***Gymnema montanum* H. Protects Against Alloxan-induced Oxidative Stress and Apoptosis in Pancreatic β -cells**

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Key Words

Gymnema montanum • Pancreatic β -cells • Diabetes • Apoptosis • Medicinal plants • Free radicals

pancreatic β -cells against reactive oxygen species (ROS) by counteracting with mitochondrial membrane permeability and inhibition of the apoptotic pathway.

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Abstract

The present study evaluated the molecular mechanism of antidiabetic property of *G. montanum* leaf extract (GLEt) against alloxan-induced apoptotic cell death in rat insulinoma cells (RINm5F). The pre-treatment of GLEt (5 μ g and 10 μ g/ml) resulted in significant decrease in intracellular Ca^{2+} concentration, nitric oxide (NO) production along with increase in mitochondrial membrane potential in alloxan (7mM/ml) treated cells. Further GLEt reduced apoptosis by inhibiting the release of cytochrome c and subsequent cleavage of PARP and caspase-3. The immunohistochemical staining of 8-hydroxydeoxyguanosine (8-OHdG) also evidenced the suppression of oxidative stress by GLEt. The cell cycle analysis, annexin-V labelling assay and TUNEL assay showed the suppression of apoptosis by the treatment of GLEt. Moreover, GLEt significantly increased the cellular antioxidant levels and decreased the lipid peroxides in alloxan-treated RINm5F cells. Taken together, these findings suggest that *G. montanum* protects

Introduction

Oxidative stress-induced pancreatic β -cell death plays an important role in the pathogenesis of diabetes mellitus. The chronic hyperglycemia in diabetes leads to overproduction of ROS which causes cellular injury through non-specific modification and disruption of proteins, phospholipids and nucleic acids [1, 2]. Glucose auto-oxidation [3], nonenzymatic glycation [4], the interaction between glycated products and their receptors [5], and the polyol pathway [6, 7] are the potential sources of hyperglycemia-induced oxidative stress. The ROS and the resulting oxidative stress lead into the activation of caspases [8] and eventually apoptosis [9].

The pancreatic β -cells are vulnerable to oxidative stress, possibly due to low levels of antioxidant enzyme expression [10]. In addition to increased production of free radicals, antioxidant defence systems are markedly disturbed in pancreas of diabetic animals [11, 12]. Previous reports provided evidence that high ambient glucose can

promote apoptosis *in vitro*, suggesting potential cellular damage as a result of hyperglycemia in diabetes [13]. Strategies aimed at interfering the oxidative stress induced apoptotic pathways could therefore be of potential therapeutic value. Medicinal plants and phytochemicals have been reported for their preventive action of β -cell death [14-16].

In traditional system of Indian medicine, *Gymnema* sp. is used for diabetes treatment, as a diuretic, and a digestive stimulant [17]. Several researchers have reported that *Gymnema* extracts (*G. sylvestre*, *G. inodorum*, *G. yunnanense*) help in repairing and/or regenerating the pancreas β -cells' insulin secretion [18-20]. *Gymnema montanum* Hook, an Asclepiadaceae member is an endemic plant species found mainly in Western Ghats, India. We have previously demonstrated that the ethanol extract of *G. montanum* leaves improves hyperglycemia and its complications in alloxan induced diabetic rats [21-24]. It also helps in the prevention of cholinergic neural and retinal complications of hyperglycemia in diabetes [25]. However, the mechanism of antidiabetic effect of *G. montanum* is not yet understood. In an effort to find the cytoprotective efficacy of this plant extract on pancreatic islets, the present study was carried out to investigate the effect of *G. montanum* leaf extract on alloxan-induced oxidative damage in insulin-secreting rat insulinoma-RINm5F cells. The RINm5F cell line was chosen as an experimental model due to its expression of both the constitutive and the inducible forms of nitric oxide synthetase (NOS), which is involved in the pathogenesis of diabetes and also expresses Ca^{2+} channels [26]. To address the protective mechanism of GLEt, we evaluated the disruption of mitochondrial membrane potential, super oxide radical and NO production, release of cytochrome c from mitochondria to cytosol, activation of caspase-3 and PARP cleavage in alloxan-induced RINm5F cells. The anti-apoptotic phenomenon of the plant extract was confirmed by cell cycle analysis, TUNEL assay and annexin-V labelling assay.

Materials and Methods

Plant material and preparation of the extract

Fresh leaves of *G. montanum* Hook were collected in November 2006 from the Shola forests of Western Ghats, Gudalur, The Nilgiri Biosphere Reserve at an altitude of 900 - 1500 MSL. The plant was identified with the Herbarium of Botanical Survey of India, Southern Circle, Coimbatore, India (Accession No. 32561-65) and was deposited in the Department

of Biotechnology, Anna University Tiruchirappalli.

The fresh leaves of *G. montanum* (500 g) were air-dried for 7 days and the dried material was made to a fine powder (particle size ~ 0.25 mm) using a laboratory mill and defatted with 750 ml petroleum ether (60-80°C) for 2-3 h in a Soxhlet apparatus. The resulting residue was dried and extracted further with 95% ethanol. The ethanol extract was filtered and concentrated on a rotary evaporator at 40°-50°C under reduced pressure and lyophilized. A greenish-black powdered material was obtained (20-30 g) and stored at 0-4°C until further use.

Total phenolic content

The phenolic content of the plant extract was determined according to the method of Shetty [27]. A standard curve was established using gallic acid in 95% ethanol. The phenolic content was expressed as gallic acid equivalent ($\mu\text{g}/\text{mg}$ of the extract).

Cells and Culture conditions

The RINm5F cell line was obtained from National Centre for Cell Sciences, Pune, India. The cells were grown as adherent culture at 37°C under a humidified, 5% CO_2 atmosphere in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) and 2 mM glutamine, 10,000 U/ml of penicillin, 10 mg/ml of streptomycin and 2.5 $\mu\text{g}/\text{ml}$ of amphotericin B.

Effect of alloxan on the viability of RINm5F cells

Cells were seeded at a density of 1×10^3 cells/well suspended in 0.5 ml medium in 24-well tissue culture plates. Alloxan (ranges from 1 - 25 mM/ml), dissolved in 50 mM citrate buffer pH 3.0, was added to the cells and incubated for 1 h at 37°C. After the incubation period, the medium was removed and fresh medium was added and the cells were incubated for an additional 23 h. At the end of this incubation period, the cell viability was determined.

Effect of GLEt on alloxan-induced cytotoxicity to RINm5F cells

The RINm5F cells were treated with alloxan (7 mM) and GLEt (0.5 μg - 20 $\mu\text{g}/\text{ml}$). Three types of exposure protocols were followed to study the effect of GLEt on alloxan-induced cytotoxicity.

In the pre-treatment protocol, the cells (1×10^4) were first incubated with GLEt for 1 h at 37°C. Then the extract was removed by aspirating the medium and was replaced with fresh medium. To these fresh medium-supplemented cells, alloxan was added and incubated for an additional 1 h. After this incubation, the medium was removed and replaced with fresh medium and incubated again for another 22 h. At the end of incubation, cell viability was determined by MTT assay.

In the simultaneous treatment protocol (co-exposure), the experiment was performed similar to that for pre-treatment except that the cells were exposed to GLEt and alloxan simultaneously and incubated for 1 h at 37°C. At the end of this period, the medium was removed and fresh medium was added and incubated for 23 h and then subjected to MTT assay for the cell viability determination.

In the post-treatment schedule, the RINm5F cells were first treated with alloxan for 1 h at 37°C, at the end of which the medium was removed and replaced with fresh medium containing GLEt and incubated for additional 1 h. At the end of incubation, the cells were washed and incubated in fresh medium for 22 h, and then the cell viability was assessed by MTT assay.

Assessment of cell viability

The viability of the cells was assayed by reduction of MTT to formazan as described previously [28]. Cells in each well were incubated at 37°C in 20 µl of MTT (5 mg/ml) for 4 h. After removing the medium containing MTT, 200 µl of dimethyl sulphoxide and 25 µl of glycine buffer (pH 9.5) were added to each well. Absorbance was read at 570 nm using a microplate ELISA reader. The absorbance of untreated cells was considered as 100%. The results were determined by three independent experiments.

Measurement of intracellular reactive oxygen species

Intracellular ROS were detected by fluorescent spectrophotometry (Hitachi F4500, Japan) using a fluorescein-labeled dye, DCFH-DA. Briefly, after treatment, the cells were cultured with 10 µM DCFH-DA for 30 min at 37°C. Then the cells were washed with PBS and homogenized in 300 µl of 0.1% Triton X-100 (PBS, pH7.4) through sonication. After incubation at 4°C for 10 min, the homogenates were centrifuged and the supernatants were used for assay with excitation wavelength at 488 nm and emission wavelength at 510 nm.

Griess nitrite assay

The production of NO was estimated spectrophotometrically as a formed nitrite (NO₂⁻). Briefly, after treatment, 100 µl of culture medium was taken from each well and incubated with 100 µl of Griess reagent (1% sulfanilamide in 0.1 mol/l HCl and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride) at room temperature for 10 min. Then the absorbance was measured at 540 nm using microplate reader. The nitrite content was calculated based on a standard curve constructed with NaNO₂ [29].

Estimation of lipid peroxidation and antioxidants

For the estimation of lipid peroxidation (Thiobarbituric Acid Reactive Substances, TBARS) and antioxidants (superoxide dismutase, SOD; catalase, CAT; glutathione peroxidase, GPx and glutathione-S-transferase, GST), 1 x 10⁶/well RINm5F cells were seeded in 6-well culture plates. The cells were initially treated with GLEt (5 µg and 10 µg/ml) for 1 h at 37°C following which the medium was replaced with fresh medium. Then alloxan (7 mM/ml) was added and the cells were incubated for another 1 h. Further the medium was removed and replaced with fresh medium and incubated again for another 22 h. At the end of this incubation, the cells were centrifuged and the supernatant was removed. The cell pellet was resuspended in PBS and sonicated. The clear lysate was used for the estimation of TBARS by the method of Janero et al. [30] and antioxidant enzymes such as SOD, CAT and GPx were determined by the method of Kakkar et al. [31], Sinha [32] and

Rotruck et al. [33] respectively. The activity of GST was measured using 1-chloro-2, 4-dinitro benzene as substrate by the method of Habig et al. [34].

Measurement of mitochondrial membrane potential ($\Delta\Psi_m$) and intracellular Ca²⁺ concentration

Changes in the $\Delta\Psi_m$ during apoptosis were measured using 3,3'-dihexyloxacarbocyanine (DiOC₆(3); Molecular Probes, Eugene, OR) [35]. Cells (5 x 10⁵) in 500 µl of RPMI 1640 medium were exposed with 100 nM DiOC₆(3) during the last 30 min of treatment. The cells were then pelleted at 1000g for 10 min. The pellet was resuspended and washed twice with PBS. The pellet was then lysed by the addition of 600 µl of deionized water followed by homogenization. The concentration of the retained DiOC₆(3) was read on a spectrofluorometer (Hitachi F-2500, Japan) with an excitation wavelength of 488 nm and an emission wavelength of 500 nm.

In an independent experiment, the intracellular Ca²⁺ concentration was determined using fluo-3-AM according to the method of Chai et al. [36]. The cells were prepared as described above except that the cells were incubated with 5 µM fluo-3-AM for further 30 min at 37°C. Then, the cells were rinsed twice with Ca²⁺-free PBS and read on a spectrofluorimeter (Hitachi F-2500, Japan) with an excitation wavelength of 340 nm and an emission wavelength of 380 nm.

Immunocytochemical staining of 8-OHdG

The presence of oxidative damage was assessed using monoclonal antibody against 8-OHdG (QED Bioscience Inc., San Diego, CA). The treatment protocol was followed as described above. After the treatment, the cells were fixed with 4% paraformaldehyde for 5 min at room temperature and washed with DPBS. Then the cells were permeabilized in 0.5% Triton-X-100 for 15 min and pre-incubated with protein blocking solution (DAKO Corp., Carpinteria, CA) for 30 min and further incubated with the 8-OHdG monoclonal antibody at 1:500 dilution in antibody diluting solution (DAKO Corp., Carpinteria, CA) for overnight. The cells were then treated with anti-mouse IgG secondary antibody for 1 h. Visualization was achieved by incubation with Cy3-conjugated streptavidin (Jackson ImmunoResearch Laboratories Inc. West Grove, PA) for 1 h. Finally, nuclear localization was detected using DAPI stain (Molecular Probes, California). The cover glasses were mounted using mounting medium (DAKO Corp., Carpinteria, CA), and slides were stored at 4°C. Cells were examined using a laser scanning confocal microscope (LEICA TSD-4D equipped with an argon-krypton laser) at an excitation of 488 nm for DAPI and 647 nm for Cy3. The laser power, the voltage of the photomultiplier tube and the number of scans were kept constant so as to compare the fluorescent intensity of the samples semi-quantitatively.

Detection of apoptosis by TUNEL assay

Late apoptotic events were analyzed by terminal deoxynucleotidyl transferase-mediated uridine triphosphate (dUTP) nick-end labelling (TUNEL) assay and the number of apoptotic cells, as defined by chromatin condensation or nuclear fragmentation (apoptotic bodies). Briefly, the RINm5F

cells (3×10^3 per well) were cultured on glass cover slips in 24-well plates followed by 1 h of pre-treatment with GLEt (5 μ g/ml) at 37°C. Then the medium was replaced with fresh medium and exposed to alloxan (7mM) for 1 h. Cells were also exposed to GLEt (5 μ g/ml) and alloxan (7mM) independently. PBS exposed cells were treated as untreated control. The TUNEL assay was performed according to the manufacturer's protocol (Chemicon International, CA, USA). Apoptotic cells were visualized under fluorescent microscope and processed by AxioVision software (Carl Zeiss, Thornwood, NY, USA). Each experiment was repeated three times.

Caspase-3 activity assay

The cells were treated as described above. At the end of the experiment, the cells were lysed in a cell lysis buffer (100 mM of HEPES, pH 7.4, 0.5 mM of phenylmethylsulfonyl fluoride, 5 μ g/mL of aprotinin, 5 μ g/mL of pepstatin, and 10 μ g/mL of leupeptin) and then centrifuged at 15,000g for 20 min at 4°C. The activity of caspase-3 was estimated in the supernatant by a colorimetric method using DEVD-p-nitroanilide as substrate, according to the manufacturer's instructions (ApoAlert Caspase-3 Colorimetric Assay Kits, Clontech, Palo Alto, CA, USA).

Western blot analysis of Cytochrome c release and PARP cleavage

Release of cytochrome c from mitochondria to cytosol was measured by western blot as previously described [37] with some modifications. At the end of the experiment, cells were harvested, washed once with ice-cold PBS, gently lysed in ice-cold lysis buffer (250 mM sucrose, 1 mM EDTA, 0.05% digitonin, 25 mM Tris, pH 6.8, 1 mM dithiothreitol, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin, 1 μ g/ml aprotinin, 1 mM benzamidine, and 0.1 mM phenylmethylsulfonyl fluoride) for 30 sec and centrifuged at 12,000g at 4°C for 3 min. Protein concentration was measured in the supernatants (cytosolic fraction free of mitochondria) and the pellets (fraction that contains mitochondria) according to the method of Bradford [38] and subjected to SDS-PAGE and then analyzed by western blot using anti-cytochrome c antibody (Pharmingen, San Diego, CA, USA) or PARP antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by reaction with alkaline phosphatase conjugated secondary antibody and 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT).

Flow cytometric detection of cell cycle

Quantitative cell cycle analysis was performed using the cycleTEST PLUS DNA Reagent kit with FACS Vantage SE flow cytometer (Becton Dickinson, USA). For the determination of the cell cycle phases, a peak area of FL2-H was recorded on a linear scale. The percentages of cells in sub-G1, G1, S and G2/M cell cycle phases were determined using CELLQuest Software (Becton Dickinson). The cells ($\sim 1 \times 10^3$) were treated with GLEt (2 and 5 μ g/ml) for 1 h at 37°C followed by alloxan (7 mM/ml for 1h). Then the medium was removed and replaced with fresh medium, incubated for 22 h and subjected for the analysis. The cells incubated in RPMI-1640 medium only were used as the control. For flow cytometry analysis duplicate samples were

used for each treatment, and each experiment was repeated at least three times. Approximately 10,000 cells were evaluated for each sample. In all cytofluorometric determinations, cell debris and cell clumps were excluded from the analysis by suitable gating. The effect of GLEt on cell apoptosis was determined by the changes in the proportion of sub-G1 (hypo diploid) cells.

Measurement of annexin-V binding

Phosphatidylserine redistribution in the membrane was measured by the binding of annexin-V-fluorescein isothiocyanate (FITC) according to the manufacturer's protocol (Miltenyl Biotec, Germany). The same treatment protocol (concentration and duration of exposure) as described for cell cycle analysis was followed. After treatment and subsequent incubation, the cells were washed and resuspended in 100 μ l of HEPES buffer [10 mM HEPES (pH 7.4), 140 mM NaCl and 5 mM CaCl_2] containing annexin-V-FITC (10 μ l). Samples were mixed gently and incubated at room temperature in dark for 15 min. Immediately before analysis by flow cytometry (FACS Vantage SE, BD Biosciences, San Jose, CA USA), 5 μ l of propidium iodide (PI) (100 μ g/ml) was added to each sample. For each experiment, 10,000 events were collected and analyzed. Each experiment was repeated three times.

Statistical analysis

All the data were expressed as mean $\pm$ S.D. of number of experiments ($n = 3$) and tested using ANOVA using SPSS version 7.5 (SPSS Inc., Cary, NC, USA). The group means were compared by Duncan's multiple range test (DMRT). Values were considered statistically significant at $P < 0.05$.

Results

Our preliminary studies indicated a higher antioxidant activity and high phenolic content of the ethanol extract compared to other solvent extracts. Hence, this study was conducted using ethanol extract of this plant. The total phenolic content was found to be 156 mg of galic acid equivalent per gram of dry leaves.

Effect of the GLEt and alloxan on Cell Proliferation in RINm5F cells

In order to investigate whether GLEt alone induces cytotoxicity, the cells were treated with GLEt (5 - 25 μ g/ml) for 24 h and the number of viable cells was assessed. As shown in figure 1A, treatment with GLEt up to 20 μ g/ml dose at 37°C for 24 h did not induce significant cytotoxicity and cell viability was found to be more than 80%. When the dose was increased to 25 μ g/ml, cell viability was decreased significantly; hence, the dose up to 20 μ g/ml was selected for further studies. However, exposure of RINm5F cells to 1 - 10 mM of alloxan

Fig. 1. Effect of GLEt (A) and alloxan (B) on the viability of RINm5F cells. The cells were treated with different concentrations of GLEt and alloxan for 24 h in two independent experiments. Then, the cell viability was estimated by the MTT assay. The results were expressed as percentage of the control value from the untreated normal cells. Data were presented as mean $\pm$ S.D. of three independent experiments.

Fig. 2. Effect of GLEt on alloxan-induced cytotoxicity in RINm5F cells. The RINm5F cells were treated with alloxan (7mM) and GLEt (0.5 μ g - 20 μ g/ml) in three types of exposure protocols: pre-exposure, post-exposure and co-exposure. Then, the cell viability was estimated by the MTT assay. The results were expressed as percentage of the control value from the untreated normal cells. Data were presented as mean $\pm$ S.D. of three independent experiments. Statistical evaluation was done by ANOVA followed by DMRT. Values not sharing a common superscript differ significantly at $p < 0.05$.

significantly reduced their viability compared to control cells (Fig. 1B). The IC_{50} dose was found to be 7 mM and all further studies were performed using this concentration of alloxan.

Effect of GLEt on alloxan-induced apoptosis in RINm5F cells

In this experiment, three different modes of exposure were tried: pre-exposure, post-exposure and co-exposure. Although both co- and post-exposure of RINm5F cells with GLEt provided a significant protection from alloxan-induced cytotoxicity, the effect was less significant compared to pretreatment with GLEt (Fig. 2). The dose response study revealed that GLEt at the concentrations of 5 and 10 μ g/ml have significant protection against alloxan-induced cytotoxicity. Therefore, all further studies were carried out using these dose levels.

Protection against alloxan-induced intracellular ROS generation and nitrite formation by GLEt

In order to further elucidate the molecular basis of the antiapoptotic effect of GLEt, we determined whether GLEt exerts an effect on ROS and nitrite production. Intracellular ROS generation was increased after alloxan treatment and was significantly suppressed by GLEt pretreatment (Fig. 3A). As shown in Fig. 3B, incubation of RINm5F cells with alloxan for 24 h resulted in significant increase in nitrite formation. GLEt pretreatment diminished the alloxan-mediated nitrite formation by the cells in a concentration-dependent fashion with a half maximal inhibition observed at 5 μ g/ml. Near complete inhibition of nitrite formation was observed at GLEt concentration of 10 μ g/ml. Treatment with GLEt alone did not significantly alter nitrite production.

Fig. 3. Effect of GLEt on the intracellular ROS generation (A) and nitrite generation (B) in alloxan exposed RINm5F cells. The intracellular ROS was detected by DCFH-DA fluorescent method and the nitrite generation was estimated spectrophotometrically by Griess nitrite method. Data were presented as mean $\pm$ S.D. of three independent experiments. Statistical evaluation was done by ANOVA followed by DMRT. Values not sharing a common superscript differ significantly at $p < 0.05$.

Fig. 4. Effect of GLEt on mitochondrial membrane potential (A) and Ca^{2+} concentration (B) in alloxan exposed RINm5F cells. The mitochondrial membrane potential was assessed using the potential-sensitive dye DiOC₆(3) and intracellular Ca^{2+} was detected by fluo-3-AM. Data were presented as mean $\pm$ S.D. of three independent experiments. Statistical evaluation was done by ANOVA followed by DMRT. Values not sharing a common superscript differ significantly at $p < 0.05$.

Effect of GLEt on mitochondrial membrane potential and Ca^{2+} concentration in alloxan-treated RINm5F cells

Since, excessive ROS production leads to a decrease of mitochondrial membrane potential and apoptotic cell death, we assessed whether there was any change in the mitochondrial membrane potential in alloxan-induced cell apoptosis using the potential-sensitive dye DiOC₆(3). Alloxan treatment decreased the mitochondrial membrane potential, whereas no such decrease was observed in GLEt pre-treated RINm5F cells (Fig. 4A). Figure 4B showed that intracellular Ca^{2+} concentration was increased after alloxan treatment for 1 h as detected by fluo-3-AM. Preincubation of the cells with GLEt (5 and 10 $\mu\text{g/ml}$) dose dependently reduced the Ca^{2+} concentration to a near normal level.

Fig. 5. Effect of GLEt on lipid peroxidation in alloxan exposed RINm5F cells. The level of lipid peroxidation was assessed by TBARS. Data were presented as mean $\pm$ S.D. of three independent experiments. Statistical evaluation was done by ANOVA followed by DMRT. Values not sharing a common superscript differ significantly at $p < 0.05$.

Parameters	Control	Control + GLEt (10µg/ml)	Alloxan	Alloxan + GLEt (5µg/ml)	Alloxan + GLEt (10µg/ml)
GSH (mM/10 ⁶ cells)	9.25 ± 0.56 ^a	8.65 ± 0.96 ^b	2.31 ± 0.16 ^c	6.94 ± 0.24 ^d	8.10 ± 0.62 ^c
SOD (Units ^A /10 ⁶ cells)	0.44 ± 0.01 ^a	0.48 ± 0.12 ^a	0.26 ± 0.04 ^b	0.29 ± 0.05 ^b	0.38 ± 0.04 ^c
CAT (Units ^B /10 ⁶ cells)	6.04 ± 0.26 ^a	6.18 ± 0.16 ^a	2.41 ± 0.16 ^b	3.62 ± 0.18 ^c	4.83 ± 0.24 ^d
GPx (Units ^C /10 ⁶ cells)	105.56 ± 16.20 ^a	99.28 ± 10.10 ^a	43.64 ± 3.84 ^b	62.93 ± 2.6 ^c	88.30 ± 5.26 ^d
GST (Units ^D /10 ⁶ cells)	5.75 ± 0.68 ^a	5.46 ± 0.44 ^b	3.08 ± 0.16 ^b	3.65 ± 0.21 ^d	4.77 ± 0.26 ^c

Table 1. Effect of GLEt on antioxidant levels in alloxan treated RINm5F cells (mean ± SD, n=3). Statistical evaluation was done by ANOVA followed by DMRT. Values not sharing a common superscript letter differ significantly at p<0.05. A - One unit of activity was taken as the enzyme reaction which gave 50% inhibition of NBT reduction in one min; B - µ mole of H₂O₂ consumed/min C - µg of GSH consumed/min; D - µ moles of CDNB - GSH conjugate formed/min.

Effect of GLEt on lipid peroxidation and antioxidant levels in alloxan-treated RINm5F cells

TBARS levels were significantly increased in alloxan treated RINm5F cells when compared with untreated cells (Fig. 5). Pretreatment with GLEt at 5 and 10 µg/ml, dose dependently decreased the alloxan-induced lipid peroxidation. Similarly, while the activities of enzymic antioxidants: SOD, CAT, GPx, GST and non-enzymic antioxidant GSH were significantly (p<0.05) decreased in alloxan treated cells, in GLEt pretreated cells levels of these cellular antioxidants were not decreased significantly when compared to normal cells (Table 1).

We examined whether GLEt protects the cells from alloxan-induced oxidative stress, using Immunocytochemical staining with a monoclonal antibody against 8-OHdG (Fig. 6). This antibody can recognize oxidized nucleotides (8-OHdG, 8-hydroxyguanine, and 8-hydroxyguanosine) as markers of oxidative damage to both DNA and RNA. In alloxan-treated cells, a strong 8-OHdG signal was observed in the cytoplasm; however, no signal was detected in the nucleus at any time point. However, GLEt pre-treated cells produced very weak signal when compared to alloxan alone treated cells.

Effect of GLEt on cytochrome c release, caspase-3 activity, and PARP cleavage in alloxan- treated RINm5F cells

In the present study, the cytosol levels of cytochrome c were examined using western blot analysis. Our results revealed that alloxan induces cytochrome c release from mitochondria (Fig. 7). Since cytochrome c is reportedly involved in the activation of caspases that trigger apoptosis, we investigated the role of caspase-3 in the cell response

Fig. 6. Immunocytochemical staining of RINm5F cells with an 8-OHdG monoclonal antibody treated with alloxan and/or GLEt. A- untreated cells; B- cells treated with 7 mM of alloxan; C- cells treated with GLEt alone (5 µg); D - cells pretreated with GLEt (5 µg) followed by 7 mM of alloxan.

to alloxan. Caspase-3 is a cytosolic protein that normally exists as a 32-KDa inactive precursor. It is cleaved proteolytically into a heterodimer when the cell undergoes apoptosis [39]. As shown in Fig. 8A, alloxan treatment induced the activation of caspase-3 in RINm5F cells. PARP-specific proteolytic cleavage by caspase-3 is considered a classical biochemical characteristic of apoptosis [39]. In our experiment, in RINm5F cells, after the exposure to alloxan, PARP was found to be cleaved into an 85-kDa fragment (Fig. 8B). However, activation of caspase-3 and PARP cleavage were not observed in GLEt pretreated cells.

Fig. 7. Effects of GLEt on cytochrome c release from mitochondria into cytosol in RINm5F cells. Cytosolic and mitochondrial fractions were prepared and analyzed by Western blotting as described in Materials and Methods. Lane-1, Control; Lane-2, 7 mM alloxan; Lane-3, GLEt (5 µg/ml) pre-treated cells exposed with alloxan; Lane-4, GLEt (10 µg/ml) pre-treated cells exposed with alloxan. Experiments were repeated three times with similar results and typical data were presented. Actin was used for control of equal protein loading.

Effect of GLEt on cell cycle arrest and apoptosis in alloxan-treated RINm5F cells

The protective effects of GLEt on alloxan-induced apoptosis were assessed using TUNEL assay. TUNEL signal was highly detectable in alloxan treated cells and was reduced significantly by GLEt pre-treatment (Fig. 9). However, no apoptotic signal and significant changes in cell shape or number was observed in either GLEt alone treated or untreated cells.

Cell cycle analysis indicated that in the alloxan-treated RINm5F cells, the proportion of sub-G1 cells was found to be high (27.14%) when compared with untreated cells (1.20%) and pretreatment of RINm5F cells with GLEt (5 and 10 µg/ml) significantly reduced the sub-G1 cells in a dose dependent manner (20% and 13% respectively) (Fig. 10). The annexin-V analysis showed that pretreatment of RINm5F cells with GLEt (5 and 10 µg/ml) for 1 h before exposure to alloxan effectively reduced the proportion of apoptotic cells from 32 % (alloxan alone treated cells) to 21 % and 16 % respectively (Fig. 11).

Discussion

Reduced pancreatic β-cell mass caused by apoptotic death has been suggested as the fundamental cause for type-1 diabetes mellitus [40]. Alloxan is selectively toxic to pancreatic β-cells and has direct effect on islet cell permeability and thus a very good model for experimental type-1 diabetes [41]. Morphologic abnormalities in alloxan-

Fig. 8. Inhibitory effects of GLEt on activation of caspase-3 and PARP cleavage in alloxan-treated RINm5F cells. The cells were pretreated with GLEt or Ac-DEVD-CHO and then incubated with alloxan, for caspase-3 activity (A) and PARP cleavage (B) as described in materials and methods. Lane-1, Control; Lane-2, 7 mM alloxan; Lane-3, GLEt (5 µg/ml) pre-treated cells exposed with alloxan; Lane-4, Ac-DEVD-CHO pre-treated cells exposed with alloxan. Data were presented as mean±S.D. of three independent experiments. Statistical evaluation was done by ANOVA followed by DMRT. Values not sharing a common superscript differ significantly at $p < 0.05$.

Fig. 9. Characterization of apoptosis using TUNEL (panel B) staining in RINm5F cells treated with alloxan and/or GLEt. A- untreated cells; B- cells treated with 7 mM of alloxan; C- cells treated with GLEt alone (5 µg); D - cells pretreated with GLEt (5 µg) followed by 7 mM of alloxan.

Fig. 10. Cell cycle analysis of RINm5F cells treated with alloxan and/or GLEt measured by flow cytometry. The distribution of cells was analyzed by flowcytometry as described in materials and methods. Each experiment was carried out in triplicates.

A- untreated cells; B- cells treated with 7 mM of alloxan; C- cells pretreated with GLEt (5 μ g) followed by 7 mM of alloxan; D - cells pretreated with GLEt (10 μ g) followed by 7 mM of alloxan.

treated cells have suggested the disruption of β -cell membrane by apoptosis [42]. All the deleterious effects of alloxan on membrane permeability, transport, intracellular energy generating pathways and insulin secretion are mediated through the formation of free radical. Increase in cytosolic Ca^{2+} also plays an important role in the diabetogenic action of alloxan [43]. It was reported that alloxan causes DNA strand breaks, activation of PARP, depletion of cellular NAD^+ content and ultimately β -cell death [44]. Numerous evidences clearly demonstrated the importance of medicinal plants in the treatment of oxidative stress induced apoptosis or β -cell death in experimental diabetes [16, 45].

In our earlier studies, leaf extract of *G. montanum* was found to have modulatory effect on rate-limiting enzymes of glycolysis and gluconeogenesis in diabetic rats [21]. However, no reports demonstrated the mechanism of antidiabetic activity of *G. montanum*. This study for the first time explains the cytoprotective effect of GLEt in rat pancreatic β -cell line, RINm5F. In this study, it is clearly indicated that GLEt prevented alloxan-induced cell death in RINm5F cells as assessed by MTT assay. Exposure to GLEt alone was not found to be toxic to RINm5F cells at the tested concentrations. Among the three different modes of exposures (pre-, co- and post- alloxan exposure), pretreatment to GLEt showed higher protective effect.

Excessive superoxide levels are a well known cause of cellular dysfunction and apoptotic cell death [46]. Enhanced ROS production occurred during prolonged exposure to glucose is also implicated in apoptosis of pancreatic β -cells [47]. In this study, the exposure of RINm5F cells to alloxan alone elevated the intracellular ROS levels. However, in GLEt pretreated cells, alloxan was not found to induce ROS levels. Lipid peroxidation is the most extensively investigated process induced by ROS that participates in the toxic actions that lead to necrosis or apoptosis of the insulin-secreting cells.

Fig. 11. Analysis of apoptotic frequencies of GLEt in alloxan treated RINm5F cells measured by flow cytometry using annexin-V labelling. The intensity of the annexin-V-Fluos signal is represented on the X-axis and the intensity of the propidium iodide (PI) signal is represented on the Y-axis. A- untreated cells; B- cells treated with 7 mM of alloxan; C- cells pretreated with GLEt (5 μ g) followed by 7 mM of alloxan; D - cells pretreated with GLEt (10 μ g) followed by 7 mM of alloxan. The lower left quadrant contains viable cells; the upper left quadrant contains PI- positive cells and the two right quadrants contain annexin-V positive cells. Apoptotic cells are located in the two right quadrants.

In RINm5F cells, although alloxan treatment resulted in an increase in lipid peroxidation, this was not observed in GLEt pretreated cells. These results indicate that GLEt pretreatment was effectively protecting RINm5F cells from alloxan-induced ROS and subsequent lipid peroxidation related damages.

NO is believed to be an effector molecule that mediates interleukin (IL)-1 β -induced dysfunction and destruction of pancreatic β -cells [48]. The cytotoxicity of NO is attributed to its ability to react with iron-containing enzymes of the respiratory cycle and interfere with the synthesis of DNA [49]. In addition, NO reacts with and depletes glutathione, which increases the susceptibility of β -cells to oxidative stress [50, 51]. Our findings show that GLEt pretreatment is effectively inhibiting alloxan-induced NO generation and glutathione depletion. Pancreatic β -cells have low levels of antioxidant enzymes, in particular GPx and CAT [52]. In the present study, alloxan treatment decreased the activity of antioxidant enzymes in RINm5F cells and making them sensitive to alloxan toxicity [53]. However, GLEt pretreatment resulted in an increase in the activities of these enzymes and was able to reduce alloxan-induced oxidative stress related toxicity.

Previous findings demonstrated that alloxan caused an increase in cytosolic free Ca²⁺ in rat pancreatic β -cells [54, 55] as well as in pancreatic cell line [15]. The pretreatment of RINm5F cells with GLEt completely inhibited the increase of cytosolic free Ca²⁺ induced by alloxan. It is likely that the cytoprotective action of GLEt could be attributed to its ability to reduce the cytosolic Ca²⁺ content.

Mitochondrial damage is an important early event in apoptosis [56, 57] and is consistent with intracellular ROS production and changes in mitochondrial membrane potential [58]. Our results also show that alloxan treatment upregulates intracellular ROS production leading to loss of mitochondrial membrane potential. Several studies have suggested that increased ROS production, preceding the loss of mitochondrial membrane potential, is correlated with the release of cytochrome c and caspase-3 activation in various cell types which can lead to the induction of the apoptosis pathway [59]. Cytochrome c acts in concert with other cytosolic factors to cause the activation of executioner caspase-3 leading in turn to downstream apoptotic events. Once in the cytosol, cytochrome c binds to APAF-1 and procaspase-9 in the presence of dATP to form the apoptosome [60]. This complex activates caspase-9, which in turn cleaves and thereby activates caspase-3. In this study, cytochrome

c was detected in the cytosol of alloxan-treated cells as early as 1 h after incubation which was followed by activation of caspase-3. This was evidenced by both the increased protease activity of caspase-3 and the cleavage of PARP in alloxan-treated RINm5F cells. The specific caspase-3 inhibitor, Ac-DEVD-CHO, prevented the alloxan-induced cleavage of PARP of RINm5F cells, confirming an essential role for caspase-3 activation in alloxan-induced apoptosis. GLEt pre-treatment effectively blocked cytochrome c release from mitochondria to cytosol. Both the activation of caspase-3 and the PARP cleavage were not induced by alloxan in GLEt pretreated cells. These data suggested that GLEt exerted the protective effects on alloxan-induced cell death through the inhibition of cytochrome c release into cytosol, activation of caspase-3, and PARP cleavage.

Further, flow cytometric studies indicated that GLEt pretreatment has significantly lowered apoptotic cell death caused by alloxan as shown by the proportion of sub-G1 DNA content and annexin-V positive cells. The cell cycle analysis data clearly revealed the protective effects of GLEt on apoptotic and growth inhibitory effects of alloxan in RINm5F cells.

The underlying mechanism of the anti-apoptotic action of GLEt could be the inhibition of oxidative stress and caspase pathway, possibly due to the phenolic phytochemicals present in the extract. Numerous laboratory and epidemiological studies have shown that the phenolic phytochemicals have antioxidative, antimicrobial, antidiabetic and anti-inflammatory activities [61]. Among the numerous polyphenolics, gallic acid, resveratrol and quercetin are widely distributed in the plant kingdom and reported to possess antioxidant, antidiabetic and radical scavenging properties [61]. GLEt was found to contain 11.57% w/w of Carvacrol, 6.77% of Erythritol, 4.58% of Gallic acid and 3.09% of Quercetin [24]. These results suggest that the phenolic compounds present in GLEt possibly scavenge oxidative free radicals and play a significant role in protection of pancreatic cells against oxidative damage.

In summary, we report here that *G. montanum* treatment prevents β -cell apoptosis and thereby maintains the pancreatic insulin secretion capacity against oxidative stress. Since, oxidative stress induced cell death is caused by increased NO generation, altered mitochondrial membrane potential and/or activation of caspases, interference with these events has been suggested to protect pancreatic β -cells from oxidative stress induced apoptosis [62]. Our results suggest that GLEt contains chemical substances which could possibly interfere with

these events and thus able to protect β -cells from alloxan-induced apoptosis. Earlier, *G. sylvestre* was shown to stimulate insulin release by increasing membrane permeability in various pancreatic cell lines [18], this is the first report demonstrating the protective effects of *G. montanum* on pancreatic β -cells. Further studies on the isolation and characterization of the compounds from the extract may pave the way for the discovery of new diabetic drug which improves not only β -cell function, but also insulin stimulation.

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