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SHORT COMMUNICATION

Protective Effect of Grape Seed Extract against Oxidative Stress-Induced Cell Death in a Staurosporine-Differentiated Retinal Ganglion Cell Line

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ABSTRACT

Objective: Grape seed extract (GSE) is a potent antioxidant. We examined the effect of GSE on oxidative stress-induced cell death in a transformed retinal ganglion cell line, RGC-5.

Methods: Staurosporine-differentiated RGC-5 (ssdRGC-5) cells obtained by treating RGC-5 cells with 1 μM staurosporine were incubated with GSE for 2 h and then exposed to buthionine sulfoximine plus glutamate (B/G) for 24 h. Cell death was detected using the LIVE/DEAD viability assay and the type of cell death was evaluated using fluorescein isothiocyanate-conjugated Annexin–V/propidium iodide staining. To investigate the mechanism underlying cell death, we determined the caspase-3 activity and level of reactive oxygen species (ROS) formation.

Results: Treatment of ssdRGC-5 cells with B/G increased intracellular ROS and induced apoptosis (not necrosis) with increasing caspase-3 activity. GSE rescued the ssdRGC-5 cells from oxidative stress-induced cell death by inhibiting both intracellular ROS production and caspase-3 activation.

Conclusion: GSE had a neuroprotective effect against oxidative stress-induced apoptotic death in ssdRGC-5 cells.

Keywords: Grape seed extract, Retinal ganglion cell, Oxidative stress, Apoptosis, Glaucoma

INTRODUCTION

Oxidative stress is thought to be an important mechanism of cell death in various neurodegenerative diseases, including glaucoma.[1] Glaucoma is characterized by a specific pattern of optic nerve head and visual field damage, which, if not controlled, leads to blindness via the death of retinal ganglion cells (RGCs). Increased free radicals and oxidative stress have been reported in the retina and optic nerve in animal models of glaucoma and glaucoma patients[2]; consequently, the suggestion has been made that antioxidants can prevent or ameliorate the progression of neuronal death in patients with glaucoma.[1] Recent studies have shown that the administration of *Ginkgo biloba* extract, a well-known antioxidant, improved preexisting visual field damage in glaucoma patients,[3] supporting the promising therapeutic effect of antioxidants on glaucoma.

Grape seed extract (GSE) from *Vitis vinifera* is widely used as a food additive for its beneficial effects on health and chronic illness[4] or for therapeutic effects[5] due to its potent antioxidation effects. GSE is rich in polyphenols, primarily proanthocyanidins.[6] In the rat central nervous system, GSE showed neuroprotective effects against oxidative stress.[7] Moreover, aloe-emodin, a plant polyphenol, has been suggested as a potential agent for neuroprotective therapy in glaucoma due to its anti-apoptotic effect on N-methyl-D-aspartate-induced death in RGCs.[8] Based on these reports, we hypothesized that GSE may play a neuroprotective role in glaucoma, and investigated this hypothesis by examining the effect of GSE on oxidative-stress-induced neuronal death in RGC-5

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cells[9] and whether the antioxidant properties of GSE are related to its neuroprotective effect.

MATERIALS AND METHODS

Cell Cultures and Oxidative Stress

The RGC-5 was a kind gift from Prof. Abbot Clark (North Texas Eye Research Institute, Fort Worth, TX). RGC-5 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum and 1% penicillin–streptomycin in a humidified 5% CO_2 atmosphere at 37°C. For all experiments, no RGC-5 cells were passed for more than 30 passages. The RGC-5 cells were seeded at a confluence of approximately 10% (1.5×10^4 cells/cm^2) onto plastic culture plates and grown to between 80% and 85% confluence. To obtain staurosporine-differentiated RGC-5 (ssdRGC-5), cells were exposed to 1 µM staurosporine (Sigma-Aldrich, St. Louis, MO) for 6 h and recovered in the culture medium for 1 day. Then, the ssdRGC-5 cells were plated and incubated with GSE or (–)-epicatechin-3O-gallate (Sigma-Aldrich) for 2 h. GSE (Leucoselect; Indena, Milan, Italy) was kindly provided by Hanlim Pharmaceutical (Seoul, Korea). GSE was dissolved with phosphate-buffered saline. The effect of GSE was compared with 100 µM trolox (Tocris, Ballwin, MO) and 1 µM z-DEVD-fmk (Biomol International, Plymouth Meeting, PA).[10,11] Then, oxidative stress was induced by 5 mM glutamate and 0.5 mM L-buthionine-(*S*,*R*)-sulfoximine (Sigma-Aldrich) (B/G) for 24 h.[12]

Live and Dead Assay

Cell death was evaluated using the LIVE/DEAD viability assay kit (Invitrogen) according to the manufacturer's instructions. Briefly, cells were incubated with 2 µM calcein-AM (live cells fluoresce green) and 4 µM ethidium homodimer-1 (EthD-1; dead cells fluoresce red) for 30 min at room temperature and examined under a fluorescence microscope (Carl Zeiss, Jena, Germany).[13]

FITC–Annexin-V and PI Analysis

Both apoptotic and necrotic indices were estimated in a given sample by dual labeling with fluorescein isothiocyanate-conjugated annexin V (Annexin-V; for apoptosis) and propidium iodide (PI; for necrosis) using the Annexin-V Apoptosis Detection Kit I (BD PharMingen, San Diego, CA). The stained cells were subjected to fluorescence-activated cell sorter analysis (FACS) using a FACSCalibur flow cytometer (FACSVantage; Becton Dickinson, Franklin Lakes, NJ).

Caspase-3 Activity Assay

The caspase-3 activity was measured by detecting the cleavage of a colorimetric caspase-3 substrate, Ac-DEVD-p-Na (Biomol International), as described previously.[14]

Reactive Oxygen Species (ROS) Measurement

The amount of intracellular ROS was measured fluorometrically using the nonfluorescent dye 2′,7′-dichlorofluorescein diacetate (DCF-DA; Invitrogen) as described.[14] The intensity of fluorescent 2′,7′-dichlorofluorescein (DCF) was quantified using the AxioVision™ image analysis software program (Carl Zeiss).

Statistical Analysis

All data are expressed as the mean ± SEM for at least three different experiments. Comparisons were made using Student's *t*-test. A p-value of <0.05 was considered significant.

RESULTS

Effect of GSE on B/G-Induced Cell Death in ssdRGC-5 Cells

As shown in Figure 1A and 1B, B/G significantly increased the number of EthD-1-positive cells (red, dead cells) (22.4 ± 1.1%) compared to the control (9.6 ± 1.2%). Incubation with GSE before B/G treatment reduced the number of dead cells in a concentration-dependent manner, with the maximum efficacy at 100 ng/ml of GSE (15.1 ± 1.7%). The protective effect of GSE was comparable to that of trolox and DEVD-fmk (15.4 ± 2.5% and 14.4 ± 1.2%, respectively). As shown in Figure 1C and 1D, after treatment with B/G for 24 h, the proportion of Annexin-V-positive cells increased significantly, by about 1.7-fold, compared to the control, while Annexin-V-negative/PI-positive cells did not increase significantly, indicating that the B/G-induced neuronal cell death mainly involved apoptosis, rather than necrosis. In agreement with Figure 1B, pretreatment with 100 ng/ml of GSE significantly attenuated the B/G-induced apoptosis of ssdRGC-5 cells (113.9 ± 6.9%) with a potency comparable to that of trolox (112.3 ± 5.8%) and DEVD-fmk (129.3 ± 6.9%).

Effect of GSE on B/G-Induced Caspase-3 Activation in ssdRGC-5 Cells

The caspase-3 activity peaked after treatment with B/G for 20 h (180.6 ± 4.6%) (Figure 1E, inset). As

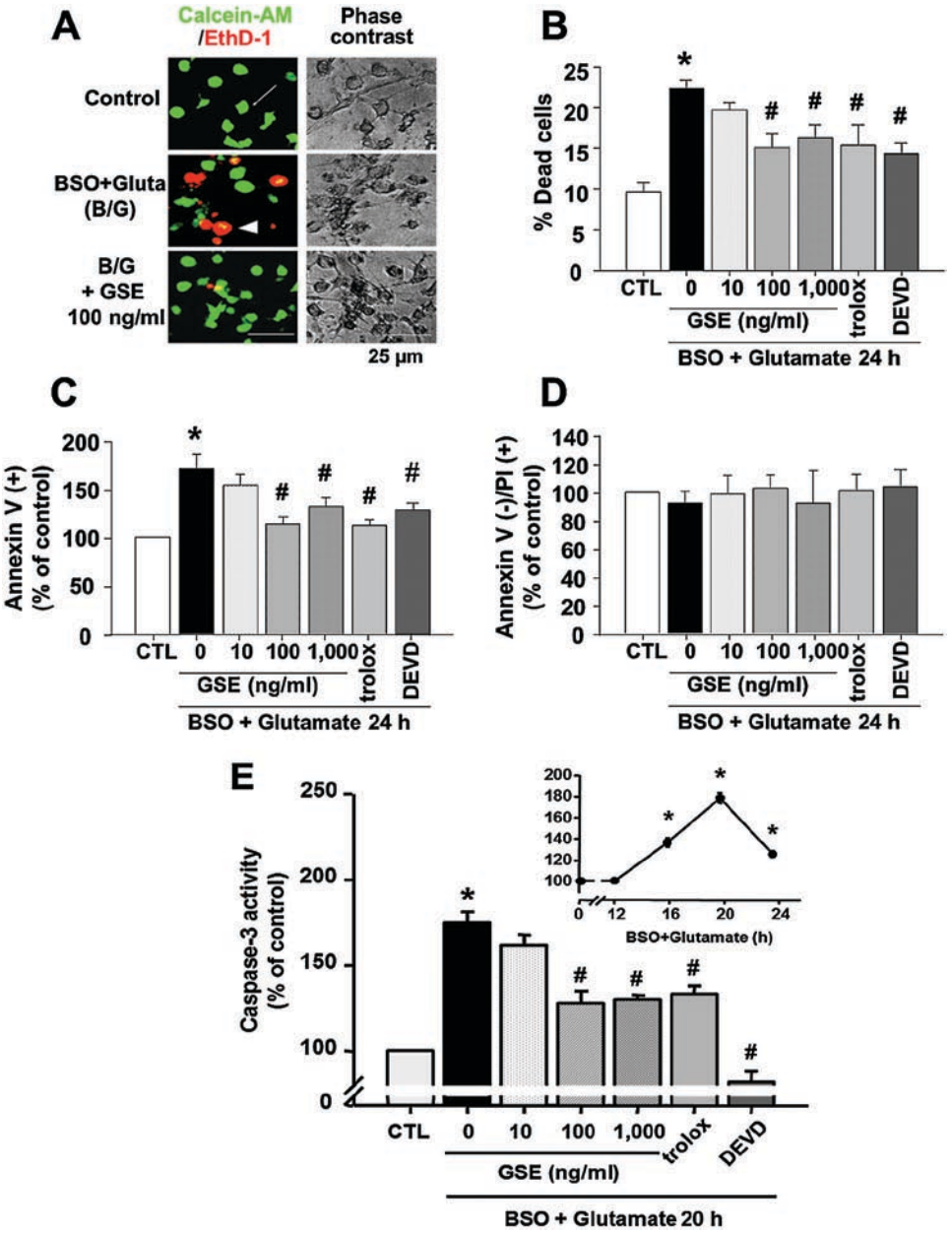

FIGURE 1 Effect of GSE on B/G-induced cell death in ssdRGC-5. ssdRGC-5 cells were incubated with or without GSE (10–1,000 ng/ml) for 2 h before adding 0.5 mM BSO plus 5 mM glutamate, and incubated for 24 h. Trolox (100 μM) or DEVD-fmk (1 μM) was used to compare the protective potency. (A) Live and dead cells were stained with calcein-AM (green, arrow) and EthD-1 (red, arrowhead), respectively. Scale bar, 25 μm. (B) The number of dead cells was counted and the percentage of total cells was analysed. (C and D) FACS analysis of cells after Annexin-V–PI staining. The percentages of (C) apoptotic [Annexin-V (+)] and (D) necrotic [Annexin-V (–)/PI (+)] cells were analysed. (E) The effect of GSE on B/G-induced caspase-3 activation in ssdRGC-5 cells. B/G was added to the medium and incubated for 24 h (inset). The percentage of caspase-3 activity was compared to the control. Data represent the mean ± SEM of at least five independent experiments. *$p < 0.05$ vs. untreated control (CTL); #$p < 0.05$ vs. B/G only, without pretreatment with GSE.

shown in Figure 1E, B/G increased the caspase-3 activity (175.2 ± 6.1%) compared to the control (100%). Pretreatment with 100 or 1,000 ng/ml of GSE significantly attenuated the B/G-induced increase in caspase-3 activity at this time point (128.1 ± 6.8 or 130.9 ± 2.5%, respectively). The efficacy of GSE was comparable to that of trolox (133.8 ± 4.6%), and DEVD-fmk showed the greatest potency in reducing the caspase-3 activity (82.1 ± 6.5%).

Effect of GSE on B/G-Induced Intracellular ROS Accumulation in ssdRGC-5 Cells

Compared to the control, the intracellular ROS accumulation increased almost maximally to about 2.7-fold after a 20-h exposure to B/G (Figure 2B, inset). As shown in Figure 2B, the B/G-induced intracellular ROS accumulation (272.1 ± 26.1%) was inhibited by GSE in a concentration-dependent manner, with maximum

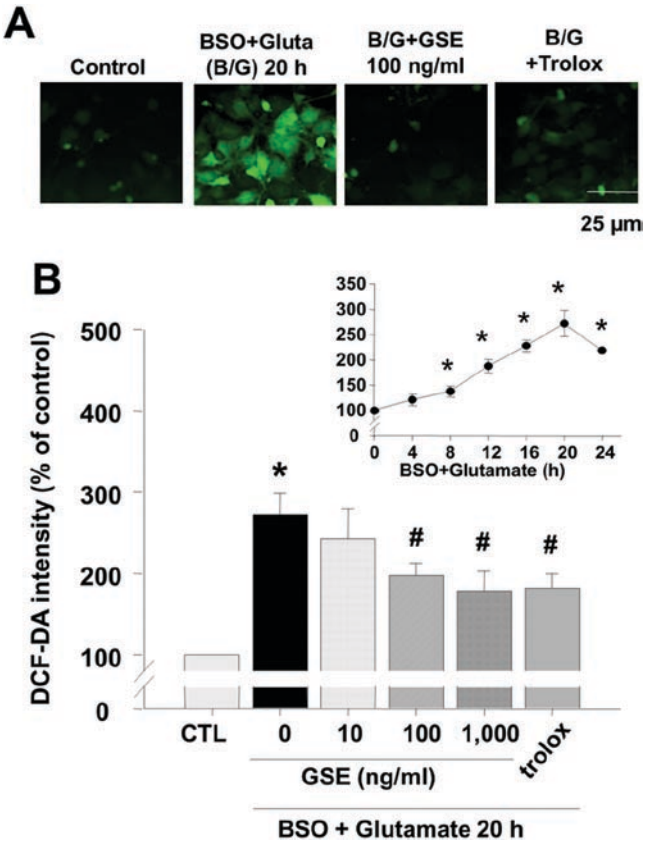

FIGURE 2 The effect of GSE on B/G-induced ROS accumulation in ssdRGC-5 cells. ssdRGC-5 cells were incubated with or without GSE (10–1,000 ng/ml) for 2 h, as described in Figure 1, and B/G was added to the medium and incubated for 24 h (B, inset). (A) The amount of intracellular ROS was evaluated using the fluorescent dye DCF-DA (green). Scale bar, 25 µm. (B) The intensity of DCF was quantified. Data represent the mean ± SEM of at least four experiments. *$p < 0.05$ vs. untreated control (CTL); #$p < 0.05$ vs. B/G only, without pretreatment with GSE.

inhibition by 1,000 ng/ml GSE (178 ± 24.8%) and its effect was similar to that of trolox (181.7 ± 17.8%) at this time point.

Effect of Epicatechin Gallate on Cell Death and ROS Accumulation in ssdRGC-5 Cells

As shown in Figure 3A, after treatment with B/G for 24 h, the proportion of Annexin-V-positive cells increased significantly (222.3 ± 7.0%) compared to the control. Pretreatment with epicatechin gallate significantly attenuated the B/G-induced apoptosis of ssdRGC-5 cells in a dose-dependent manner, with maximum inhibition by 100 µM epicatechin gallate (61.4 ± 12.8%). As shown in Figure 3C, B/G-induced intracellular ROS accumulation (290.3 ± 26.2%) was inhibited by epicatechin gallate in a concentration-dependent manner, with maximum inhibition by 100 µM epicatechin gallate (112 ± 7.1%).

DISCUSSION AND CONCLUSIONS

A transformed RGC line, RGC-5, has been used as an *in vitro* model of glaucoma because it has many of the characteristics of primary RGCs.[9] Since RGC-5 cells differentiate into neuron-like, non-mitotic cells on exposure to the protein kinase inhibitor staurosporine,[15] ssdRGC-5 cells have been adopted as an *in vitro* model of glaucoma.[16] Based on the previous studies reporting the neuron-like differentiation of RGC-5 cells induced by staurosporine, we exposed RGC-5 to 1 µM of staurosporine for 6 h and selected 24 h as the recovery time to show evident differentiation.[15,17] Similar to previous reports, the RGC-5 cells differentiated into neuron-like cells after treatment with staurosporine (Figure 1A, phase contrast images).

It has been reported that cell death induced by B/G in RGC-5 is accompanied by increased DNA fragmentation, not the activation of caspase-3.[12] In this study, however, ssdRGC-5 underwent apoptosis with increasing caspase-3 activity (Figure 1E), which implies that staurosporine alters the features of RGC-5 with regard to cell death mechanisms. The major finding in this study is that GSE can inhibit apoptosis induced by B/G in ssdRGC-5 with decreasing caspase-3 activity. Although DEVD-fmk reduces caspase-3 activity more than GSE and trolox, even below the level of the control (Figure 1E), the effect of GSE on cell death is similar to that of DEVD (Figure 1B and C). Perhaps GSE inhibits caspase-3 less strongly than DEVD; however, it possesses other mechanisms to compensate for this shortcoming and to protect RGCs against oxidative stress. Because a similar antioxidant, (–)-epigallocatechin-3-gallate, a major green tea polyphenol, was reported to downregulate Bad protein levels,[18] further study is needed to examine whether the underlying mechanisms for the anti-oxidative effect of GSE include modulation of Bcl-2 family.

B/G-induced cell death was thought to be initiated by the depletion of GSH. After the loss of GSH, the intracellular ROS increases gradually and the peak in ROS is followed by an increase in intracellular calcium and then cell death.[12] Therefore, we attempted to estimate cell death 4 hours (24 h) after the ROS peak (20 h) in B/G exposed cells. Consistent with our study, ROS accumulation was detected at 18 h and cell death was measured at 24 h after B/G induced injury in a previous study.[19]

Proanthocyanidins, including epicatechin, are the main components of GSE. Proanthocyanidins are known to have neuroprotective effects against β-amyloid-induced neuronal death via their antioxidant and free radical scavenging activity.[20] The effects of flavanols, including catechin and epicatechin, against B/G-induced oxidative stress have been evaluated in RGC-5, and only epicatechin appeared to be effective.[19] However, no reports exist on epicatechin gallate, especially in ssdRGC-5. In the present study, we

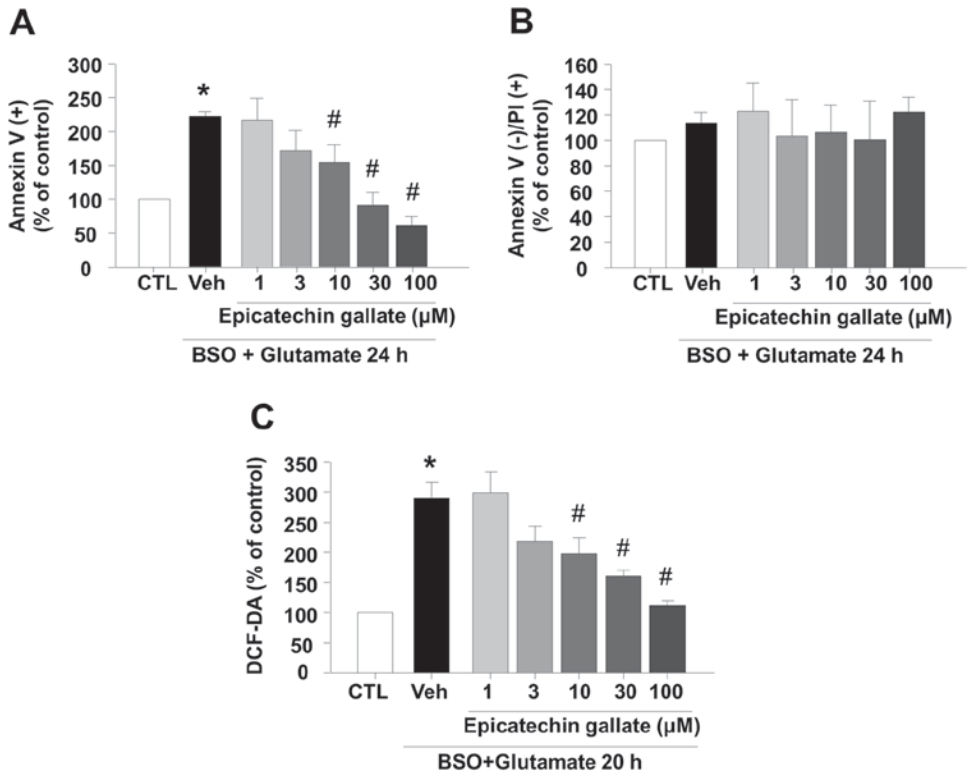

FIGURE 3 Effect of epicatechin gallate on B/G-induced cell death and ROS accumulation in ssdRGC-5. ssdRGC-5 cells were incubated with or without epicatechin gallate (1–100 μM) for 2 h before adding 0.5 mM BSO plus 5 mM glutamate, and incubated for 24 h. (A and B) FACS analysis of cells after Annexin-V–PI staining. The percentages of (A) apoptotic [Annexin-V (+)] and (B) necrotic [Annexin-V (–)/PI (+)] cells were analysed. Data represent the mean ± SEM of at least five experiments. (C) The amount of intracellular ROS was evaluated using the fluorescent dye DCF-DA at 20 h. The intensity of DCF was quantified. Data represent the mean ± SEM of at least four experiments. *$p < 0.05$ vs. untreated control (CTL); #$p < 0.05$ vs. B/G only without pretreatment with epicatechin gallate.

found that epicatechin gallate had a protective effect on Annexin-V/PI staining and the ROS assay in a concentration-dependent manner. This result suggests that the GSE-induced anti-apoptotic effect is associated with that of epicatechin gallate, although whether the mixture of proanthocyanidins in GSE potentiates the antioxidant effect of each component is not clear. The effective dosage of GSE for preventing cell death is related to the dosage with an ROS-reducing effect (Figure 2). The most effective concentration of GSE for protection was 100 ng/ml (Figure 1) and significant cell death was apparent at concentrations over 10 μg/ml (data not shown). Taken together, this study demonstrates that GSE protects ssdRGC-5 against oxidative stress-induced apoptotic death by reducing ROS and inhibiting caspase-3 activation. The protective effect of GSE should be investigated further in an animal model of glaucoma to determine whether GSE is a potential neuroprotective agent in glaucoma management.

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