutagenic activity against adriamycin and STZ induced clastogenicity.[34,35] In comparison to Met, we found that GL did not show potent anti-mutagenic property. Further, glimepiride (Gmp) was also found to possess the anti-clastogenic effect in experimental diabetes.[36] Comparison between Gmp and GL indicated that both the compounds showed similar activities in terms of reduction in MN frequency, sperm shape abnormality and antioxidant status. These observations suggested that Met being a better

Table 1: Effect of glibenclamide 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 + Glibenclamide (0.5 mg/kg)	NA-STZ + Glibenclamide (5 mg/kg)	NA-STZ + Glibenclamide (50 mg/kg)	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]	1.32 ± 0.07[a]	1.29 ± 0.05[a*]	1.21 ± 0.07[a**]	1.17 ± 0.14[a***]	1.47 ± 0.02[a]
% MN in NCEs	0.41 ± 0.02	1.24 ± 0.12[a]	1.29 ± 0.12[a]	1.19 ± 0.16[a]	1.10 ± 0.10[a*]	0.96 ± 0.15[a**]	1.27 ± 0.10[a]
P/N ratio	1.08 ± 0.03	0.79 ± 0.02[a]	0.79 ± 0.04[a]	0.81 ± 0.06[a]	0.80 ± 0.05[a]	0.88 ± 0.07[a***]	0.76 ± 0.04[a]

Values are expressed as Mean ± SD, NA – Nicotinamide, STZ – Streptozotocin, 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 glibenclamide on the sperm morphology and sperm count in NA-STZ induced diabetic rats

Sperm abnormality test	Treatment and dose (mg/kg)						
	Control (Saline-0.5ml / kg)	NA (230 mg) + STZ (65 mg)	NA-STZ + Glibenclamide (0.5 mg/kg)	NA-STZ + Glibenclamide (5 mg/kg)	NA-STZ + Glibenclamide (50 mg/kg)	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]	1.18 ± 0.03[a]	1.20 ± 0.03	1.21 ± 0.03	1.19 ± 0.07	1.24 ± 0.27
Total % abnormality	1.04 ± 0.07	1.64 ± 0.07[b]	1.56 ± 0.05[c]	1.47 ± 0.08[c**]	1.39 ± 0.12[c***]	1.21 ± 0.34[**]	1.59 ± 0.08[b]
Sperm count (106)	33.18 ± 1.36	27.77 ± 1.31[b]	27.62 ± 1.99[c]	27.06 ± 2.74[c]	26.37 ± 1.79[c]	31.87 ± 2.76[**]	27.69 ± 2.23[b]

Values are expressed as Mean ± SE, NA – Nicotinamide, STZ – Streptozotocin, N=8, Statistics: Mann-Whitney U test, a $P<0.05$, b $P<0.01$, c$P<0.001$ compared with the control, **$P<0.01$, ***$P<0.01$ compared with the Diabetic group

Table 3: Effect of glibenclamide 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-5ml / kg)	NA (230 mg) + STZ (65 mg)	NA-STZ + Glibenclamide (0.5 mg/kg)	NA-STZ + Glibenclamide (5 mg/kg)	NA-STZ + Glibenclamide (50 mg/kg)	NA-STZ + α-Tocopherol (20 mg/kg)	NA-STZ + Insulin (1 IU/kg)
Lipid peroxidation (η mol/ mg protein)	2.39 ± 0.20	3.35 ± 0.22^{c}	3.50 ± 0.39^{c}	3.59 ± 0.28^{c}	2.86 ± 0.41^{a*}	2.40 ± 0.37***	3.36 ± 0.11^{c}
Catalase (units / mg protein)	6.39 ± 0.34	3.12 ± 0.38^{c}	3.17 ± 0.29^{c}	3.20 ± 0.41^{c}	3.84 ± 0.19^{c**}	5.27 ± 0.66^{b***}	3.11 ± 0.05^{c}
SOD (units / mg protein)	0.46 ± 0.05	0.22 ± 0.06^{c}	0.21 ± 0.04^{c}	0.27 ± 0.02^{c}	0.31 ± 0.03^{c**}	0.43 ± 0.05***	0.23 ± 0.09^{c}
Glutathione (GSH) (µg / mg protein)	3.27 ± 0.12	1.748 ± 0.27^{c}	1.70 ± 0.21^{c}	2.02 ± 0.12^{c*}	2.03 ± 0.11^{c*}	2.84 ± 0.52**	1.74 ± 0.15^{c}
Glutathione peroxidase (GPx) (µg of glutathione consumed / mg protein)	1.52 ± 0.04	1.19 ± 0.17^{c}	1.19 ± 0.24^{c}	1.21 ± 0.16^{c}	1.41 ± 0.17^{a*}	1.41 ± 0.10^{a*}	1.17 ± 0.86^{a}
Blood glucose (mg/l)	92.3 ± 3.44	174.3 ± 6.32^{c}	159.1 ± 7.34^{c**}	134.6 ± 6.39^{c***}	120.1 ± 7.32^{c***}	157.4 ± 6.47^{c*}	143.8 ± 5.93^{c***}

Values are expressed as Mean ± SD, NA – Nicotinamide, STZ – Streptozotocin, N=8, Statistics: One way ANOVA, $^{a}P<0.05$, $^{b}P<0.01$, $^{c}P<0.001$ compared with the Control, $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ compared with the Diabetic group

antioxidant compared to Gmp/GL, might have shown better protection against the oxidative-stress induced nuclear damages.

Compounds possessing antioxidant property are known to produce the anti-mutagenic effect. Vitamins such as vitamin E, vitamin C and vitamin A have all been found to reduce the mutagenic complications due to their antioxidant activity.[22,37,38] Besides, Met and Gmp have also been reported to exhibit the antimutagenic activity due to the antioxidant effect.[34,36] The importance of antioxidant activity in reducing the mutagenesis can be ascertained from the observations of insulin treatment to the NA-STZ diabetic rats. Although insulin in this group significantly reduced the hyperglycemia, it did not avert the oxidative stress meditated increased MN frequency and sperm abnormalities [Tables 1-3]. Moreover, the mild anti-diabetic effect of α-tocopherol observed in this study [Table 3] is also related to the antioxidant potential.[39]

The antioxidant activity of GL is well documented in the literature. Earlier studies indicated that GL possess direct mechanism to enhance the levels of SOD, CAT, glutathione-s-transferase (GST) besides reducing the LPO in the STZ diabetic animals.[9,10,40,41] In another study, it was suggested that the inhibition of ATP sensitive potassium channels decreases the generation of · OH through xanthine oxidase reaction.[8] Considering these information, it can be suggested that GL in this study could have exerted similar mechanism to decrease the oxidative stress responsible for nuclear damage in erythropoietic cells and sperm abnormalities in diabetic animals. Further, the antidiabetic action of GL is well documented in the literature and is due to the inhibition of ATP-sensitive potassium channels that triggers the release of insulin from β-cells.[1]

Another observation of this study is that the administration of GL did not enhance the P/N ratio and sperm count in diabetic animals [Tables 1 and 2]. The data suggests that GL did not enhance the proliferation of fast multiplying cells in the body. This property can be linked to the anticancer potential of GL. The possible mechanism for anti-tumor activity include inhibitory effect on the voltage gated potassium channel which otherwise implicated in cellular proliferation by causing the opening of tumor micro vessels or capillaries.[4,5] In addition, research also suggests that potassium channel inhibitors like GL induce apoptotic cell death by increasing the Ca^{2+} influx in the cell.[42] We also observed that both GL and α-tocopherol did not increase the diminished weight of testis in diabetic condition [Table 2], suggesting that both the agents in the tested dose and duration did not stimulate the microtubular proliferation of Leydig cells.[33]

The observations from this study suggest that anti-diabetics possessing antioxidant property could be beneficial in reducing the oxidative stress mediated health complications such as neurological defects, aging, infertility, birth abnormalities including childhood cancer. A more detailed research involving different anti-mutagenic models is suggested to find the precise role of GL in diabetes and oxidative stress mediated nuclear deformities.

CONCLUSION

The experimental type-2 diabetes after the administration of NA-STZ increased the somatic and germinal cell damage besides increasing oxidative stress. GL treatment at 50 mg/kg decreased the frequency of micronuclei, sperm shape abnormality and enhanced the antioxidant status but did not increase the P/N ratio and sperm count. The data suggest that

though GL reduced the incidence of MN in PCEs and NCEs, and defects in the sperm morphology, it also possesses anti-proliferative effect.

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