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Protective effects of a compound isolated from *Alnus japonica* on oxidative stress-induced death in transformed retinal ganglion cells

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ABSTRACT

Here, we investigated whether hirsutenone, a compound isolated from *Alnus japonica*, was able to attenuate oxidative stress-induced death in transformed retinal ganglion (RGC-5) cells.

Hirsutenone effectively protected RGC-5 cells from oxidative insult induced by, l-buthionine-(S,R)-sulfoximine (BSO) plus glutamate in a concentration-dependent manner, as demonstrated by propidium iodide (PI)/Hoechst 33342 double staining, flow cytometry, and MTT assays. Moreover, hirsutenone inhibited the increase in apoptotic protein expression resulting from BSO plus glutamate.

Hirsutenone also effectively inhibited sodium nitroprusside (SNP)-induced lipid peroxidation in rat brain homogenates. To investigate the effects of hirsutenone in vivo, we used *N*-methyl-D-aspartate (NMDA) as a negative insult on the retinas of rats. NMDA affects the thinning of the inner plexiform layer (IPL) and causes an increase in the number of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)-positive ganglion cells in the ganglion cell layer (GCL). Hirsutenone treatment led to a reduction in NMDA-induced IPL and TUNEL staining of the GCL.

In conclusion, hirsutenone isolated from *A. japonica* may act as neuroprotective agent for conditions such as glaucoma.

1. Introduction

Glaucoma is a group of chronic degenerative optic neuropathies in which degeneration of retinal ganglion cells (RGCs) leads to gradual obstruction of vision and ultimately blindness. Raised intraocular pressure (IOP) has been shown to be an important risk factor for optic nerve damage in glaucoma (Foster et al., 2002).

IOP-reducing agents are commonly used as a treatment option for glaucoma (Weinreb and Khaw, 2004), however, decreasing IOP to acceptable levels does not effectively inhibit glaucoma disease progression.

Therefore, identification of new compounds that directly target RGCs damaged as a result of glaucoma may be a successful strategy (Levin, 2003; Levin and Peeples, 2008). Neuroprotection of RGCs has been indicated as a treatment option for glaucoma and has been shown to prevent the progression of this chronic neurodegenerative disease (Levin and Peeples, 2008).

Neuroprotective agents have been shown to protect against RGC death by stopping or preventing apoptosis, preventing tumor necrosis factor activation, stabilizing Ca^{2+} homeostasis, blocking glutamate excitotoxicity, inhibiting nitric oxide production, modulating expression of heat shock proteins, supplying neurotrophins, and improving blood flow to the optic nerve, etc. (Marcic et al., 2003).

Natural products may be used to enhance neuroprotection due to their anti-oxidative capacity, which can potentially be used to protect RGCs. Several reports have described the neuroprotective effects of the constituents of food on RGCs, such as extracts from *Ginkgo biloba*, tea, red wine, dark chocolate and coffee (Agte and Tarwadi, 2010). Moreover, the protective effects of naturally occurring substances on RGC death caused by oxidative insults have been demonstrated both in vitro and in vivo (Kim et al., 2011; Li and Lo, 2010; Nakajima et al., 2007; Zhang and Osborne, 2006).

Alnus japonica is an Asian herb traditionally used for fever, hemorrhages, diarrhea, and alcoholism. Diarylheptanoids have been shown as major components of *A. japonica* and have been reported to exhibit antioxidative properties that contribute to hepatoprotective activity in certain situations (Park et al., 2010; Tung et al.,

Abbreviations: BSA, bovine serum albumin; BSO, l-buthionine-(S,R)-sulfoximine; FBS, fetal bovine serum; GCL, ganglion cell layer; GSH, glutathione; IOP, intraocular pressure; IPL, inner plexiform layer; MMP, mitochondria membrane potential; MTT, 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide; NAC, N-Acetyl-L-cysteine; NADPH, nicotinamide adenine dinucleotide phosphate; NMDA, *N*-methyl-D-aspartate; PI, propidium iodide; PVDF, polyvinylidene difluoride; RGCs, retinal ganglion cells; ROS, reactive oxygen species; SDS, sodium dodecyl sulfate; TMRE, tetramethylrhodamine ethylester; TUNEL, terminal deoxynucleotidyl transferase dUTP nick-end labeling.

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2010). Moreover, diarylheptanoids from *A. japonica* also seemed to have significant low-density lipoprotein-antioxidant and radical scavenging activities (Lee et al., 2005). Substantial evidence shows that oxidative stress plays a key role in the pathogenesis of many chronic diseases through direct oxidation and damage to DNA, proteins, and lipids (Lonkar and Dedon, 2011). However, despite the protective properties of *A. japonica*, it has not yet been studied for its ability to protect RGCs in the context of glaucoma.

In this study, the neuroprotective properties of *A. japonica* were investigated using oxidative stress-induced RGC in vitro and an *N*-methyl-D-aspartate (NMDA)-induced excitotoxicity model in vivo.

2. Materials and methods

2.1. Chemicals

Hoechst 33342 and propidium iodide (PI) were from Molecular Probes (Eugene, OR, USA). Anti-poly (ADP-ribose) polymerase (PARP), cleaved caspase-3 and NMDA receptor (NMDAR) were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-β-actin, anti-rabbit IgG and anti-mouse IgG antibodies were purchased from Santa Cruz Biotechnology (CA, USA).

All other chemicals and reagents were purchased from Sigma–Aldrich (St Louis, MO, USA).

2.2. Animals

All animal studies were carried out in a pathogen-free barrier zone at the KIST Gangneung Institute and were done in accordance with the procedure outlined in the Association for Research in Vision and Ophthalmology (ARVO) Statement for the Use of Animals in Ophthalmic and Vision Research. Procedures used in this study were approved by the Animal Care and Use Committee of KIST.

Male adult Sprague–Dawley rats weighing between 250 and 300 g (8 weeks of age) were used in the present study, and were acclimated for at least one week, caged in groups of five or less, and were fed with a diet of animal chow and water *ad lib*. They were housed at 23 ± 0.5 °C and 10% humidity with a 12 h light–dark cycle (lights on at 7 am and off at 7 pm).

2.3. Plant materials

A. japonica was purchased in the Kyungdong market, Korea and authenticated by emeritus Prof. H.J. Chi, Seoul National University, South Korea. A voucher specimen (D-AJ) was deposited at the Herbarium of KIST, Gangneung Institute, Korea. The dried stem of *A. japonica* (3.6 kg) was extracted three times with hot 94% EtOH (8.0 L) for 3 h. This residue was evaporated *in vacuo* with a Model EYELA N-1000 rotary evaporator (Tokyo Rikakikai Co., Tokyo, Japan) to yield the brown residue total extract (201.5 g, 5.6%).

2.4. Culture of RGC-5 cells

RGC-5 cells have been previously shown to express ganglion cell markers and exhibit ganglion cell like behavior in culture. RGC-5 cells were kindly gifted by Alcon Research, Ltd. (Fort Worth, TX, USA) (Takeoka et al., 2001).

Cells were cultured in DMEM containing 5 mM glucose, 100 U/mL penicillin/streptomycin, 2 mM glutamine, 5 mg/mL active geneticin and 10% (v/v) heat inactivated fetal bovine serum (FBS) in a humidified incubator with 5% CO_2 at 37 °C. To examine the effects of the samples, RGC-5 cells were seeded at a density of 5×10^3 cells per well into 96-well plates. After incubating for 24 h, cells were washed in DMEM containing 1% FBS. Cells were then exposed to DMEM containing 1% FBS plus samples or plus vehicle. After 1 h pre-treatment with samples, 1-buthionine-(S,R)-sulfoximine (BSO) (0.5 mM) plus glutamate (10 mM) (glutamate/BSO) or hydrogen peroxide (H_2O_2) was added to cultures, which were then maintained for 24 h. The combination of glutamate/BSO, a glutamate cysteine ligase inhibitor, was used to enhance excitotoxicity as previously reported. N-Acetyl-L-cysteine (NAC) has been shown to significantly reduce apoptosis in RGC-5 caused by glutamate/BSO. In this study, NAC was used as positive control.

2.5. Cell viability

Cell viability was determined by a modified version of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Mosmann, 1983). Briefly, cells (in 96-well plates) were subjected to the appropriate treatment and the MTT was added at a final concentration of 0.5 mg/mL for 1 h at 37 °C. After this time, the medium was removed and reduced MTT (blue formazan product) was solubilized by adding 100 μl of DMSO to each well. After agitation of the plates for 15 min, the optical density of the solubilized formazan product in each well was measured using spectrophotometer (BioTek Instruments, VT, USA) with a 570 nm test wavelength and a 690 nm reference wavelength.

2.6. Microscopic analysis for cell viability by PI and Hoechst 33342 double staining

Apoptotic or necrotic cell death caused by glutamate/BSO was characterized by double staining the cells with Hoechst 33342 and PI (Kim et al., 2011). Cells were stained with 8 μM Hoechst 33342 and 1.5 μM PI for 20 min at 37 °C. After being washed twice with serum free media, cells were imaged with fluorescence microscope (Olympus, Tokyo, Japan). PI positive cells were counted using a cell counter under a fluorescence microscope at 100 times magnification. The total number of PI positive cells in four different representative fields per well were quantified for each treatment group to estimate the percent of PI positive cells out of the total cell number (Minimum 200 cells/well counted and four wells were sampled for each treatment group).

2.7. Electrophoresis and western blotting

For immunoblot analysis, the RGC-5 cells were treated with different concentrations of hirsutenone for 1 h. After the pre-treatment, the cells were incubated with glutamate/BSO for 24 h. After 24 h, the medium was removed from the dish and washed once with cold D-PBS. The cells were scraped using a cell scraper and

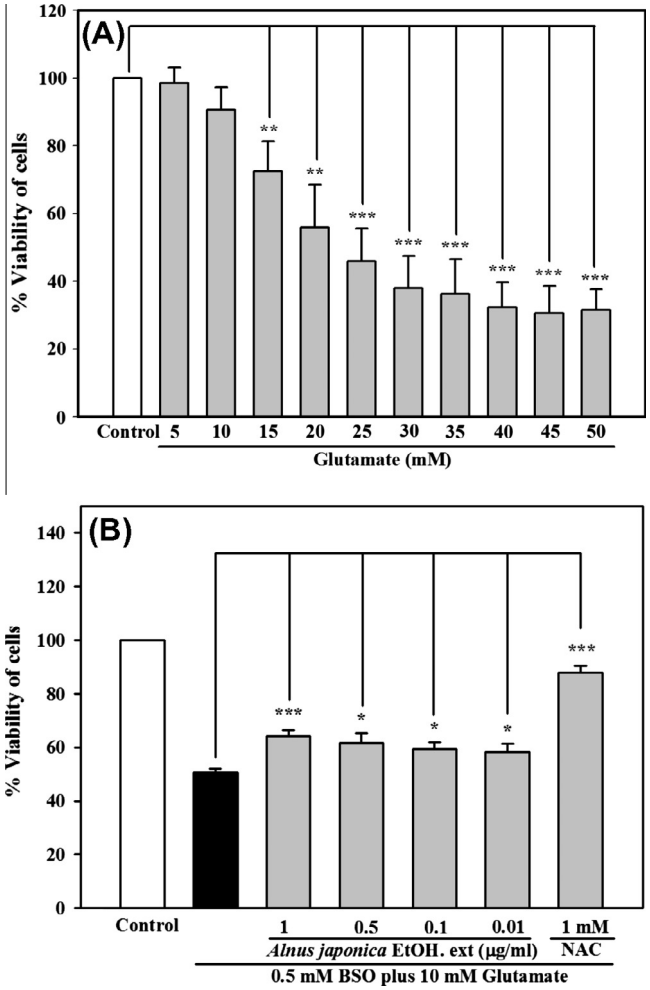

Fig. 1. (A) Glutamate sensitivity of RGC-5 cells. Viability of cells after treatment with 5 to 50 mM of glutamate for 24 h, as measured by the MTT assay. Experimental values are expressed as a percentage of the viable cells with error bars indicating the S.E.M. from three independent experiments. (B) Effects of *A. japonica* on glutamate/BSO induced retinal ganglion cell viability. Glutamate/BSO induced a decrease in cell viability to approximately 50% of the control. *A. japonica* at 1, 0.5, 0.1 and 0.01 μg/mL (and also NAC at 1 mM) significantly inhibited glutamate/BSO induced cell damage. Experimental values are expressed as the percentage of viable cells with error bars indicating the S.E.M. from four independent experiments ($^{*}p < 0.05$ and $^{***}p < 0.001$).