

Original Article

GENISTEIN ALLEVIATES HIGH GLUCOSE INDUCED TOXICITY AND ANGIOGENESIS IN CULTURED HUMAN RPE CELLS

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ABSTRACT

Objective: Genistein, an iso flavonoid has been shown to possess many biological activities including anti-inflammatory, antioxidant and anti-angiogenic property. It has been shown to be protective in dampening diabetes induced retinal inflammation *in vivo*. Therefore, the purpose of the present study is to investigate the effect of genistein on glucose induced toxicity in cultured human RPE cells (ARPE-19).

Methods: ARPE-19 cells were challenged with normal glucose (NG 5 mM) and high glucose (HG1 25 mM & HG2 50 mM) concentrations with or without genistein (20 µM) for 24 h. The mRNA expression of aldose reductase (ALR) and VEGF was measured by real-time PCR using SYBR green. Inhibitory effects upon ALR activity were performed. The VEGF levels of cell supernatant were estimated by sandwich ELISA. Cellular viability and mitochondrial function upon genistein treatment were assessed using dye exclusion method and MTT assay respectively.

Results: Genistein at the studied concentration showed 93% of cell viability and no marked toxicity was observed on cell growth. ARPE-19 cells challenged with HG1 and HG2 showed (2.32) and (2.48) fold increase in ALR expression as compared to NG. Significant increase in VEGF₁₆₅ expression was observed in ARPE-19 cells with HG1 and HG2 as compared to NG. The genistein treated cells significantly reduced the mRNA expression of both ALR and VEGF₁₆₅. No significant increase in VEGF level was observed in ARPE-19 cell supernatant with HG1 and HG2 (220.68±5.24 and 228.96±7.19 pg/ml) as compared to NG (220.30±2.04 pg/ml), however, significant reduction was observed in response to treatment with genistein in HG1 and HG2 (155.51±9.33 and 122.85±4.76 pg/ml). Also ALR activity significantly reduced in genistein treated cells when compared with HG1 and HG2 concentrations.

Conclusion: The results of the present study clearly demonstrate that genistein protects RPE cells from glucose toxicity. Genistein could be a prospective potent agent for treating complications linked with diabetes mellitus, such as diabetic retinopathy.

Keywords: Aldose reductase, Blood retinal barrier, Retinal Pigment epithelial (RPE) Cells, Diabetic retinopathy, Genistein, VEGF

INTRODUCTION

The retinal pigment epithelium (RPE), outermost layer of the retina plays vital role like nutrients transport from the vascular choroid, development of the blood-retinal barrier (BRB) and scattered light absorption [1, 2]. In diabetic retinopathy (DR) RPE cells involved in the pathologic pathway of neovascularization and retinal vascular permeability that leads to visual loss [3-5] through polyol accumulation, formation of advanced glycation end products (AGEs), oxidative stress and production of vascular endothelial growth factor (VEGF) [6-8]. In the polyol pathway aldose reductase (ALR) is the first rate-limiting enzyme which catalyzes the conversion of excess glucose to sorbitol. The accumulated sorbitol disrupts cellular homeostasis and depletes NADPH leading to osmotic swelling and oxidative damage [9], extracellular fluid within the retina, distorting the retinal structural design leading to breakdown of BRB and leakage in the RPE [10, 11]. The increased oxidative stress possibly raises the synthesis of diacylglycerol (DAG) followed by activation of the protein kinase C, which results in increasing the expression of endothelial nitric oxide synthetase (eNOS), endothelin-1 (ET-1) and VEGF [12-14].

Many studies have revealed that VEGF plays a prominent role in the angiogenesis in eye. The human VEGF gene makes up to five different isoforms having amino acid at different positions (121, 145, 165, 189 and 206 amino acids) that code for VEGF proteins [15, 16]. Among these VEGF₁₆₅ is the principal molecular species produced by a range of normal and transformed cells [3, 17]. It is also postulated that some VEGF are important for the normal physiological homeostasis of the eye, and it can be useful to diminish VEGF in these pathologies without abolishing its expression [18, 19].

Soybeans (*Glycine max*) enclose massive amounts of the isoflavones genistein and daidzein. Genistein (4', 5', 7'-trihydroxyisoflavone) is a strong antioxidant and tyrosine kinase inhibitor [20, 21]. Genistein offers protection against a number of diseases such as cancer, diabetes, cardiovascular diseases, post-menopausal disturbances and osteoporosis [22-24].

Previous studies on experimental animals have shown that genistein has excellent antidiabetic, anti angiogenic and anti-inflammatory properties [25-27] and is beneficial in preventing and treating ocular toxicity associated with high glucose. The main objective of the present study to investigate the effect of genistein on high glucose induced ALR and VEGF expression in cultured human retinal pigment epithelial cells (ARPE-19).

MATERIALS AND METHODS

Human RPE cell cultures

ARPE-19 cells were grown in DMEM/F12 (1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12; Gibco-Life Technologies, Rockville, MD, USA) and 10% Fetal bovine serum (Gibco-Life Technologies, Rockville, MD, USA) and Penicillin-streptomycin (containing 10,000 U/ml penicillin and 10 mg/ml streptomycin; Penicillin-streptomycin). Cell cultures were maintained in a humidified incubator at 37 °C with 95% air and 5% CO₂. The medium was changed every other day. Cultures were dissociated using 0.05% (w/v) trypsin (Gibco-Life Technologies, Rockville, MD, USA) in phosphate buffered saline (PBS), pH 7.4 after its confluency. Then they were sub cultured at a splitting ratio of 1:4. Near-confluent, cells were challenged with normal (NG-5 mM), high (HG1-25 mM) and (HG2-50 mM) glucose with or without genistein for 24 h.

Cell viability assay

The effect of genistein on cell viability was assessed by dye exclusion method [28]. Briefly, serum starved ARPE-19 cells were exposed to various concentrations of genistein (5µg/ml-100 µg/ml) for 24 h. After the treatment, the cells were washed three times with ice-cold PBS. The cells were then dissociated with trypsin-EDTA and the resulting pellet was treated with trypan blue and live cells were counted using hemo cyometer.

Cell viability percentage calculated by: (live cells/total no. of cells) x 100.

Measurement of mitochondrial damage by MTT assay

The mitochondrial damage by genistein was assessed using a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay kit (*In vitro* toxicological assay kit Sigma, USA), which has been described previously [29]. ARPE-19 cells seeded in 96-well plates at a density of 1×10^4 cells/well were grown to 80% confluency. The cells were serum starved for 3 h and treated with different concentrations of genistein (5µg/ml-100µg/ml) in DMEM/F12 medium containing 1% FBS for 24 h. After incubation, MTT (20 µl) was added and incubated for 3 h. The levels of formazan reaction product from each well were determined at 570 nm using the Spectramax M3 multiplate reader (Molecular Device, California, USA).

RNA isolation and real-time PCR

Total RNA was isolated from ARPE-19 cells challenged with NG, HG1 and HG2 in the presence or absence of 50µg/ml Genistein for 24 h using Trizol Reagent (Sigma, USA). cDNA template from total RNA was synthesized using cDNA reverse transcription kit (High Capacity cDNA Reverse Transcription Kit, Thermo, USA). ALR, VEGF165 and GAPDH were amplified by gene-specific primers (Bioserve, Hyderabad, India). The sequences of primers (F): 5'-TTTTCCCAT TGGATGAGTCGG-3' and (R): 5'-ACGTGTCCAGAATGTTGGTGT-3' for human ALR (60bp); F: 5' GCTACTGCCATCCAATCGAG-3' and R: 5'-TCTTTCTTTGGTCT GCATTCAC-3' for human VEGF-165 (255bp) and GAPDH Primer (131bp) F: 5'-GTCTCCTCTGACTTCAACAGCG-3' and R: 5'-ACCACCCTGTGCTGTAGCAA-3' used as an internal control for experiment. ALR and VEGF₁₆₅ expression was quantified by quantitative real-time PCR (ABI 7900 HT, CA, USA) using SYBR green (F-410L, Thermo, USA). The cycle parameters consisted of an initial denature step of 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The PCR samples were analyzed with 1.2% agarose gel electrophoresis along with DNA ladder, stained with ethidium bromide and visualized below UV light.

Effect of genistein on VEGF secretion by sandwich ELISA

The quantity of VEGF secreted into the culture medium post exposure to

different concentrations of glucose with or without genistein conditions was estimated using sandwich ELISA (VEGF ELISA DuoSet, R&D System, USA) according to the manufacturer's protocol.

ALR activity

ALR activity was assessed spectrophotometrically as described previously by Reddy [30]. The assay was carried out in UV transparent 96 well plate with 300 µl capacity. The assay mixture contained 100 mM ALR buffer (135 mM sodium phosphate buffer (pH 7.0), 0.5 mM PMSF and 10 mM beta mercaptoethanol containing 0.2 mM LiSO₄, 5 mM DL-glyceraldehyde and enzyme preparation (Cell lysate). The reaction was initiated by the addition of 0.15 mM NADPH and the decrease in the optical density at 340 nm was recorded for 20 min.

Statistical analysis

All experiments were performed in duplicate. Values are expressed as mean±standard deviation (SD) in the results and calculations. The data were analyzed by student's *t* test for comparisons between groups using Prism 6 software; GraphPad. Values of *p*<0.05 were considered statistically significant.

RESULTS

Effect of genistein on cell viability and cytotoxicity

In order to demonstrate the safety of genistein in RPE cells, we have carried out cell viability and toxicity assay after incubation with different concentrations for 24 h.

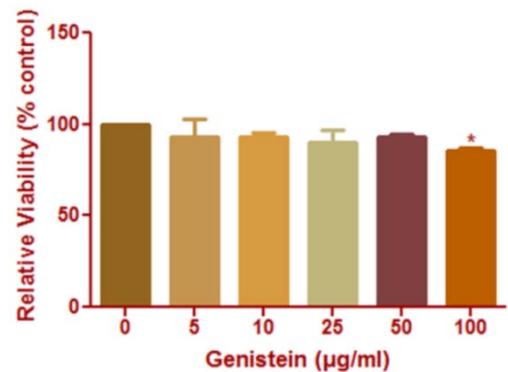


Fig. 1: Effect of genistein on the viability of human RPE cells. The results are presented as the mean±SD (n=4). **P*<0.05 vs. control

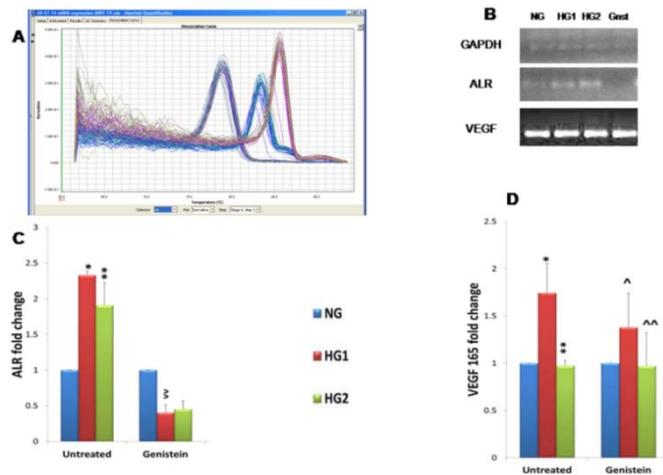


Fig. 2: Genistein inhibits HG induced ALR and VEGF expression at mRNA level. ALR and VEGF165 mRNA expression analyzed by RT-PCR (A) Melting Curve analysis from a real time PCR assay (B) The gel electrophoresis represent wholesome gene amplification of our interest without any primer dimer formation. (C and D) ALR and VEGF 165 mRNA expression was increased with high glucose and inhibited by genistein, Data are presented as mean±SD (n=3) **p*<0.001 vs. NG; ***P*<0.0001 vs. NG; >>*p*<0.05 vs. HG1;>*p*<0.001 vs. HG2 with genistein treated cells

The results suggest that genistein does not significantly alter viability of RPE cells upto 50µg/ml. Genistein at 100µg/ml showed 14.4% toxicity by dye exclusion method ($p<0.05$) (fig.1). However, genistein at all studied concentrations did not show any cell toxicity by MTT assay (Data not shown).

Effect of genistein on ALR and VEGF₁₆₅ expression by real time PCR

ALR expression was found to be 2.3 fold and 2.5 fold high in RPE cells challenged with HG1 and HG2 respectively. Genistein at studied concentration (50µg/ml) showed the marked reduction in ALR mRNA expression induced by HG1 and HG2 concentration ($p<0.05$).

VEGF₁₆₅ expression was found to be 1.7 fold increases in RPE cells challenged with HG1. Genistein showed marked reduction in VEGF mRNA expression with RPE cells challenged with HG1 and HG2 concentration of glucose ($p<0.05$).

Genistein inhibits HG induced VEGF secretion

No significant increase in VEGF secretion with high glucose was observed for 24 h. The presence of genistein significantly reduced VEGF secretion under the given experimental conditions.

Effect of genistein on ALR activity

RPE cells were grown in various concentrations of glucose for 24 h and ALR activity was determined. At HG1 and HG2 concentrations, dose dependent increase in ALR activity was observed and these changes were restored by genistein treated cells ($P<0.05$).

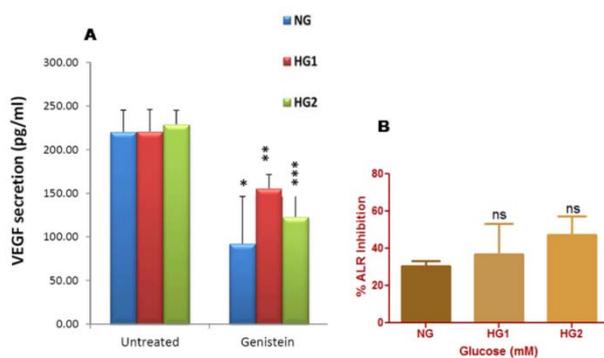


Fig. 3: (A) Genistein inhibits HG induced VEGF Secretion in human RPE cells. Data are presented as mean±SD of three experiments. * $p<0.05$ vs. NG; ** $p<0.005$ vs. HG1; * $p<0.001$ vs. HG2 Compared with treated cells (B) Effect of genistein on % ALR inhibition, At HG1 and HG2 concentrations, there was a dose dependent increase in ALR activity was observed and these changes were restored by genistein treated cells**

DISCUSSION

Retinal pigmented epithelium constitutes an important component of blood-retinal barrier (BRB) where in conjunction with endothelial cells it provides tight junction which restrict the flow of solutes and fluid from the subretinal space across BRB to the choroid in retina. In diabetes, the integrity of BRB is disrupted due to damage to RPE by oxidative stress associated with high glucose level. Corresponding to damage of RPE; vascular leakage causes excessive water influx to the retina, which leads to DR and diabetic macular edema [31].

In the present experiments, we screened the protective effect of Genistein on high glucose induced ALR and VEGF expression in cultured human RPE cells. Genistein, an iso flavonoid that is extracted from the seeds of soya bean considered to be potent anti-diabetic agents, such as preservation of insulin β -cells and restoration of the glucose metabolic enzyme activities independently from its tyrosine kinase activity and intracellular cAMP accumulation, which subsequently activates PKA [25, 27, 32, 33].

Aldose reductase (ALR) is a cytosolic enzyme which is responsible for conversion of blood glucose into fructose. During hyper glycaemic

condition, the increased glucose level enhances the activity of ALR by increasing glucose flux through this pathway. The increased expression of ALR under hyper-osmotic stress was subsequently reported in cultured human retinal pigment epithelial cells. In fact, ALR mRNA is highly expressed in the rat lens, retina and sciatic nerve, the major target organs of diabetic complication. Increased activity of ALR results in decreased NADPH/NADP⁺ ratio, which has impact on antioxidant enzyme. The decreased activity of the antioxidant enzymes causes oxidative stress under high glucose conditions followed by increased formation of advanced glycation end products (AGEs). They cause pathological changes by trouble making protein function and interfering with cellular receptors [8, 34, 35].

ALR inhibitors, such as ARI-809, TMG, minal restat, and epalrestat, have been shown to prevent or reverse early abnormalities associated with diabetes, especially eye disease, in animal models [36-38]. Many natural compounds have been tested for ALR inhibitory activity. In addition to its antioxidant properties, genistein, a tyrosine kinase inhibitor, has an inhibitory effect on the formation of advanced glycation end products [21, 39].

Our results show that genistein suppresses high glucose induced ALR expression and ALR Enzyme secretion by inhibiting the action of ALR and increases the level of cellular antioxidant. Therefore, genistein mediated inhibition of ALR and increases in antioxidants levels may help to prevent high glucose induced diabetic vision loss. However, Kim *et al.* [40] reported that genistein significantly inhibits xylose induced lens opacity, ex vivo, via inhibition of ALR and its antioxidant effects.

VEGF is recognised as a major contributor to the development of diabetic retinopathy. The protein iso forms of VEGF vary in length from 111 to 206 aa (VEGF111, 121, 145, 148, 165, 183, 189 and 206). Among these isoforms, VEGF165 is believed to be highly associated with ocular leakage and neovascularisation [41].

Stimulated by hyperglycaemia and oxidative stress, VEGF was expressed by vascular endothelial, retinal pigment epithelial, Müller glial cells, pericytes and astrocytes as well as retinal neurons [42, 43], highlighting its multiple pathological functions in diabetic retinopathy.

Hyperglycaemia-induced ischemia responsible for the production of a DNA binding protein called hypoxia-inducible factor 1 (HIF-1). HIF-1 binds to the VEGF gene and initiates the transcription process. This leads to the generation and accumulation of VEGF mRNA by both increased mRNA transcription and decreased mRNA degradation [44, 45], eventually leading to the intracellular accumulation of VEGF molecules. VEGF levels were also found to be markedly elevated in diabetic rat retinas and patients (plasma, vitreous, excised proliferative membranes) with diabetic retinopathy [42, 46, 47].

Activation of protein kinase C and phosphorylation of occludin, both induced by VEGF by altering retinal capillary permeability by increasing the phosphorylation of proteins involved with tight junctions such as zonula occludens [48]. The VEGF induction activates mitogen-activated protein (MAP), resulting in endothelial cell proliferation. This cascade coincides with the activation of the phosphatidylinositol 3-kinase (PI3)/Akt pathway after VEGFR-2 induction [49]. Activation of VEGF-R2 receptors on the apical surface of the RPE is responsible for the VEGF-induced increase in epithelial permeability which result breakdown of the blood-retinal barrier (BRB), angiogenesis [50].

Results from these studies demonstrate that the pathological response from VEGF is blocked by the protein tyrosine kinase inhibitor, genistein. However, genistein showed significantly reduced VEGF levels; indicative of potent anti-angiogenic property of genistein which is in conformity with earlier work [50, 51].

CONCLUSION

In conclusion, ALR and VEGF expression increased in human RPE cells treated with HG1 and HG2. However, HG induced ALR enzyme, VEGF secretion and mRNA expression of ALR and VEGF were

inhibited by pretreatment with genistein. The inhibitory effect of genistein on ALR and VEGF expression is mediated by inhibition of the AGEs and PKC signaling pathway.

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CONFLICT OF INTERESTS

No author has any conflict of interest in the publication of this report

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