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Neuroprotective Potential of Genistein in Mitochondrial Dysfunction Mediated Apoptosis in Diabetic Neuronal Complications

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ABSTRACT

Mitochondria are subjected to direct attack by large amounts of reactive oxygen species and compromised mitochondrial function ultimately causes neuronal apoptosis and neurodegeneration causing cognitive dysfunctions and neuropathy. Therefore, therapies based on maintaining mitochondrial integrity might be highly beneficial in such conditions. The motive behind present study is to investigate the outcome of genistein treatment against mitochondrial function impairment in brain of streptozotocin-induced diabetic mice. The mitochondrial suspension from cortex of diabetic mice was evaluated for any dysfunction aligned with levels of mitochondrial respiratory chain enzymes, thiols and oxidative stress. Mitochondrial apoptosis has been examined by investigating caspase-3 activation and cell viability by MTT assay. Chronic genistein treatment (2.5, 5.0 and 10.0 mg/kg, i.p., twice daily) significantly restored levels of mitochondrial respiratory chain enzymes (complex I, II, III and IV), reversed oxidative stress (malondialdehyde and superoxide dismutase levels), increased mitochondrial thiols (protein, non-protein and total thiols), decreased neuronal apoptosis (caspase-3 activity) and increased cellular viability (MTT assay) in the mitochondrial suspension from the cortex of streptozotocin-induced diabetic mice. Treatment with metformin also improved diabetes induced mitochondrial function impairment and significantly dragged these biochemical parameters towards respective controls. The study signifies that genistein supplementation might be a prospective therapeutic approach for the treatment and/or avoidance of diabetic linked neuronal dysfunction.

Keywords: Apoptosis; Caspase-3; Diabetes; Mitochondrial complex; MTT; Thiols

INTRODUCTION

Chronic hyperglycemia leads to an assortment of macro and micro vascular complications leading to injure, dysfunction and collapse of assorted organs, especially eyes, kidneys, brain, nerves, heart and blood vessels [1]. The contribution of hyperglycemia in pathogenesis of diabetic neuronal degradation and impairment in learning and memory is beyond controversy, which eventually leads to accumulation of advanced glycation end-products, protein kinase C isoform activation, mitochondrial dysfunction and altered activities of other enzymes like cholinesterase, Na^+-K^+-ATPase, catalase, NTPDase and 5′-nucleotidase [2]. All these pathways converge in the production of oxidative stress. A consistent feature common to all cell types that are damaged by hyperglycemia is increased reactive oxygen species (ROS) production [3].

Damaged mitochondria by direct ROS attack, progressively become less efficient, losing their functional integrity and release more ROS [4]. Other consequences of mitochondrial dysfunction include reduction in mitochondrial ATP production, increased mitochondrial DNA mutations, increase in abnormal mitochondrial criste structures and impaired intracellular calcium levels [5]. Increased ROS generation with compromised mitochondrial function ultimately affects neurons and accelerates neurodegenerative process [6]. Besides being target of cellular oxidative damage, the mitochondria are found out to be the major resource of ROS in brain tissue, followed by neuron specific enzymes [7]. Mitochondrial oxidative injure has been anticipated to outcome in neuronal apoptosis and neuro-degeneration causing cognitive dysfunctions and diabetic neuropathy [8]. Therefore, therapies based on altering mitochondrial oxidative stress might be highly beneficial in such conditions.

Genistein (5,7-dihydroxy-3-(4-hydroxyphenyl)chromen-4-one) is the key isoflavone phytoestrogen in soybean (Glycine max) known to exhibit anxiolytic, neuroprotective, tyrosine kinase inhibitory, anti-inflammatory, antioxidant, estrogenic, antidiabetic and anti-proliferative effect [2]. Recently, the curative effect of genistein on Alzheimer's disease and Parkinson's disease has been established using amyloid beta$_{(1-40)}$ rat model and 6-hydroxydopamine hemi-Parkinsonian rat model [9]. Moreover, genistein ameliorates a diabetes established situation of oxidative stress, allodynia and neuro-inflammation, improves nerve growth factor content and vascular dysfunction, suggesting its likely therapeutic application for diabetic neuronal complications [10]. In addition, genistein has been shown to be beneficial against ROS generation, mitochondrial dysfunctions and in mitochondrial dependent and -independent apoptotic cell death in cancer [11]. Hence, the present study was aimed to test the hypothesis if hyperglycemia-induced oxidative stress results from mitochondrial oxidative stress and dysfunctions leading to the activation of apoptotic pathways and to evaluate the potential beneficial effect of genistein in preventing hyperglycemia related mitochondrial alterations in brain of streptozotocin (STZ)-induced diabetic mice.

EXPERIMENTAL SECTION

Subjects

Swiss albino male mice (25-30 g and 8 weeks or older) were utilized in present study. Animals were grouping housed (six mice per cage) and maintained at standard conditions of humidity (55 ± 5%), temperature 24 ± 2 °C under 12:12 h light (08:00–20:00 h)/dark cycle. Animals have free access to rodent chow and tap water *ad* libitum. Approval for animal studies was gained by the Institutional Animal Ethics Committee (protocol no. IAEC/MIP/2013/05), constituted as per CPCSEA and were in agreement with the globally acknowledged Principles for Laboratory Animal Use and Care as indicated by the National Institutes of Health Guide (NIH Publication No. 85-23, revised 1985). Animals were naive to drug treatments and experimentation at the beginning of all studies. All tests were conducted between 09:00 and 18:00 h.

Drugs and solutions

Genistein (purity: ≥ 98%), STZ (Sigma-Aldrich Chemicals Pvt. Ltd., Bangalore India) and metformin hydrochloride (Dr. Reddy's Laboratories, Hyderabad, India) were used in the present study. All other chemicals and reagents used were of analytical grade. STZ was solubilized in citrate buffer (pH 4.5) and genistein in dimethyl sulfoxide (DMSO). All other drugs were dissolved in double distilled water. The doses for all freshly prepared drug solutions were expressed in terms of their free bases.

Experimental introduction of diabetes

Induction of diabetes was done in mice by using an earlier reported method [12]. In brief, STZ was allowed to dissolve in 0.1M sodium citrate buffer (pH 4.5) and injected in mice at the dose of 200 mg/kg through i.p. route. To resist death due to hypoglycemic shock 5% of glucose solution instead of water was given to animals for 24 h after injection of STZ. After 48 h of STZ injection, blood samples obtained from the tail vein of mice observed for blood glucose levels to confirm diabetes induction. Animals with fasting blood glucose concentration greater than 300 mg/dl were spotted as diabetic and utilized for further study. Blood glucose concentrations were verified four times during whole experiment tenure (2, 10, 20, and 30 days after the beginning of treatment).

Treatment schedule

Following the confirmation of diabetes, separate groups of mice (n=6) were administered genistein (2.5, 5.0 and 10.0 mg/kg, i.p.) and metformin (500 mg/kg) or vehicle (1 ml/kg) once daily, orally for next 30 days. Two groups of non diabetic mice received vehicle (1 ml/kg, p.o.) (non diabetic control group) and genistein (10.0 mg/kg, i.p.) respectively once daily. Genistein doses were based on previous results obtained from the neuroprotective studies with genistein on diabetic mice [10]. After completion of the treatment, the animals were fasted overnight, anaesthetize with ether and sacrificed by decapitation. Their brains were quickly removed and the cerebral cortices were dissected and stored at −80 °C for further biochemical assessment.

Isolation of mitochondria

Isolation of mitochondria from the cerebral cortex was done as explained by Puka-Sundvall et al. (2000) [13] with slight adjustments. Briefly, the cerebral cortex was homogenized in ice-cold buffer containing 10 mM Tris–HCl (pH 7.4), 320 mM sucrose, 5 mM EDTA and 0.1% bovine serum albumin, to prepare 10% w/v homogenate using mechanically driven suitable homogenizer. The homogenate was centrifuged at 2100 × g for 15 min at 4 °C. The pellet was discarded and the supernatant was re-centrifuged at 14,000 × g for 15 min at 4 °C. The crude mitochondrial pellet was separated and washed with buffer and again centrifuged at 7000 × g for 15 min at 4 °C. The final mitochondrial pellet was re-suspended in buffer containing 10 mM Tris–HCl (pH 7.4),

0.44 M sucrose and the purity of mitochondrial preparation was checked by measuring the activity of succinate dehydrogenase [13]. Determination of protein content in mitochondrial preparation was accomplished by the method of Lowry et al. (1951) [14].

Estimation of mitochondrial respiratory chain enzymes

Estimation of NADH dehydrogenase (complex I): The process engages catalytic oxidation of NADH to NAD^+ with successive reduction of cytochrome C. Briefly, mitochondrial preparation was added to a reaction mixture containing 6 mM NADH, 0.2 M glycyl glycine (pH 8.5) and 1 mM oxidized cytochrome c. The reaction was initiated by adding 0.02 M sodium bicarbonate to the mixture. The increase in absorbance was tracked at 550 nm for 3 min. Results were stated as nmol NADH oxidized/min/mg protein, using molar extinction coefficient of reduced cytochrome c at 550 nm as 19.6 mM^{-1} cm^{-1} [15].

Estimation of succinate dehydrogenase (complex II): The method engages oxidation of succinate by successive reduction of an artificial electron acceptor, potassium ferricyanide. Briefly, the reaction mixture contained 0.2 M sodium phosphate buffer pH 7.8, 0.6 M succinic acid, 1% bovine serum albumin and 0.03 M potassium ferricyanide. The reaction was started by the addition of mitochondrial preparation. The change in absorbance was observed at 420 nm for 3 min. The results were expressed as nmol succinate oxidized/min/mg protein [16].

Estimation of cytochrome-bc_1 complex (complex III): Activity of cytochrome c reductase was determined as the rate of antimycin A-dependent reduction of cytochrome c. Mitochondrial suspension was added to the reaction mixture containing 35 mM potassium dihydrogen phosphate, 5 mM magnesium chloride, 2 mM potassium cyanide and 0.05 % Triton X-100 (pH 7.02). The reaction was started with adding 60 μM reduced decylubiquinone and 50 μM of cytochrome c to the mixture. The change in absorbance was tracked at 550 nm for 3 min. Results were stated as nmol cytochrome c/min/mg protein, using molar extinction coefficient of reduced cytochrome c at 550 nm as 19.6 mM^{-1} cm^{-1} [17].

Estimation of cytochrome-c oxidase (complex IV): Cytochrome c was first reduced by adding sodium borohydride and then 0.1 M HCl was added to it to neutralize the pH to 7.0. The reaction mixture contained 0.3 mM of reduced cytochrome c in 75 mM phosphate buffer (pH 7.4). The reaction was initiated by the addition of mitochondrial suspension. The increase in absorbance was tracked at 550 nm for 3 min. Results were stated as μmol cytochrome c/min/mg protein, using molar extinction coefficient of reduced cytochrome c at 550 nm as 19.6 mM^{-1} cm^{-1} [18].

Estimation of mitochondrial oxidative stress

Estimation of lipid peroxidation: As an index of lipid peroxidation, content of malondialdehyde (MDA) was accessed as per formerly explained procedure by Ohkawa et al. (1979) with trivial adjustment. Briefly, the sample of 0.1 ml supernatant was mixed with 1.5 ml glacial acetic acid (20%), 0.2 ml sodium dodecyl sulphate (8.1%) and 1.5 ml thiobarbituric acid (0.8%). The mixture containing tubes were agitated, heated for 1 h at 95 °C on a water bath and cooled below tap water. Now, 5 ml mixture of pyridine and n-butanol (1:15) and 1 ml distilled water was added to each tube and centrifuged at 4000 rpm for 10 min. The absorbance of upper organic pink layer was measured at 532 nm in order to estimate the quantity of MDA formed. Using malondialdehyde bis-(dimethoxy acetyl) as standard, a calibration curve was plotted. The data were expressed as nmol of MDA/mg protein [19].

Estimation of superoxide dismutase: Briefly, 10 µl of mitochondrial suspension, 90 µl of 30 mM sodium tetra pyrophosphate buffer (pH 8.3), 30 µl of 0.3 mM nitro blue tetrazolium (NBT), 10 µl of 0.96 mM phenyl methyl sulphonate (PMS) and 40 µl of deuterium depleted water (DDW) was added. The reaction was initiated by the addition of 20 µl of 0.72 mM NADH. The reaction was stopped by adding 50 µl of glacial acetic acid. Absorbance was measured at 560 nm. A single unit of enzyme is expressed as 50% inhibition of NBT reduction/min/mg protein. Results were expressed as a unit of SOD/min/mg protein [20].

Estimation of mitochondrial thiols as oxidative stress markers

Estimation of total thiols (TSHs): The reaction mixture containing 0.2 M Tris–HCl and 0.02 M EDTA (pH 8.2), mitochondrial suspension and 0.01 M Ellman's reagent in methanol was incubated for 15 min at room temperature. The contents were centrifuged at 1200 × g for 5 min. The absorbance was measured at 412 nm and the results were expressed as nmoles TSHs/mg protein [21].

Estimation of non-protein thiols (NPSHs): Proteins in the mitochondrial sample were precipitated by 4% sulphosalicylic acid followed by centrifugation at 1200 × g for 5 min. To the supernatant 0.1 mM Ellman's

reagent (in 0.1 M phosphate buffer pH 8.0) was added and after 2 min the absorbance was read at 412 nm. Results were expressed as nmol NPSHs/mg protein [22].

Estimation of protein thiols (PSHs): Levels of protein thiols (PSHs) were calculated from the difference between the values of total thiols and non-protein thiols levels.

Estimation of mitochondrial function

Assessment of neuronal apoptosis (Caspase-3 activity assay): Caspase-3 activity was detected with a commercially available Caspase-3 Activity Kit (Biovision Inc., California), according to manufacturer's instructions. Mitochondrial suspension was incubated for 7h at 37°C, with 5 µl caspase-3 substrate (DEVDpNA, 4 mM/l). Substrate cleavage was measured with a spectrofluorometer at 405 nm. Results were expressed as the percentage of the caspase-3 activity of the control group [23].

Assessment of cellular viability (MTT assay): The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) reduction was used to assess the activity of the mitochondrial respiratory chain as an indicator for cellular viability. Mitochondrial suspension was incubated with MTT (45 µg/ml) for 45 min at 37°C. Active mitochondrial dehydrogenases of living cells cause cleavage and reduction of the soluble yellow MTT dye to the insoluble purple formazan. The formazan crystals formed were solubilized in iso-propanol and the absorbance was measured at 595 nm and the results were compared to those obtained with control samples to which 100% viability was attributed [24].

Statistical analysis

Results were expressed as mean ± S.E.M. The data were analyzed by one way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. Probability values less than 0.05 were considered statistically significant in all the cases.

RESULTS

Estimation of mitochondrial respiratory chain enzymes

Activity of NADH dehydrogenase (complex I) was significantly decreased in the cortex [$F(6, 35) = 1027$, $p < 0.0001$] of diabetic group as compared to the non diabetic control group. Chronic treatment with genistein (5.0 and 10.0 mg/kg) and metformin (500 mg/kg) significantly ($p < 0.0001$) increased NADH dehydrogenase concentration in diabetic mice cortex as compared to diabetic control mice. Chronic treatment with genistein (2.5 mg/kg) in diabetic mice did not influence NADH dehydrogenase activity (Figure 1A).

Activity of succinate dehydrogenase (complex II) was significantly decreased in the cortex [$F(6, 35) = 316.1$, $p < 0.0001$] of diabetic group as compared to the non diabetic control group. Chronic genistein treatment (5.0 mg/kg) significantly ($p < 0.001$) increased succinate dehydrogenase concentration in diabetic mice cortex as compared to diabetic control mice. Furthermore, chronic treatment with genistein (10.0 mg/kg) and metformin (500 mg/kg) significantly ($p < 0.0001$) increased succinate dehydrogenase concentration in diabetic mice cortex as compared to diabetic control mice. Chronic treatment with genistein (2.5 mg/kg) in diabetic mice did not influence succinate dehydrogenase activity (Figure 1B).

Activity of cytochrome-bc_1 complex or cytochrome c reductase (complex III) was significantly decreased in the cortex [$F(6, 35) = 855.6$, $p < 0.0001$] of diabetic group as compared to the non diabetic control group. Chronic genistein treatment (2.5 mg/kg) significantly ($p < 0.001$) increased cytochrome c reductase concentration in diabetic mice cortex as compared to diabetic control mice. Furthermore, chronic treatment with genistein (5.0 and 10.0 mg/kg) and metformin (500 mg/kg) significantly ($p < 0.0001$) increased cytochrome c reductase concentration in diabetic mice cortex as compared to diabetic control mice. Chronic treatment with genistein (2.5 mg/kg) in diabetic mice did not influence cytochrome c reductase activity (Figure 1C).

Activity of cytochrome c oxidase (complex IV) was significantly decreased in the cortex [$F(6, 35) = 2394.0$, $p < 0.0001$] of diabetic group as compared to the non diabetic control group. Chronic genistein treatment (2.5 mg/kg) significantly ($p < 0.001$) increased cytochrome c oxidase concentration in diabetic mice cortex as compared to diabetic control mice. Furthermore, chronic treatment with genistein (5.0 and 10.0 mg/kg) and metformin (500 mg/kg) significantly ($p < 0.0001$) increased cytochrome c oxidase concentration in diabetic mice cortex as compared to diabetic control mice. Chronic treatment with genistein (2.5 mg/kg) in diabetic mice did not influence cytochrome c oxidase activity (Figure 1D). Genistein *per se* does not reveal any change in any mitochondrial complex activity in normal mice.

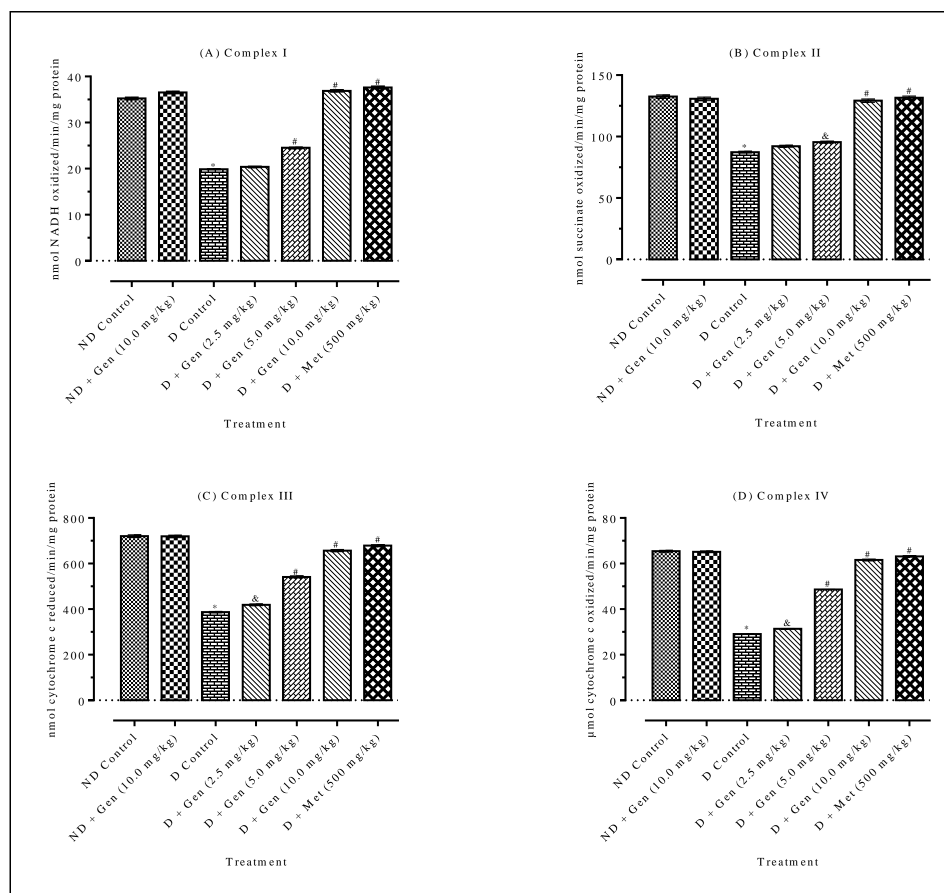

Figure 1: Effect of chronic treatment with genistein on mitochondrial respiratory chain enzymes in diabetic mice (A) complex I (B) complex II (C) complex III (D) complex IV

Results are expressed as Mean ± S.E.M.; n = 6 in each group. Data was analyzed by one way repeat measure ANOVA followed by Tuckey's multiple comparison test. Significance: $^*p < 0.0001$ when compared with non diabetic control group; $^{\&}p < 0.001$ when compared with diabetic control group; $^{\#}p < 0.0001$ when compared with diabetic control group. D: diabetic group; ND: non diabetic group; Gen: genistein treated group; Met: metformin treated group.

Estimation of mitochondrial oxidative stress

Diabetic condition resulted in enhanced lipid peroxidation in terms of rise in MDA level in the cortex [$F(6, 35) = 24.78$, $p < 0.0001$] compared to the non diabetic control group. Chronic genistein treatment (2.5 mg/kg) significantly ($p < 0.01$) decreased MDA level in diabetic mice cortex as compared to diabetic control mice. Furthermore, chronic treatment with genistein (5.0 and 10.0 mg/kg) and metformin (500 mg/kg) significantly decreased MDA level in cortex ($p < 0.0001$) of diabetic mice as compared to diabetic control mice. Genistein *per se* does not exhibit any decrease in MDA level in normal mice (Table 1).

Table 1: Effect of chronic treatment with genistein on lipid peroxidation (MDA) and superoxide dismutase level in mitochondrial suspension of cortex of diabetic mice

Group	Treatment	Lipid peroxidation (nmol MDA/mg protein)	Unit of SOD/min/mg protein
ND control	Vehicle (1 ml/kg)	2.32 ± 0.17	17.08 ± 0.14
ND + Gen	Gen (10.0 mg/kg)	2.34 ± 0.19	16.93 ± 0.15
D control	Vehicle (1 ml/kg)	4.83 ± 0.36*	8.81 ± 0.09*
D + Gen	Gen (2.5 mg/kg)	3.55 ± 0.21$	9.54 ± 0.12$
	Gen (5.0 mg/kg)	2.48 ± 0.16#	11.05 ± 0.10#
	Gen (10.0 mg/kg)	2.10 ± 0.13#	16.98 ± 0.17#
D + Met	Met (500 mg/kg)	1.98 ± 0.14#	16.50 ± 0.15#

Results are expressed as Mean ± S.E.M.; n = 6 in each group. Data was analyzed by one way repeat measure ANOVA followed by Tuckey's multiple comparison test. Significance: $^{*}p < 0.0001$ when compared with non-diabetic control group; $^{\$}p < 0.01$ when compared with diabetic control group; $^{\#}p < 0.0001$ when compared with diabetic control group. D: diabetic group; ND: non diabetic group; Gen: genistein treated group; Met: metformin treated group.

There was a significant drop in levels of SOD in cortex [$F(6, 35) = 820.00$, $p< 0.0001$] of diabetic mice as compared to non diabetic control mice. Chronic genistein treatment (2.5 mg/kg) significantly ($p< 0.01$) increased SOD level in cortex of diabetic mice as compared to diabetic control mice. Furthermore, chronic treatment with genistein (5.0 and 10.0 mg/kg) and metformin (500 mg/kg) significantly ($p< 0.0001$) increased SOD level in cortex of diabetic mice as compared to diabetic control mice. Genistein *per se* does not exhibit any effect on SOD level in normal mice (Table 1).

Estimation of mitochondrial thiols as oxidative stress markers

Diabetic condition resulted in a significant drop in total thiol concentration in the cortex [$F(6, 35) = 269.20$, $p < 0.0001$] compared to the non diabetic control group. Chronic genistein treatment (2.5 mg/kg) does not have any effect on total thiol concentration in cortex of diabetic mice. Furthermore, chronic treatment with genistein (5.0 and 10.0 mg/kg) and metformin (500 mg/kg) significantly increased total thiol concentration in cortex ($p < 0.0001$) of diabetic mice as compared to diabetic control mice (Table 2). There was a significant drop in levels of non protein thiols in cortex [$F(6, 35) = 2440.00$, $p < 0.0001$] in diabetic mice as compared to non diabetic control mice. Chronic genistein treatment (2.5 mg/kg) does not exhibit any effect on non protein thiols level in cortex of diabetic mice. Furthermore, chronic treatment with genistein (5.0 and 10.0 mg/kg) and metformin (500 mg/kg) significantly ($p < 0.0001$) increased non protein thiols level in cortex of diabetic mice as compared to diabetic control mice (Table 2). There was a significant drop in levels of protein thiols in cortex [$F(6, 35) = 10.75$, $p < 0.0001$] in diabetic mice as compared to non diabetic control mice. Chronic genistein treatment (2.5 and 5.0 mg/kg) does not exhibit any effect on protein thiols level in cortex of diabetic mice. Furthermore, chronic treatment with genistein (10.0 mg/kg) and metformin (500 mg/kg) significantly ($p < 0.05$ and 0.001 respectively) increased protein thiols level in cortex of diabetic mice as compared to diabetic control mice (Table 2). Genistein *per se* does not exhibit any effect on total, non protein and protein thiol levels in normal mice.

Table 2: Effect of chronic treatment with genistein on total thiols, non protein thiols and protein thiols levels in mitochondrial suspension of cortex of diabetic mice

Group	Treatment	Total thiols (nmoles TSHs/mg protein)	Non protein thiols (nmoles NPSHs/mg protein)	Protein thiols (nmoles PSHs/mg protein)
ND control	Vehicle (1 ml/kg)	26.18 ± 0.20	3.91 ± 0.02	22.27 ± 0.18
ND + Gen	Gen (10.0 mg/kg)	27.06 ± 0.21	3.86 ± 0.02	23.20 ± 0.19
D control	Vehicle (1 ml/kg)	19.64 ± 0.15*	1.82 ± 0.01*	17.82 ± 0.14*
D + Gen	Gen (2.5 mg/kg)	20.17 ± 0.15	1.89 ± 0.01	18.28 ± 0.14
	Gen (5.0 mg/kg)	21.94 ± 0.16#	2.17 ± 0.02#	19.77 ± 0.14
	Gen (10.0 mg/kg)	23.49 ± 0.17#	2.41 ± 0.02#	21.08 ± 0.15@
D + Met	Met (500 mg/kg)	24.55 ± 0.18#	2.67 ± 0.02#	21.88 ± 0.16&

Results are expressed as Mean ± S.E.M.; n = 6 in each group. Data was analyzed by one way repeat measure ANOVA followed by Tuckey's multiple comparison test. Significance: $^{*}p < 0.0001$ when compared with non-diabetic control group; $^{@}p < 0.05$ when compared with diabetic control group; $^{\&}p < 0.001$ when compared with diabetic control group; $^{\#}p < 0.0001$ when compared with diabetic control group. D: diabetic group; ND: non diabetic group; Gen: genistein treated group; Met: metformin treated group.

Estimation of mitochondrial function

A constant outcome for caspase-3 activity was noticed in different groups. It was observed that hyperglycemia produced a significant increase in caspase-3 activity in cortex of diabetic mice compared to their respective control groups [$F(6, 35) = 40.15$, $p < 0.0001$]. Geintein (5.0 mg/kg) significantly ($p < 0.001$) reduced caspase-3 activity in diabetic group as compared to the respective control group. Moreover, high dose of genistein (10.0 mg/kg) and metformin (500 mg/kg) caused a significant ($p < 0.0001$) reduction of caspase-3 activity to nearly a normal level in diabetic mice. It has been validated by these results that the mitochondrial-dependent apoptotic pathway was triggered during hyperglycemia and the suppression of caspase-3 by genistein might be due to reversed cytochrome C release. Genistein *per se* does not exhibit any effect on neuronal apoptosis in normal mice (Figure 2A).

The exposure of brain to hyperglycemia resulted in marked changes in cellular viability in terms of decreased MTT reduction in brains diabetic groups compared to their respective controls [$F(6, 35) = 24.91$, $p < 0.0001$].

Geintein (5.0 mg/kg) significantly ($p < 0.05$) increased MTT reduction in diabetic group compared to the respective control group. Furthermore, high dose of genistein (10.0 mg/kg) and metformin (500 mg/kg) caused a significant ($p < 0.0001$) increase in MTT reduction of brain samples in diabetic groups as compared to diabetic group treated with vehicle. Genistein *per se* does not have any effect on cellular viability in normal mice (Figure 2B).

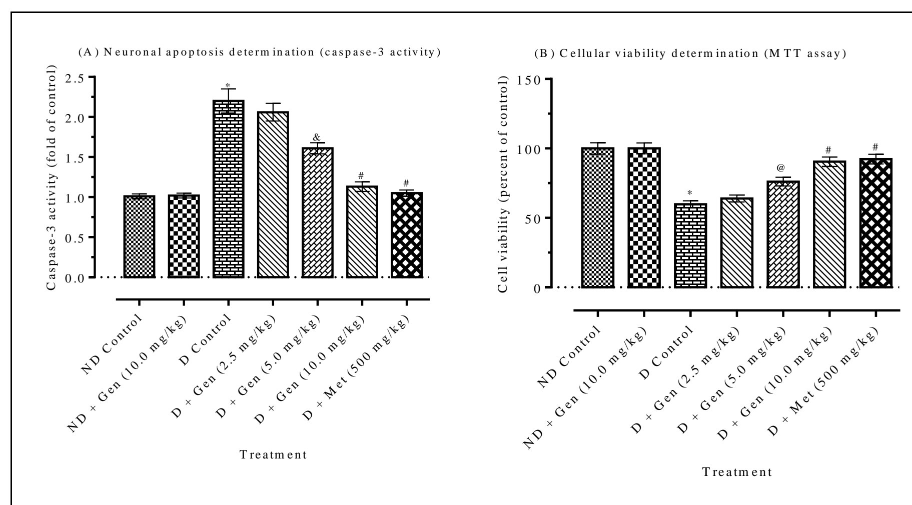

Figure 2: Effect of chronic treatment with genistein on mitochondrial function (A) determination of neuronal apoptosis in cortex of diabetic mice using caspase-3 activity assay; (B) determination of cellular viability in cortex of diabetic mice using MTT assay

Results are expressed as Mean ± S.E.M.; n = 6 in each group. Data was analyzed by one way repeat measure ANOVA followed by Tuckey's multiple comparison test. Significance: $^{*}p < 0.0001$ when compared with non-diabetic control group; $^{@}p < 0.05$ when compared with diabetic control group; $^{\&}p < 0.001$ when compared with diabetic control group; $^{\#}p < 0.0001$ when compared with diabetic control group. D: diabetic group; ND: non diabetic group; Gen: genistein treated group; Met: metformin treated group.

DISCUSSION

In present study, STZ-induced diabetes produced obvious impairment in mitochondrial respiratory chain enzymes (complex I-IV) which was coupled with marked raise in increased oxidative stress, increased neuronal apoptosis and decreased mitochondrial viability. Continuing treatment with genistein improved mitochondrial function as impaired in diabetic mice.

It is postulated that the mutilation of glucose metabolism and energy imbalance caused by STZ may be a probable supply for this oxidative stress resulting in inhibition of several mitochondrial enzymes (aconitase, α-ketoglutarate dehydrogenase, pyruvate dehydrogenase) and enzyme complexes (I, II, III and IV) [25]. In our study, in diabetic conditions the activities of mitochondrial electron transport chain (ETC) enzymes (complex I-IV) were found to be reduced which are in agreement with the earlier studies in diabetic rats [26]. Function of ETC complexes depends also on structural integrity of membrane lipid bi-layer. Cardiolipin is the important component of the inner mitochondrial membrane and it constitutes about 20% of total lipid composition. Lipid peroxidation caused due to hyperglycemia results in loss of cardiopolin, thus altering mitochondrial integrity. Cytochrome c preferentially binds to cardiolipin and is liberated upon oxidation of cardiolipin. Released cytochrome c is the key factor for apoptotic cascade. Decreased antioxidants and increased lipid peroxidation are combined with decreased complex IV activity, causing apoptosis. Furthermore, chronic diabetic condition grounds for loss of thiols that contributes to diminished activities of mitochondrial ETC enzymes [27]. Since brain has high energy requirement, any down regulation in mitochondrial complex activities cause brutal collapse on brain functions. Therefore, ETC components come into sight to be the major mitochondrial targets of chronic hyperglycemia. The protection of activity of mitochondrial complexes by genistein might be the consequence of augment in the intracellular thiols which might offer protection by scavenging ROS or protecting thiols in mitochondrial proteins or both.

In present study, a noteworthy increase in malondialdehyde levels along with obvious decline in superoxide dismutase activity in the mitochondrial suspension of brain of STZ injected mice was observed. Hyperglycemia, associated with production of free radicals, lowers the level of natural antioxidant defense compounds such as glutathione, ascorbic acid and superoxide dismutase present in the body, thus resulting in major antioxidant imbalance [28]. Hence, management and sustenance of antioxidant status can be an effective remedy for diabetes and associated complications. Imbalance among ETC complexes along with distorted energy

transduction causes enhancement in electron leakage from ETC and oxidative stress. Complex I and complex III of ETC are the chief sites for ROS production. Under standard circumstances, ETC produces very low levels of ROS, which are removed by scavenging mechanisms so that no substantial oxidative damage occurs. Due to imbalance in ETC in mitochondria, gathering of peroxidation products and ROS is coupled with a decline in ETC activity, resulting in mitochondrial swelling, decreased transmembrane potential, decreased respiratory control ratio, uncoupling of oxidative phosphorylation and reduced cytochrome c oxidase activity [29]. Genistein treatment returned lipid peroxidation and levels of superoxide dismutase towards their control values in mitochondrial suspension of cortex of diabetic mice. This is supported by similar antioxidant potential of genistein in previous reports [10]. Therefore, genistein might protect hyperglycemia associated mitochondrial dysfunction by ameliorating oxidative stress in diabetic mice.

Management of mitochondrial ROS production as well as its sensitivity for apoptotic pathways is the key role of mitochondrial non protein thiols. Mitochondrial non protein thiols keep the thiol groups of its membrane in the reduced state, thus regulating mitochondrial inner membrane permeability and maintain cell viability [30]. Non protein thiols are transported from the cytosol in mitochondria through a carrier protein, as the synthesis of non protein thiols do not take place in mitochondria [31]. The decline in the mitochondrial non protein thiols content might be a reflection of decreased cellular non protein thiols levels. The reduction in protein thiols may also affect confirmation/catalysis of thiol group containing proteins/enzymes which may be detrimental to the function of the brain mitochondria. A change in the mitochondrial non protein levels might make the mitochondria vulnerable to oxidant species generated due to chronic hyperglycemia and change in protein thiols might alter mitochondrial function [32]. In present study low levels of mitochondrial total thiols, non protein thiols and protein thiols have been observed. Low levels of mitochondrial non protein thiols observed in the present study can be attributed to enhanced ROS generation. Genistein significantly increased non protein thiols and protein thiols levels, thus increasing total thiols level in mitochondrial suspension of brains of diabetic mice. Genistein by increasing mitochondrial protein thiols might contribute in the protection of mitochondrial dysfunction by defending various proteins/enzymes and by increasing mitochondrial non protein thiols might play a key role in the protection of mitochondrial components against hyperglycemia induced oxidative damage.

Hyperglycemia induced oxidative damage to inner membrane proteins induces membrane permeability transition and alteration in mitochondrial bioenergetics, that follows cytochrome c release into cytosol and commences apoptotic cascade and conclude in neuronal damage [33]. Secondary cell death during apoptosis is originated through releasing numerous apoptogenic factors such as apoptosis inducing factor, cytochrome C and caspases. Caspase-3 is supposed as a final killer of apoptosis [34]. In our study mitochondrial apoptosis has been examined by investigating caspase-3 activation and cell viability by MTT assay. The increased caspase-3 activity reaches the high level, accompanied with increased cell death. Genistein administration inhibited caspase-3, prohibited neuronal apoptosis in brain of diabetic mice. Also, prohibition of neuronal cell death by genistein resulted in outcome of more viable cells as evident from MTT assay. The anti-apoptotic activity of this antioxidant genistein is in agreement with previously published studies [2]. The mechanism involved in the attenuation of hyperglycemia induced apoptosis might involve the restoration of cellular redox status.

CONCLUSION

In conclusion, the results from the present study confirmed that treatment with genistein was able to prevent mitochondrial dysfunction as provoked in diabetic mice, signifying that this compound restored the altered levels of mitochondrial respiratory chain enzymes, reversed oxidative stress, increased mitochondrial thiols, decreased neuronal apoptosis and increased cellular viability in the mitochondrial suspension prepared from the cortex of STZ-induced diabetic mice. Genistein could find clinical implication in treatment and/or management of neural impairment in diabetics, but further studies are necessary to examine its potential therapeutic effects and to elucidate the exact mechanism underlying these modulator cause.

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