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Original Research Article

Artemesia annua extract prevents glyoxal-induced cell injury in retinal microvascular endothelial cells during glaucoma

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Abstract

Purpose: *To investigate the effect of Artemesia annua extract on glyoxal-induced injury in retinal microvascular endothelial cells (HRECs).*
Methods: *HRECs were cultured in a medium containing 500 µM glyoxal or glyoxal plus 50µM Artemesia annua extract, or in the medium alone for 24 h. Apoptosis was analysed by flow cytometry using annexin V and propidium iodide staining. Changes in mitochondrial membrane potential were determined by JC-1 staining.*
Results: *When HRECs were cultured in a medium of 500 µM glyoxal, a significant ($p < 0.05$) decrease in caspase-3 expression was observed. However, treatment of HRECs with Artemesia annua extract (50 µM) inhibited the glyoxal-mediated decrease in caspase-3 expression. The extract also inhibited caspase-3 proteolysis, as was evident from the reduction in the level of cleaved caspase-3. Up-regulation of ROS production by glyoxal in HRECs was inhibited by treatment with the extract. The viability of HRECs was significantly decreased by glyoxal ($p < 0.05$), but the decrease in viability was significantly reversed by Artemesia annua extract ($p < 0.05$). The extract also reduced gyloxal-induced apoptosis in HRECs from 17.3 to 2.6 % ($p < 0.001$). Results from JC-1 staining showed significantly ($p < 0.001$) higher level of green fluorescence in HRECs cultured with glyoxal. However, the glyoxal-induced increase in green fluorescence level was significantly ($p < 0.01$) reduced on exposure to Artemesia annua extract.*
Conclusion: *Artemesia annua extract prevents oxidative damage to HRECs via inhibition of ROS production, up-regulation of caspase-3 expression and suppression of caspase-3 proteolysis. Therefore, Artemesia annua can potentially be used for the development of a new drug for the prevention of retinal injury in glaucoma.*

Keywords: *Artemesia annua, Retinal injury, Glaucoma, Green fluorescence, cleaved caspase-3, ROS production*

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INTRODUCTION

Diabetic retinopathy is a common cause of vision loss in people with diabetes mellitus throughout the world [1]. It causes cellular degradation of retina, and is characterised by hyperglycaemia [1]. The level of glyoxal, which is a toxic chemical, increases during hyperglycaemia,

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leading to oxidative damage through up-regulation of generation of reactive oxygen species (ROS) [2,3]. In hyperglycaemic patients, the development of diabetic retinopathy is facilitated by ROS production through increased capillary permeability [4-6]. The high level of blood glucose promotes O-linked β-N-acetyl glucosamine expression which plays a crucial role in the development of diabetes [7,8].

High intraocular pressure is considered a major pre-disposing factor for glaucoma, and it leads to optic nerve shrinkage and retinal ganglion cell apoptosis [9-11]. This disorder cannot be remedied even after transplantation of stem cells or ganglion cells [9,12]. Current treatments for glaucoma focus mainly on neuro-protection. Identification of novel and efficient neuro-protective agents is of immense importance for the prevention of retinal injury during glaucoma. Studies have been carried out to investigate the protective effects of various compounds against retinal injury during glaucoma in animal models. The extracts which have been investigated in animal models for this purpose include *Lycium barbarum* Lynn and *Salvia miltiorrhiza* [13-16].

In the present study, the effect of *Artemesia annua* extract on the viability of HRECs was investigated. Glyoxal was added to HRECs cultures to induce oxidative stress, and the protective effect of *Artemesia annua* extract was investigated.

EXPERIMENTAL

Culturing of HRECs

The HRECs were obtained from Cell Systems Corp. (CSC; Kirkland, WA, USA) and expression of ROS was analysed by immunofluorescence staining. The cells were grown in DEM medium containing 5 % FBS and 1 % penicillin and streptomycin in an atmosphere of 5 % CO_2 at a temperature of 37 ˚C.

Western blot assay

The HRECs were cultured in a medium containing 500 µM glyoxal alone (control) or glyoxal and *Artemesia annua* extract (50 µM) for 24h, and washed twice with phosphate-buffered saline (PBS). The cells were then treated with lysis buffer supplemented with protease inhibitor cocktail. The proteins from the cell extract were separated by subjecting equal amounts (30 µg) to fractionation on 10 % polyacrylamide gel. The separated proteins were subsequently transferred at 200 mA to polyvinylidene fluoride membranes for 2 h. The membranes were blocked by incubation with 0.05 % non-fat milk powder in TBST for 2h at 37 ˚C. After blocking, the membranes were incubated with primary antibodies overnight at 4 ˚C.

The antibodies used were rabbit anti-caspase-3 (catalog no. 9665; dilution 1: 1, 000) and anti-β-actin (catalog no. 8457; dilution 1:1,000; both from Cell Signalling Technology; Danvers, MA, USA). After incubation, the blots were washed twice with PBS-T (0.1 % Tween-20 in PBS), and then incubated with goat horseradish peroxidase-conjugated anti-mouse IgG (dilution 1: 5, 000; Pierce Biotechnology, Rockford, IL, USA) secondary antibody at room temperature for 1h. The blots were thereafter visualized under Odyssey (LI-COR Biosciences, Lincoln, NE, USA).

Analysis of ROS production

Analysis of ROS production in HRECs was carried out using DCFH-DA. Into the cell cultures, 5 µM of DCFH-DA was added and the cells were cultured for 30 min, and then lysed using sodium hydroxide (400 mM) for 2 h at room temperature. The lysates were used for determination of ROS production by measuring the intensity of fluorescence at absorption and emission wavelengths of 480 and 430 nm, respectively in a multi-well plate reader (Synergy 2, BioTek Instruments Inc., Winooski, VT, USA). Bicinchoninic acid (BCA) protein assay kits (Pierce Biotechnology) were used for protein determination. Quantification was performed as the ratio of the intensity of fluorescence to concentration of protein.

Evaluation of cell viability

The HRECs were seeded into 96-well culture plates at a density of 1 x 10^4 cells per well and cultured for 24 h under an atmosphere of 5 % CO_2 at 37 ˚C. The cells were then assigned to three groups: one group was cultured in a medium containing 500 µM glyoxal; the second group was cultured in a medium containing 500 µM glyoxal and 50 µM *Artemesia annua* extract, while the third group (normal control) was cultured in medium alone. After 24 h, CCK-8 solution (10 µL) was added to the plates and incubation was continued further for 4 h.

At the end of incubation, the absorbance for each plate was recorded three times in a multi-well plate reader at 450 nm. The effect of hydrogen peroxide on HRECs viability was analysed on incubation with 200 µM H_2O_2 in the medium containing 5 % of FBS.

Flow cytometry analysis

The cells were cultured in a medium containing 500 µM glyoxal or glyoxal (500 µM) plus *Artemesia annua* extract (50 µM), or medium alone, for 24 h. After incubation, the cell pellet was re-suspended in binding buffer, and incubated with 5 ml of Annexin V (conjugated with FITC; BD Biosciences) and 10 ml of PI (BD Biosciences), in the dark for 5 min. Finally, the cells were examined in a FACScan flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA).

Determination of mitochondrial membrane potential

In the analysis of changes in mitochondrial membrane potential, JC-1 stain was used. The three groups of cells cultured in different media as outlined earlier, were analysed for changes in membrane potential. The cells were stained with JC-1 (2.5 µM) for 20 min at 37 ˚C, and subsequently rinsed thrice in PBS. Images were captured at 525 nm and 557 nm absorption and emission wavelengths, respectively, using an inverted fluorescence microscope (Leica Microsystems). The intensity of fluorescence was analysed using Image-Pro Plus v6.0 software.

Statistical analysis

The experimental data are expressed as mean ± standard deviation (SD). Statistical analysis was performed with SPSS 13.0 statistical software (SPSS, Inc. Chicago, IL, USA). One-way analysis of variance (ANOVA) was used, followed by Dunnett's test for multiple comparisons. $P < 0.05$ was considered to indicate statistically significant difference.

RESULTS

Effect of *Artemisia annua* on expression of caspase-3 in HPRECs

Glyoxal caused a significant decrease in the expression of caspase-3 in HRECs. However, treatment of the glyoxal-exposed HRECs with *Artemesia annua* extract led to a significant ($p < 0.01$) increase in caspase-3 expression (Figure 1). Proteolysis of caspase-3 was inhibited by *Artemesia annua* extract, as was evident in reduction of the level of cleaved caspase-3. These findings suggest that *Artemesia annua* extract protected HRECs from glyoxal-induced damage.

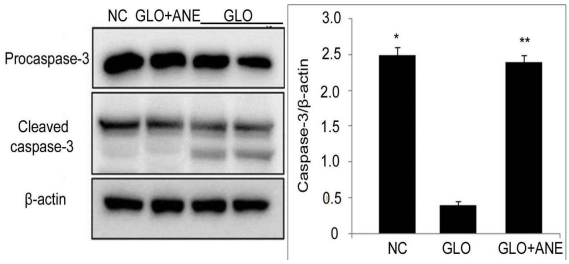

Figure 1: *Artemesia annua* extract inhibited glyoxal-induced cleavage of caspase-3 in HRECs. The quantification of caspase-3 level in HRECs following treatment with glyoxal and/or *Artemesia annua* extract was performed using Quantity One software. *$p < 0.05$ (glyoxal vs. normal control); **$p < 0.01$ (glyoxal vs. glyoxal + *Artemesia annua* extract)

Artemesia annua extract inhibited production of ROS in glyoxal-treated HRECs

Examination using fluorescence microscopy showed higher intensity of bright spots in HRECs treated with glyoxal, when compared to untreated HRECs (Figure 2). The ROS production induced by glyoxal in HRECs was inhibited by treatment with *Artemesia annua* extract ($p < 0.05$). Therefore, the extract protected HRECs from glyoxal-mediated oxidative damage.

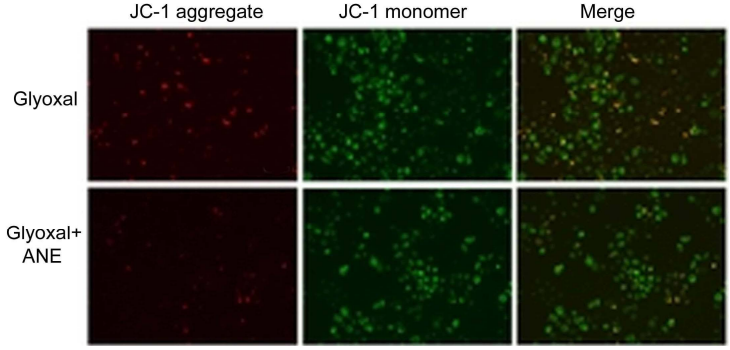

Figure 2: *Artemesia annua* extract blocked up-regulation of ROS production by glyoxal. HRECs, after treatment with glyoxal and/or *Artemesia annua* extract, were analysed for ROS content by fluorescence microscopy using DCFH-DA staining. An automatic microplate reader was used for quantitative analysis of changes in ROS

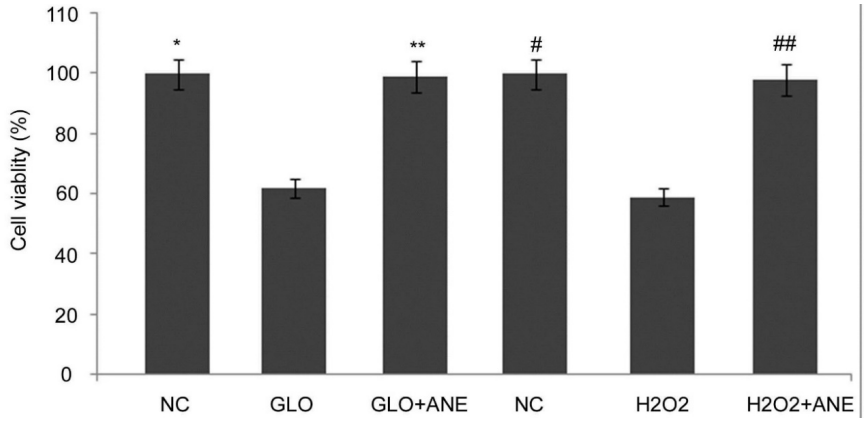

Figure 3: *Artemesia annua* extract prevented glyoxal-mediated loss of viability in HRECs. *p < 0.005 (glyoxal vs. control); **p > 0.05 (glyoxal vs glyoxal + *Artemesia annua* extract). HRECs were exposed to H_2O_2 and then treated with *Artemesia annua* extract for 24 h. $^{\#}p$ < 0.001 (H_2O_2 vs. normal control); $^{\#\#}p$ < 0.0001 (H_2O_2 vs H_2O_2 + *Artemesia annua* extract)

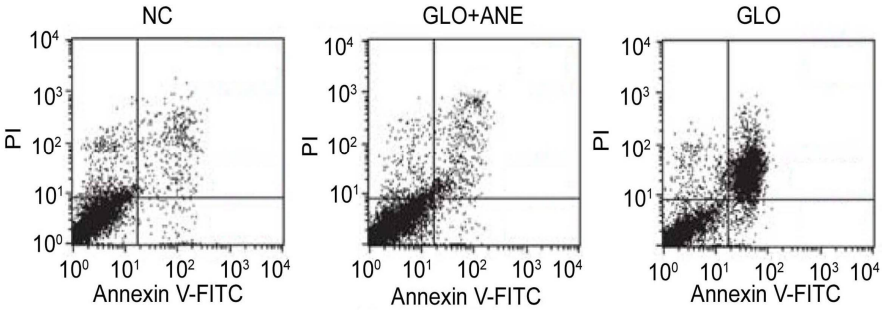

Figure 4: *Artemesia annua* extract prevented glyoxal-induced apoptosis in HRECs. Flow cytometry was used to determine the effect of glyoxal and *Artemesia annua* extract on apoptosis in HRECs after 24 h. Staining of HRECs was performed using Annexin V-FITC and PI, followed by FACS

Artemesia annua extract prevented glyoxal-mediated decrease in viability of HRECs

Changes in viability of HRECs treated with *Artemesia annua* extract were analysed after 24h. Glyoxal treatment led to a significant (p < 0.005) decrease in HRECs viability, when compared to the untreated control (Figure 3). However, after *Artemesia annua* extract treatment, the viability of HRECs exposed to glyoxal was significantly increased. In order to confirm if *Artemesia annua* extract prevented inhibition of viability through attenuation of oxidative damage, HRECs were treated with H_2O_2 (200 µM) for 2 h. The results showed that H_2O_2 reduced HRECs viability, but the inhibitory effect was reversed by *Artemesia annua* extract (Figure 3).

Artemesia annua extract blocked apoptosis in HRECs

Glyoxal treatment increased the percentage of apoptosis in HRECs to 17.3 %, when compared to 2.5 % in the untreated control (Figure 4). However, addition of *Artemesia annua* extract to the glyoxal-treated cultures of HRECs led to significant reduction of apoptosis to 2.6 % (Figure 4). Thus, it is clear that *Artemesia annua* extract prevented glyoxal-mediated increase in the percentage of apoptosis in HRECs.

Artemesia annua extract prevented glyoxal-mediated alteration in mitochondrial membrane potential

The results from JC-1 probe showed a significantly higher level of green fluorescence in HRECs cultured with glyoxal (Figure 5). However, the level of green fluorescence was significantly reduced in glyoxal-treated HRECs on exposure to *Artemesia annua* extract (Figure 5). These observations suggest that *Artemesia annua* extract blocked changes in mitochondrial membrane potential caused by glyoxal.

DISCUSSION

Reactive oxygen species (ROS) are responsible for cellular stress that results in retinal injury and loss of vision [5,17,18]. Increased blood glucose levels for prolonged durations at the initial stage

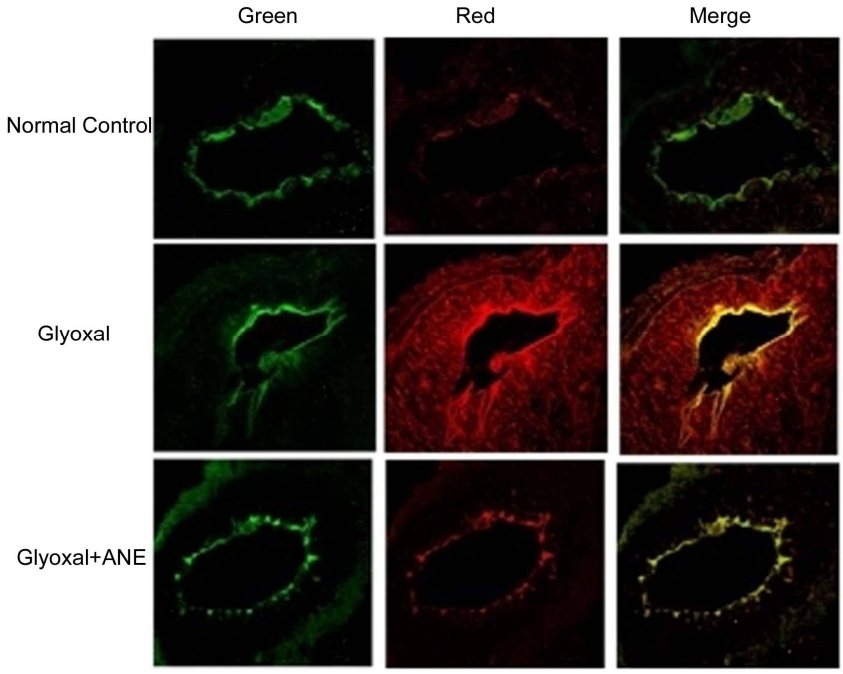

Figure 5: Effect of *Artemesia annua* extract on glyoxal-induced changes in mitochondrial membrane potential in HRECs. The cells were incubated with JC-1 after treatment with glyoxal and *Artemesia annua* extract for 24 h. Inverted fluorescent microscope and Image-Pro Plus software were used for measurement of fluorescence. The loss in membrane potential in cells treated with glyoxal was prevented by addition of *Artemesia annua* extract

of diabetic retinopathy result in generation of ROS and the development of retinal hypoxia [19]. Studies have shown that following surgical eye treatment in patients with proliferative diabetic retinopathy, the expression of hypoxia-inducible factor (HIF)-1α is high in vitreous fluid [20]. Up-regulation of HIF-1α expression is a characteristic feature of hypoxia and onset of oxidative damage [21]. Exposure of HRECs to glyoxal leads to marked increases in the rate of apoptosis [22]. It has been reported that the suppression of ROS production inhibits apoptosis and increases the viability of HRECs [22]. Indeed, several studies have shown that the production of ROS plays a vital role in the development of retinal injury and diabetic retinopathy [23-26].

The results of the present study showed that *Artemesia annua* extract inhibited glyoxal-induced ROS generation in HRECs. Apoptosis in endothelial cells which form lining of the capillary wall plays a vital role in the onset of diabetic retinopathy. The present study has demonstrated that glyoxal-mediated increase in apoptosis in HRECs was significantly inhibited by *Artemesia annua* extract. In order to understand the mechanism of *Artemesia annua* extract-mediated blockage of glyoxal-induced apoptosis in HRECs, caspase-3 and mitochondrial membrane potential were determined. Increases in the level of cleaved caspase-3 enhance the rate of apoptosis in HRECs. Caspase-3 is activated by proteolysis into cleaved caspase-3 which has an important role in inducing apoptosis [27-29]. Glyoxal treatment has been shown to increase the level of cleaved caspase-3 level and decrease overall caspase-3 activity in HRECs after 24 h [28, 29]. In the current study, the addition of *Artemesia annua* extract to the glyoxal-treated cultures of HRECs led to increased level of caspase-3. In presence of glyoxal, treatment of HRECs with *Artemesia annua* extract prevented reduction in viability. In order to confirm the anti-oxidative mode of action of *Artemesia annua* extract in blocking glyoxal-mediated loss of viability, HRECs were treated with H_2O_2. The results revealed that the H_2O_2-induced reduction in HREC viability was inhibited by the extract. Thus, *Artemesia annua* extract acted as a therapeutic agent for prevention of apoptosis in HRECs through a mechanism involving targeting of ROS production.

Mitochondria play important roles in the protection of cellular damage by inhibiting ROS production, and regulating calcium ion channels [30,31]. In the present study, results from JC-1 staining and inverted fluorescent microscopy showed that *Artemesia annua* extract prevented changes in mitochondria membrane potential induced by oxidative damages in HRECs.

CONCLUSION

The findings of the present study provide indicate

that *Artemesia annua* extract prevents glyoxal-induced apoptosis in HREC by inhibition of ROS production and blockage of caspase-3 cleavage. The extract also prevents changes in mitochondrial membrane potential. Thus, *Artemesia annua* has potentials to be developed into a new drug for the treatment of diabetic retinopathy.

DECLARATIONS

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

Shun Jiang drafted the manuscript and did all the experiments under the supervision of Jiawen Ling. Qin Wang collected materials and did statistical analysis.

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