**Gymnemasylvestre** derived compounds inhibit GSH depletion and increase cGMP and nitric oxide to attenuate advanced glycation end products induced hypertrophic growth in renal tubular epithelial cells

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**ABSTRACT**

The accumulation of advanced glycation end products (AGE) plays significant role in developing tubular hypertrophy during diabetic nephropathy (DN). Reactive oxygen species and nitric oxide (NO) are directly involved in the progression of DN. We have studied the effect of standardized Gymnemasylvestre organic extract (GE) on AGE induced cellular hypertrophy using rat renal tubular epithelial cells (NRK 52E). AGE (400 μg/ml) induced cytotoxicity to NRK 52E cells as determined by MTT assay at 0–72 h. We report cellular hypertrophy mediated cytotoxicity by AGE which was the result of significant reduction in the cellular nitric oxide and cGMP levels associated with increased lipid peroxidation and antioxidant depletion (P<0.05). Upon treatment with GE the cell viability was increased with reduced cellular hypertrophy by 1.7 folds when compared to AGE treated group. GE could significantly increase NO by 1.9 folds and cGMP by 2.8 folds and inhibited GSH depletion by 50% during AGE induced toxicity. The antioxidant enzyme activity of catalase was increased by 50% while, glutathione peroxidase and superoxide dismutase enzyme activities were significantly increased by 42% and 67% with decreased lipid peroxidation (49%) upon GE treatment. Thus, GE attenuates AGE induced hypertrophic growth by inhibiting GSH depletion and partly through increased NO/cGMP signaling.

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1. **Introduction**

Diabetic nephropathy (DN), a major microvascular complication of diabetes mellitus (DM) increases the risk of life threatening end-stage renal disease [36,1]. High glucose and advanced glycation end products (AGE) play a vital role in the development of DN [2,3]. AGE arise from glucose-derived Amadori (1-deoxy-d-fructosyl derivatives) products and act to increase oxidative stress, elevate vascular permeability, enhance protein and lipoprotein deposition, promote extracellular matrix (ECM) protein...
synthesis, and tubulointerstitial fibrosis, and exert a number of toxic effects on renal cells [3–5]. AGE mediate their effects via specific receptors (RAGE), activating diverse signal transduction cascades, and downstream pathways, including generation of generalized cellular dysfunction and via reactive oxygen species [3–5].

Nitric oxide (NO) modulates large variety of physiological functions and initiates diverse cellular signaling cascades which comprise nitrosoylation of proteins, adenosine 5′-diphosphate (ADP)-ribosylation, or stimulation of soluble guanylylcyclases which catalyze intracellular guanosine 3′,5′-cyclic monophosphate (cGMP) synthesis [6]. cGMP activates cGMP dependent protein kinase (PKG) which mediate localized and global signaling. Considerable evidence suggests that advanced DN leading to severe proteinuria, declining renal function, and hypertension is associated with a state of progressive NO deficiency [6].

Protective effects of exogenously administered antioxidants have been extensively studied in animal models [7]. N-acetylcysteine (NAC), a thiol-containing radical scavenger and glutathione precursor, has been found to protect β-cells in culture and in vivo from “glucose toxicity” preserving insulin synthesis and secretion [8]. In similar lines taurine act as an antiglycative compound, providing free amino groups that may compete for the reducing sugars [7–9].

Rat renal tubular epithelial cells (NRK-52E) are widely used to study the DN related complications in vitro [10]. NRK-52E cells exposed to AGE-modified BSA (AGE-BSA; 40 microM) or TGF-beta1 readily undergoes transition from epithelial to mesenchymal cells a characteristic feature of renal fibrosis in experimental diabetes [10] and qualifies as a good model to study DN related complications.

In this context natural compounds from herbal origin which can enhance the NO production and possess strong antioxidant might be beneficial in attenuating the undesired effects of AGE might be beneficial in retarding the risk of life threatening end-stage renal disease. Gymnema sylvestre has been extensively used to treat diabetes and related complications from time immemorial [11,12]. Apart from this it has been reported that Gymnema sylvestre is beneficial in the treatment of obesity, arthritis, hyperlipidemia, Parkinsonism, hypercholesterolemia [13,38,14]. In light of these reports in the present study Gymnema sylvestre organic extract (GE) was evaluated in vitro using rat renal tubular epithelial cells during AGE induced DN associated complications.

2. Materials and methods

2.1. Chemicals

Bradford reagent, cytochrome-C, 2,7-dichlorofluorescein diacetate, diphenylamine (DPA), Dulbecco’s Minimum Essential Medium (DMEM), p-Ribose, Fetal bovine serum (FBS), glutathione (GSH), hydrogen peroxide, 3′(4,5-dimethyl thiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT), β-Nicotinamide adenine dinucleotide phosphate (β-NADPH), Phosphate buffered saline (PBS), Perchloric acid, thiorbarbituric acid, p-Ribose, Sodium cholate, Sodium dodecyl sulphate (SDS), Tween-20, xanthine and xanthine oxidase were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were of analytical grade.

2.2. Gymnema sylvestre organic extract and marker compound analysis

Standardized Gymnema sylvestre organic extract (GE) was a gift from Phytochemistry Division, The Himalaya Drug Company, Bangalore, India. Briefly, 25 kg of the leaves of Gymnema sylvestre was pulverized to fine powder and subjected to hot water extraction. The resultant extract was spray dried and analyzed for gymemic acid an active marker found in Gymnema sylvestre bygravimetric analysis. The net yield of the standardized extract based on gymemic acid was found to be 20%. The good agricultural and collection practices (GACP) were employed during farming, harvesting and collection of plant. The plant Gymnema sylvestre was identified and certified by Botanist and a voucher specimen of the same has been archived in the herbarium of R&D, The Himalaya Drug Company, Bangalore, India.

2.3. Preparation of AGE

Aliquots of FBS were incubated for 7 days at 37°C in the presence of 50 mM p-ribose plus 1% antibiotic-antimycotic solution and then extensively dialyzed (10 kD cut off) against sodium phosphate saline buffer (PBS) 0.1 M pH 7.4, to remove surplus sugar (glycated serum, GS). Further aliquots of FBS were processed in the same way, but without ribose (non-glycated serum, NGS) [15]. GS serum aliquots were stored at 20°C until 3 days, and before each experimental procedure the presence of AGE was evaluated using CircuLex™CML/Ne-(carboxymethyl) lysine ELISA kit (MBL International Corporation, USA) according to manufacturer’s instruction. Thus prepared AGE was used in all the experiments.

2.4. Cell culture

All the experiments were performed using NRK 52E cells within 10 passages after thawing. The NRK 52E cells (Rat renal tubular epithelial cell line) was obtained from the National Centre for Cell Science (NCCS) Pune, India, were maintained in culture using 25 cm² polystyrene flasks (Tarsons) with DMEM containing 10% FBS, 1% antibiotic-antimycotic solution, and 3.7 g/L sodium bicarbonate under an atmosphere of 5% CO2 at 37°C with 95% humidity. Continuous cultures were maintained by sub-culturing cells every 4 days at 2.2 × 10⁶ cells/25 cm² flasks by trypsinization.

2.5. AGE induced cytotoxicity and protection by GE

NRK 52E cells were plated into 96-multicwell culture plates from the stock containing 1 × 10⁵ cells/ml and each well was seeded with ≈20,000 cells. To study AGE induced cytotoxicity, 24 h after plating, the medium was discarded and fresh medium containing AGE at various concentrations (100–500 μg/ml) was added. At different time points
2.7.4. In order to determine the non-toxic concentration of GE cells were incubated for 0–72 h and the cytotoxicity was determined. Based on the experimental results the effective concentration was determined as 200 μg/ml and used in all the experiments to evaluate the protective potential of GE on several cellular parameters.

2.7.5. Measurement of enzymic antioxidants

The antioxidant enzymes superoxide dismutase (SOD), catalase and glutathione peroxidase, (GPx) activities were analyzed using cytosolic fraction. Total SOD activity was determined by monitoring the inhibition of the reduction of ferricytochrome C at 550 nm, using the xanthine-xanthine oxidase system as the source of superoxide. One unit of the SOD is defined as the amount of the enzyme required to inhibit 50% of the rate of cytochrome C reduction [20]. Catalase activity was measured by following the rate of H2O2 consumption spectrophotometrically at 240 nm and expressed as μmol H2O2 oxidized/min/mg protein [21]. Glutathione peroxidase activity was determined by following the enzymatic NAPDH oxidation at 340 nm [22].

2.8. Cellular hypertrophy analysis

Cells were grown in six-well plates until 50% confluence and then made quiescent for 2 days in serum-free DMEM (High Glucose) medium. The cultures were treated with BSA, AGE and with or without GE and experimental control. At the end of experimental time the cell surface area was analyzed for at least 30 cells per condition using the public ImageJ 1.47 software. Further, the cells were trypsinized, washed twice with phosphate-buffered saline (PBS) and counted using a hemocytometer. Equal numbers of cells were lysed in buffer (0.1% [wt/vol] SDS, 0.5% [wt/vol] sodium deoxycholate, 1% [wt/vol] Tween 20 in PBS). The total protein content was measured using the Bio-Rad protein assay kit. Total protein was expressed as micrograms of protein per 10⁴ cells.

2.9. Statistical analysis

Statistical analysis was carried out using Graph Pad Prism statistical software (Graph Pad Prism, San Diego, CA, USA). Results are analyzed by t-test and one-way analysis of variance (ANOVA) wherever applicable and the significance was calculated using the Tukey–Kramer multiple comparison test and results are considered as significant at P < 0.05.

3. Results

3.1. AGE characterization by CML content and its cytotoxicity

The AGE used throughout the experimentation was characterized by analyzing CML Nε-(carboxy methyl) lysine. The results showed that AGE prepared by following the method detailed contained 16.07 ± 0.76 ng/ml of CML (Fig. 1). Cytotoxicity of AGE and GE in NRK-52E cells was evaluated using MTT assay and the results are given in Fig. 2(a) and (b). AGE was tested for its cytotoxicity with wide range of concentration for 0–72 h and the results are given in Fig. 2(a). On the other hand GE did not present any cytotoxic effect at concentration ranging from 0 to 250 μg/ml when tested for 0–72 h when tested against
cell control (data not shown). The results showed that AGE at (100–500 μg/ml) caused cytotoxicity to NRK-52E cells. The CTC50 of AGE was determined to be 400 μg/ml [AGE sample used in the experiments contained 40 ng CML/mg AGE] at 72 h. Hence, the cells were challenged against CTC50 in all the subsequent experiments for testing protective effect of GE.

3.2. Effect of GE on nitric oxide and cGMP levels upon AGE treatment

The cells exposed to AGE were evaluated for nitric oxide and cGMP levels and the results are given in Fig. 3(a) and (b). The results showed that AGE inhibited the NO and cGMP by 64% and 68.5% respectively in NRK-52E cells. Further, the cells when added with GE increased NO and cGMP levels by 1.9 folds and 2.8 folds respectively compared to AGE treated cells. Cells treated with antioxidant taurine shown to significantly inhibit depletion of NO and cGMP by 1.7 folds and 2.5 folds respectively compared to AGE group.

3.3. Effect of GE on lipid peroxidation, ROS generation and non-enzymic antioxidants

The lipid peroxidation was significantly increased by 3.2 folds upon addition of AGE to NRK-52E cells as shown in Fig. 4(a). The cells treated with antioxidant taurine could
3.4. Effect of GE on antioxidant enzyme activity

The antioxidant enzymes catalase, glutathione peroxidase (GPx) and superoxide dismutase (SOD) activities were evaluated and the results (Table 2) showed that AGE inhibited the catalase activity by 63%. The GPx activity was inhibited by 49% and SOD activity was inhibited by 45% respectively in AGE treated cells. Upon addition of GE to cells treated with AGE the catalase activity increased significantly by 2.2 folds, whereas GPx and SOD activity were increased by 42% and 67% respectively compared to AGE treated group. In similar lines when the cells challenged with AGE were treated with taurine the antioxidant enzyme activities were significantly increased.

3.5. Effect of GE on hypertrophic growth

The effect of GE during AGE-mediated cellular hypertrophy was studied and the results are presented in Fig. 5(a)–(d). The results showed that AGE significantly reduced the cell viability by 52% and inhibited mitogenesis compared to control cells Fig. 5(a). Apart from cell viability, cell number and relative cell surface area (%) was determined and the results are given in Fig. 5(b) and (c). The results showed that AGE treatment significantly decreased the cell number by 38.2% with increased relative cell surface area by 25%. While in GE and taurine treated groups decreased relative cell surface area by 18.1% and 21.1% and increased cell number by 51% and 34.8% respectively compared to AGE treated group. The increased ratio of cellular protein content to cell number represents the cellular hypertrophy and the results showed that AGE increased significantly the cellular hypertrophy by 79% compared to control cells Fig. 5(d). While GE and taurine treated cells significantly inhibited cellular hypertrophy. The cell morphological characteristics were analyzed by microscopy and the results are given as photomicrographs Fig. 6. The results clearly showed that cells treated with AGE exhibited profound hypertrophic characteristics while, treatment with GE and taurine successfully inhibited the morphological changes associated with AGE induced hypertrophy.

4. Discussion

DN is alarmingly increasing and is regarded as the common cause for the end-stage renal failure requiring organ replacement [22,23]. The molecular mechanism associated with DN has been one of the most desired research topics to be studied. In the present study it has been showed that AGE mediates cytotoxicity to NRK-52E cells when incubated over a period of 0–72 h in an incremental manner. Our observations are in agreement with earlier reports wherein it has been experimentally shown that AGE alter the function and structure of tissue matrix proteins and this result in triggering expression of various pathogenic mediators through increased oxidative stress, ECM, TGF-β1, etc. which are implicated in the development and induction of DN [3,4,24,25]. Clinically it has been consistently shown that AGE are involved in
Table 1
Effect of GE on non-enzymic antioxidants during AGE induced cellular hypertrophy in NRK-52E cells. Cells were co-incubated with BSA (400 μg/ml), AGE (400 μg/ml) in presence or absence of GE (200 μg/ml), or Taurine (100 μM) and non-enzymic antioxidants were determined at 24 h as described in Section 2. Values are mean ± SEM of three independent experiments carried out in triplicates. *Statistically significant at P<0.05 compared to control. †Statistically significant at P<0.05 compared to BPA.

| Groups       | GSH (nmol/mg protein) | GSSG (nmol GSH equiv/mg protein) | GSH/GSSG ratio |
|--------------|-----------------------|----------------------------------|----------------|
| Control      | 94.65 ± 3.22‡         | 11.58 ± 1.35§                    | 8.18           |
| BSA          | 91.84 ± 2.82‡         | 14.32 ± 2.80§                    | 6.41           |
| AGE          | 22.25 ± 1.15**        | 20.37 ± 2.98**                   | 0.53           |
| AGE+Taurine  | 49.85 ± 4.12**        | 12.64 ± 3.41‡                    | 1.81           |
| AGE+GE       | 69.45 ± 3.22**        | 11.85 ± 2.32‡                    | 4.42           |

ND—not detected.

Table 2
Effect of GE on antioxidant enzymes catalase, glutathione peroxidase and superoxide dismutase activity during AGE induced cellular hypertrophy in NRK-52E cells. Cells were co-incubated with BSA (400 μg/ml), AGE (400 μg/ml) in presence or absence of GE (200 μg/ml), or Taurine (100 μM) and antioxidant enzyme activities were determined at 24 h as described in Section 2. Values are mean ± SEM of three independent experiments carried out in triplicates. *Statistically significant at P<0.05 compared to control. †Statistically significant at P<0.05 compared to ethanol.

| Groups       | Catalase a | Glutathione peroxidase b | Superoxide dismutase c |
|--------------|------------|--------------------------|------------------------|
| Control      | 3.24 ± 0.21† | 39.19 ± 2.75† | 40.22 ± 2.56† |
| BSA          | 2.98 ± 0.16† | 40.23 ± 3.65‡ | 44.52 ± 3.22† |
| AGE          | 1.21 ± 0.35** | 19.84 ± 1.38** | 21.97 ± 1.31** |
| AGE+Taurine  | 2.10 ± 0.32** | 24.38 ± 3.68** | 25.44 ± 1.08** |
| AGE+GE       | 2.73 ± 0.49† | 28.17 ± 2.12 † | 36.71 ± 2.17 † |

a µmoles of H2O2 decomposed/min/mg protein.
b µmoles of NADPH oxidized/min/mg protein.
c Units/mg protein.

Fig. 5. Effect of GE on AGE mediated cellular hypertrophy. Serum-deprived cells were treated with BSA (400 μg/ml), AGE (400 μg/ml) in presence or absence of GE (200 μg/ml), or Taurine (100 μM) for 3 days, and then assayed for (a) cell viability (b) cell number (c) relative cell size and (d) hypertrophy index as described in Section 2. Values are mean ± SEM of three independent experiments carried out in triplicates. *Statistically significant at P<0.05 compared to control. †Statistically significant at P<0.05 compared to AGE.
the development and progression of glomersulosclerosis and tubulointerstitial fibrosis in patients with diabetes [3–5].

Hyperglycemia is associated with the decrease in NO production and the loss of NO-mediated repression of aldose reductase is a significant factor in the activation of the polyol pathway and the development of several diabetic complications [26,27]. We report that AGE significantly depletes NO and cGMP in NRK 52E cells which might have been resulted due to the activation of polyol pathway. Thus, compounds which increase NO generation during AGE induced pathogenesis may inhibit oxidative stress induced by hyperglycemia. Earlier, nitric oxide synthase cofactor tetrahydrobiopterin has been successfully shown to inhibit the generation of reactive oxygen species (ROS) [28–30]. In concurrence to the reports, cells exposed to AGE and treated with GE significantly increased the NO measured as nitrate in cells followed with increased cGMP levels to increase the cell viability. Hence, compounds present in GE was mainly responsible in bringing about increase cell viability through enhanced NO production and increased cGMP levels.

Several studies have suggested that AGE stimulates production of ROS, including superoxide anions, hydrogen peroxide and hydroxyl radicals, in a variety of cell types [3–5,7,31]. Antioxidants such as taurine can increase the intracellular concentrations of glutathione and augment cellular antioxidant capacity and can also directly scavenge ROS [7,8,31]. Our findings show that AGE increased intracellular generation of ROS which is reported as increased lipid peroxides. Incubation of cells with GE and taurine significantly decreased generation of lipid peroxides and ROS which is in agreement with earlier reports [32]. These observations suggest for the first time that GE acts as a strong antioxidant during AGE induced complications.

It is known that, GSH a non-enzymic antioxidant plays an important role in cellular defense against ROS, free radicals and electrophilic metabolites [33,34]. Hence, severe GSH depletion leaves cells more vulnerable to oxidative damage by radicals and increases protein thiolation or oxidation of SH groups that may lead to alterations in cellular calcium homeostasis [34]. A sustained increase in cytosolic calcium levels results in activation of enzymes (phospholipases, non-lysosomal proteases, endonucleases) and cytoskeletal damage, which ultimately causes cell death [34]. The potential of GE to maintain GSH at reasonably high levels is of importance against AGE induced toxicity. Therefore, the ability of GE in preventing AGE induced GSH depletion by 80% is very significant in restoring the cell viability. The GSSG formation was inhibited by GE and this may be attributed to the formation of GSH conjugates rather than oxidation to GSSG in AGE induced toxic conditions.

Beside these, cells secrete strong antioxidant enzymes such as superoxide dismutase, catalase and glutathione peroxidase to combat severe oxidative stress during diseased/toxic conditions. Earlier it has been shown that AGE augment formation of ROS through depletion of glutathione peroxidase [37]. We also report in similar
lines that AGE significantly inhibited the antioxidant enzyme activities of catalase, superoxide dismutase and glutathione peroxidase. While AGE induced inhibition of antioxidant enzyme activities was restored by treatment with GE with reduced ROS generation in cells. The increased antioxidant enzyme activities were observed in cells treated with taurine and diclofenac too. These results add to the fact that compounds in GE extract acts in similar lines with taurine to bring pharmacological action.

It has been clearly shown in our study that cellular hypertrophy is the characteristic feature in renal tubular epithelial cells treated with AGE with reduced NO and cGMP levels. Our observation is further substantiated with the earlier reports that AGE induced cellular hypertrophy through iNOS/PGK inactivation followed with expression of ERK/JNK/p38 MAPK pathway and fibronectin/p21 Waf1/Cip1 synthesis [35]. In addition we also report that treatment with GE and taurine could effectively reduce the relative markers of cellular hypertrophy suggesting that GE effectively restores NO/cGMP levels to inhibit cellular differentiation observed during DN complications. Thus, it is inferred that standardized Gymnemysylesthe organic extract derived compounds inhibit GSH depletion and increase cGMP and nitric oxide to attenuate advanced glycation end products induced hypertrophic growth in renal tubular epithelial cells and warrants further studies.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.toxrep.2014.08.015.

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