Neuroprotective effect of a dietary supplement against glutamate-induced excitotoxicity in retina

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Abstract

- AIM: To evaluate the neuroprotective effect of a dietary supplement (ClearVision EX®; CV) against glutamate-induced excitotoxicity in retina.
- METHODS: We evaluated the protective effects CV on glutamate-induced cell toxicity of an immortalized mouse hippocampal cell line (HT-22) in vitro and N-methyl-D-aspartate (NMDA) induced retinal injury in vivo. Once-daily oral administration of CV or vehicle (5% Arabic gum) was started the day before the NMDA injection and continued until the end of the study. Electroretinograms (ERGs) were recorded to evaluate the retinal function at 2d after NMDA injection. Furthermore, a histological evaluation, Western blot analysis, and immunohistochemistry were performed for assessing the signal transduction pathway.
- RESULTS: HT-22 cell death was induced by the addition of glutamate and co-incubation with CV protected against it. Oral administration of CV inhibited the decrease in scotopic threshold response amplitudes induced by the intravitreal injection of NMDA and those of the thickness of the inner retinal layer in the histological evaluation. The increased phosphorylated levels of extracellular signal-regulated kinase (ERK) but not cAMP response element binding protein (CREB) or Akt were observed 1h after NMDA injection in both the vehicle- and CV-treated rats; however, pERK activation was no more upregulated at 3h after NMDA injection. pERK upregulation was observed in Müller cells.

- CONCLUSION: CV shows a protective effect against both glutamate-induced HT-22 cell death and NMDA-induced retinal damage. pERK upregulation in the Müller cells plays a key role in the protective effect of CV against glutamate-induced retinal toxicity.

- KEYWORDS: glutamate-induced toxicity; retinal ganglion cells; HT-22 cells; pERK

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INTRODUCTION

Retinal ganglion cells (RGCs), which are the third-order neuron in the visual pathway in the retina, play a role in transmitting visual signals from the retina to the brain. RGCs are injured in various types of retinal diseases such as glaucoma, ischaemic neuropathy, and hereditary optic neuropathy. The progression of these diseases has a possibility of loss of vision. However, critical treatments have not been developed yet.

There are various types of glaucoma such as primary open angle glaucoma, primary angle-closure glaucoma, and normal-tension glaucoma (NTG). Glaucoma is a neurodegenerative condition characterized by a progressive loss of RGCs and associated with visual field defects and thin optic nerve fiber layer. One of the causes of glaucomatous retinal injury is thought to be related to ocular hypertension, which is the major risk factor for glaucoma and causes the death of RGCs. The molecular mechanisms underlying the death of RGCs, such as changes in axonal flow including neurotrophic factors, oxidative stress, ischaemia, and excitotoxicity, have been reported as potential contributors to glaucomatous injury. Recently, inflammatory reactions related to the progression of RGC death have been reported. Thus, the mechanisms involved are very complicated and effective neuroprotective agents have not been explored.

Neuroprotective effects of various types of antioxidants on neuronal cell death have been reported. We have reported that...
Neuroprotective effect of a dietary supplement

thioredoxin-2, a small redox-active protein\(^{14}\), protected retinal epithelial cells that played a critical role in retinal degeneration from oxidant-induced apoptosis\(^{15-16}\). However, it takes a long time to develop gene therapies for application in the clinical setting.

Dietary supplementation is a candidate approach for protecting neurons from neurodegenerative conditions. One of the benefits of using a dietary supplement is to apply it for human easily because the safety data of all of contents in the dietary supplement have been established. ClearVision EX\(^ {\text{®}}\) (CV) is a commercialized Japanese dietary vitamin supplement based on the Age-Related Eye Disease Study (AREDS) formulation, containing β-carotene, vitamin C, vitamin E, lutein, zeaxanthin, niacin, zinc, and copper. From detailed description of contents in CV, it is expected to have some anti-oxidant effects. Thus, in this study, we evaluated the efficacy of CV for managing N-methyl-D-aspartate (NMDA)-induced retinal degeneration.

MATERIALS AND METHODS

Ethical Approval  The use of animals in these experiments was in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and conducted with the consent of the Animal Research Committee of Rohto Pharmaceutical Co., Ltd., Japan.

Cell Culture  The immortalized mouse hippocampal cell line HT-22 was cultured in Dulbecco’s modified Minimum Essential Medium (Thermo Fisher Scientific, Tokyo, Japan) supplemented with 10% foetal bovine serum under a 5% CO\(_2\) atmosphere at 37°C. The culture medium was changed every 3d and cells were passaged using a 0.02% ethylaminediaminetetraacetic acid/phosphate-buffered saline (PBS) solution. We investigated appropriate glutamate concentrations and cell densities to induce glutamate toxicities because cell toxicities depend on cell densities. We determined the glutamate concentration to 1.5 mmol/L at the cell density of 5×10\(^{3}\) cells/well that showing about IC\(_{50}\) (data not shown). The HT-22 cells were plated at a density of 5×10\(^{3}\) cells/well in a 96-well plate and incubated with glutamate (1.5 mmol/L) and CV (1.6-200 μg/mL). After incubation for 24h, cell viability was evaluated using an MTS cell proliferation colorimetric assay kit (Funakoshi, Tokyo, Japan).

Cell toxicity was evaluated using a lactate dehydrogenase (LDH) cytotoxicity assay kit (Funakoshi, Tokyo, Japan).

Animals  Sprague-Dawley rats (SLC Japan Inc., Shizuoka, Japan) aged 8wk were maintained under a 12-hour/12-hour light-dark cycle (light intensity: 300 lx) with unlimited access to laboratory chow and water. Once-daily oral administration of CV (30, 100, or 300 mg/kg) or vehicle (5% arabic gum) was started on the day before NMDA injection and continued until the end of the study. Thirty or fourteen rats were used for electoretinogram (ERG) recordings and histological analysis or Western blot analysis, respectively.

Reagents  CV was supplied by Rohto Pharmaceutical Co., Ltd (Osaka, Japan). Antibodies against extracellular signal-regulated kinase (ERK) (Cat No. 4695S), phospho-ERK (pERK) (Cat No. 9101S), Akt (Cat No. 9272), pAkt (Cat No. 9271L), cAMP response element binding protein (CREB) (Cat No. 9197S), pCREB (Cat No. 9198S), and β-actin (Cat No. sc-69879) were obtained from Cell Signaling Technology (Tokyo, Japan). Glutamine synthetase (GS) antibody (Cat No. MAB302) was obtained from Millipore (Tokyo, Japan). Secondary antibodies against rabbit (Cat No. S3731) and mouse immunoglobulin G (IgG) (Cat No. S3721) were obtained from Promega (Tokyo, Japan).

N-methyl-D-aspartate-induced Retinal Damage  Retinal damage was induced using NMDA (Sigma, St. Louis, MO, USA). Under anesthesia by continuous inhalation of isoflurane (Pfizer Japan Inc., Tokyo, Japan), 5 μL of NMDA solution (1 mmol/L) was injected into the vitreous by entering the eye at the ora serrata by using a 32-gauge needle on a Hamilton syringe.

Electroretinogram Recordings  ERGs were recorded to evaluate the retinal function at 2d after NMDA injection as described previously\(^{[17]}\). In brief, the rats were dark-adapted for a minimum of 12h. A small contact lens with an electrode was then mounted on the cornea and a reference electrode was placed under the tongue. The amplitude of the scotopic threshold response (STR, -5 log cd/m\(^2\)) of ERG was determined.

Histological Analysis  Ten days after the NMDA injection, the rats were euthanized by CO\(_2\) inhalation. The eyes were enucleated and immersed in Karnovsky fixative solution and then embedded in paraffin. Serial sections (4 μm) of whole eyes were cut horizontally through the cornea and parallel to the optic nerve and stained with haematoxylin and eosin. Light microscopy images of the histological sections were photographed (Olympus IX71, Osaka, Japan), and the ratio of the thickness of the whole retinal layer (WRL) and the inner plexiform layer (IPL) was calculated (IPL/WRL).

Western Blot Analysis  Western blot analysis was performed using the methods described previously\(^{[18]}\). Briefly, the retinas were isolated from the eyes 1 and 3h after NMDA injection. Protein was extracted with a lysis buffer containing 10 mmol/L Tris-HCl (pH 7.5), 1 % Triton X-100, 0.5 % NP-40, 1 mmol/L EDTA, 150 mmol/L NaCl, 1× protease inhibitor cocktail (Thermo Scientific, Tokyo, Japan), and 1× Halt™ phosphatase inhibitor cocktail (Thermo Scientific, Tokyo, Japan). The lysate was quantified using a bicinechonic acid assay (BCA) protein assay kit (Pierce, Rockford, IL). Thirty micrograms of protein was electrophoresed on 4%-15% Mini-PROTEIN TGX gels (Bio-Rad, Tokyo, Japan) and transferred onto a polyvinylidene difluoride (PVDF) membrane. After blocking with a membrane, the membrane was incubated with the first
antibody. Following washing, the membrane was incubated with the secondary antibody, an alkaline phosphatase-conjugated antibody. Chemiluminescence detection (CDP-Star Detection Reagent; GE Healthcare, Tokyo, Japan) was performed according to the standard procedure. Band densities were measured using ImageQuant software (GE Healthcare, Tokyo, Japan) and the density of each band was normalized to that of β-actin.

Immunohistochemistry
Immunohistochemistry was performed on the retina obtained 3h after NMDA injection. Paraffin-embedded sections were deparaffinized according to the standard procedure. The sections were treated in citrate buffer (pH 6.0) in a microwave oven as an antigen retrieval method. After blocking, sections were incubated in a solution of pERK antibody (dilution 1:200) and GS antibody or in a control solution of anti-rabbit and anti-mouse IgG overnight at 4°C. The sections were then washed three times with PBS containing 0.01% Tween20 and incubated with Alexa488- or Alexa594-conjugated anti-rabbit and anti-mouse IgG solution at room temperature for 1h. Following washing, the sections were covered with mounting media including 4’,6-diamidino-2-phenylindole (DAPI; VECTASHIELD: Funakoshi, Tokyo).

Statistical Analysis
Statistical analyses for in vitro experiments were performed using GraphPad Prism 4 (GraphPad Software, San Diego, CA, USA). Data are expressed as mean±SD values. The statistical method used was Tukey’s multiple comparison test. Statistical analyses in vivo experiments were performed using StatLight (Yukms Corp., Tokyo, Japan). Data are expressed as mean±SD values. Statistical comparisons were made using Student’s t-test or Dunnett’s test after checking for homoscedasticity.

RESULTS
Effect of CV on Glutamate-induced Toxicities in HT-22 Cells
Cell viability decreased upon the addition of glutamate; however, after the addition of CV, the viability increased and was higher than that of the control cells (Figure 1A). The increased cell viabilities might indicate that CV affected cell proliferation. We tested the effects of CV on cell proliferation (Figure 1B). CV treatment at a concentration of 1.6 μg/mL positively affected cell proliferation. To evaluate cell toxicity excluding the proliferative activities, we measured LDH release from cells (Figure 1C). Increased LDH levels were observed in the glutamate-treated cells. On the other hand, the increase in LDH levels was inhibited by the addition of 8 μg/mL of CV.

Effects of CV on NMDA-induced Retinal Toxicity
RGC function was evaluated by measurements of STRs by ERG recordings 2d after NMDA injection (Figure 2A). The amplitudes of the STRs recorded in rat retinas treated with an intravitreal injection of NMDA markedly decreased compared to those recorded in non-injected rats. On the other hand, the decrease in the STRs tended to increase in a CV dose-dependent manner, and a significant increase was observed in rats administered a CV dose of 300 mg/kg compared to that in the vehicle-administered rats. The thickness of the IPL of the histological sections was evaluated (Figure 2C). The thickness of the inner retinal layer clearly decreased in the NMDA-injected rats. CV administration had a protective effect against inner retinal damage. The percentage of IPL thickness to that of the WRL in the NMDA-injected retinas markedly decreased compared to that in the non-injected retina. The decreased value of IPL/WRL in the vehicle-administered rats was significantly higher than that in the rats administered CV at 300 mg/kg (Figure 2B).

Western Blot Analysis
To investigate the effect of CV on the early response related to the cell death pathway, we performed the Western blot analysis (Figure 3). Phosphorylated or non-phosphorylated ERK, CREB and Akt, normalized by β-actin and phosphorylated levels were investigated. pERK levels increased at 1h after the treatment with vehicle and CV. The significant difference was also detected in pERK of 3h after the treatment with CV and the level of ERK in the CV-administered rats increased at 3h after the NMDA injection. Significant differences in the levels of pERK/ERK, as a result, were only detected at 1h after the NMDA injection in the vehicle group (Figure 3A). On the other hand, the levels of
phosphorylated and non-phosphorylated CREB and Akt were unchanged both in the vehicle- and CV-administered rats (Figure 3B, 3C).

**Immunohistochemistry** To determine the area of pERK activation, immunohistochemical studies were performed. In the normal retinas (untreated retinas and retinas without...
NMDA injection), only weak pERK immunoreactivity was observed in the whole retina (Figure 4A: green). Increased pERK immunoreactivity was observed 3h after the NMDA injection (Figure 4B, 4C). In the vehicle-treated rat retinas, pERK immunoreactivity was mainly observed in the ganglion cell layer (GCL) and IPL (Figure 4B). In the CV-treated rat retinas, the inner nuclear layer (INL) also showed immunoreactivity (Figure 4C).

DISCUSSION
The aim of this study was to evaluate the effects of CV, a vitamin supplement based on AREDS formulation that has been commercialized in Japan, on glutamate-induced excitotoxicity and to investigate the mechanisms underlying its protective effects. Various kinds of factors such as glutamate excitotoxicity, nitric oxide, generation of reactive oxygen species (ROS), and reduction of blood flow are related to the induction of glaucomatous injury, although elevated intraocular pressure (IOP) is the main risk factor for glaucoma[19]. Among these causes, we focused on retinal injury induced by ROS. The two main results obtained were as follows: pre-administration of CV protected the retina from NMDA-induced toxicity. The major effect underlying the protective mechanism of CV is radical scavenging by vitamins. NMDA-induced neuronal toxicities are implicated in the pathological mechanisms of Alzheimer’s disease[20], Huntington disease[21], and Parkinson’s disease[22]. One possible mechanism of NMDA-induced neurotoxicity has been well identified as follows: prolonged NMDA receptor activation causes the opening of a cationic channel and increases Ca\(^{2+}\) concentration in depolarized cells, which results in destabilization of mitochondrial membrane potentials. The release of ROS and cytochrome c from the mitochondria lead to cell death[23]. To elucidate the possible mechanism underlying the protective effect of CV, we used HT-22 cells. HT-22 cells, which lack an ionotropic glutamate receptor such as an NMDA receptor under undifferentiated conditions[24], are a good model for evaluating neuronal oxidative stress. It has been reported that glutamate-induced cell death is induced by inhibition of the
cystine/glutamate exchanger. The inhibition leads to decreased glutathione production and subsequently causes intracellular ROS production [25-26]. CV clearly showed a protective effect against glutamate-induced toxicity in HT-22 cells, indicating that CV acted as a radical scavenger during oxidative stress. In in vivo experiments, we observed decreased STR amplitudes indicating dysfunction of RGCs 2d after the intravitreal injection of NMDA. It is known that STRs are a representative marker of RGC function [27-28]. Histological examinations indicated that the physiological dysfunctions associated with the STRs were morphological damages but not transient. In the histological examinations, we calculated the percentage of the inner retinal layer to evaluate the damage to the inner retinal neurons. The protective effects of CV were observed in both evaluations, ERGs and histological examinations. We also analysed the signal transduction pathways related to NMDA-induced retinal toxicity. There was a significant increase in pERK/ERK level in the vehicle-administered rats. However, pERK level in CV-administered rat retinas continuously increased and a significant difference was observed at 3h after NMDA injection. The upregulation of pERK is well identified as a marker of NMDA-induced retinal toxicity [29-30]. We previously reported that pERK has a protective role in ischaemia-induced retinal damage [31]. Some studies have reported that co-injection of U0126, an ERK inhibitor, exacerbated NMDA toxicity [30,32]. Therefore, we hypothesized that continuous pERK activation in Muller cells, but not the level of pERK/ERK, was important for protecting the retina from NMDA-induced toxicity.

In summary, CV had protective effects against NMDA-induced retinal damage and cell death induced by oxidative stress. We showed that Müller cells had a key role in the protective effect. Our results indicate that oral vitamin supplementation may prevent retinal cell death caused by oxidative stress. However, the data showing here is the protective role of CV on ROS-related toxicities. The ganglion cell death caused by glaucoma is not simple, as indicated above. Further studies using other models such as increased IOP or optic nerve crush model is needed to confirm the efficacy of CV to use as a daily supplementation for patients with glaucoma.

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Conflicts of Interest: Kurose T, Kato M, Mitsuguchi Y, Takai Y, Homma Y, are employed by the Rohto Pharmaceutical Co., Ltd. Sugano E, None; Sugai A, None; Shiraiwa R, None; Tabata K, None; Tomita H, None.

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