Neuroprotective effect of maltol on experimental glaucoma model

Yookyung Song

Department of Medicine

The Graduate School, Yonsei University

Neuroprotective effect of maltol on experimental glaucoma model

Directed by Professor Gong Je Seong

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Yookyung Song

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This certifies that the Doctoral Dissertation of Yookyung Song is approved.

Thesis Supervisor : Gong Je Seong

Thesis Committee Member#1 : Jung Il Moon

Thesis Committee Member#2 : Jong Eun Lee

Thesis Committee Member#3: Kyung Soo Park

Thesis Committee Member#4: Samin Hong

The Graduate School
Yonsei University

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Finally, I give thanks to God for helping and strengthening me during the Ph.D. journey. As I move on to the next stage of my career, I will look back on it and smile.

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ABSTRACT

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Purpose: Maltol (3-hydroxy-2-methyl-4-pyrone), formed through thermal degradation of starch, is found in caramelized foods, baked cereals, coffee, and Korean red ginseng. This study investigated the neuroprotective effects of maltol and its underlying mechanism in R28, rat embryonic precursor neuroretinal cells and retinal ganglion cells (RGCs) of optic nerve crush (ONC) mice.

Methods: R28 cells were exposed to hydrogen peroxide (H_2O_2, 0.0 to 1.5 mM) with or without maltol (0.0 to 1.0 mM). Cell viability was monitored with the lactate dehydrogenase (LDH) assay and apoptosis was examined by the terminal deoxynucleotide transferase-mediated terminal uridine deoxynucleotidyl transferase nick end-labeling (TUNEL) method. ONC was performed unilaterally in adult male C57BL/6 mice without or with pre- or post-treatment of intraperitoneal (IP) maltol injection (100 mg/kg) for 5 consecutive days. Each eye was enucleated after 1 day (n=15), 3 days (n=15), and 7 days (n=15). RGCs of ONC mice were assessed by H & E stain,

immunohistochemistry and TUNEL method. To investigate the neuroprotective mechanism of maltol, phosphorylation of nuclear factor-kappa B (NF-κB), and mitogen-activated protein kinases (MAPK) were evaluated.

Results: R28 cells exposed to H_2O_2 were found to have decreased viability in a dose- and time-dependent manner. However, H_2O_2-induced cytotoxicity was decreased with the addition of maltol. This H_2O_2-induced cytotoxicity caused apoptosis of R28 cells, characterized by DNA fragmentation. Apoptosis of oxidative stressed R28 cells with 1.0 mM $H_2O_2$was decreased with 1.0 mM maltol. Also, in RGCs of ONC mice, maltol not only increased cell viability but also attenuated DNA fragmentation. Western blot analysis of R28 cells and immunohistochemistry of ONC mice showed that maltol reduced phosphorylation of NF-κB and MAPKs.

Conclusions: The present study demonstrates that maltol inhibits apoptosis of R28 cells induced by H_2O_2 and RGCs induced by crush injury of optic nerve in mice. The mechanisms underlying this protection are not fully understood, but are associated with NF-kB and MAPK signaling pathways. This study provides evidence that maltol could be an innovative neuroprotective therapeutic agent for glaucoma.

Key words : maltol, neuroprotection, oxidative stress, rat embryonic precursor neurosensory retinal cell, optic nerve crush mouse model

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I. INTRODUCTION

Glaucoma is a common eye condition that affects millions of individuals worldwide, making it the second-leading cause of blindness.[1] It is a generic term for a group of heterogeneous ocular neuropathies that eventually lead to gradual axonal degeneration in the optic nerve and progressive loss of retinal ganglion cells (RGCs). RGC death and optic nerve degeneration are complex processes with underlying molecular mechanisms that are only partially understood.

The most common treatment of glaucoma is the use of medications that lower intraocular pressure (IOP) because increased IOP is the single most significant risk factor for the development and progression of glaucoma. However, treatments commonly used for glaucoma have little long-term value and only prolong the period over which degeneration occurs. Therefore, a

neuroprotective medicine that can delay or prevent RGC loss is needed. Neuroprotective therapies work at the connection between damaging the optic nerve and loss of RGCs. Although recent studies of glaucoma have aimed to develop new treatment strategies to rescue the damaged RGCs with neuroprotective agents, currently there is no drug that shows significant neuroprotective effects.

Maltol (2-methyl-3-hydroxy-1,4-pyrone) is a naturally occurring substance that is widely used as a favoring agent.[2] It is formed through thermal degradation of starch and is found in baked products as well as Korean red ginseng, coffee, soybeans, and caramelized foods. Maltol is not only commonly used in breads, cakes, malt beverages, and chocolate milk as a flavor enhancer,[3] but but is also used in medications such as vanadyl maltolate for the treatment of diabetes, and ferric trimaltol for the treatment of iron deficiency anemia.[4,5] In previous studies, it has been reported that maltol has a neuroprotective effect through its antioxidant properties,[6,7] and anti-apoptotic effects.[8] Although previous studies have shown that maltol attenuates neurotoxicity and prevents oxidative damage, there has been no comprehensive study of the protective effects of maltol on experimental glaucoma models *in vitro* and *in vivo*.

This study investigated the neuroprotective effects of maltol and its underlying mechanism on oxidative stressed R28 rat embryonic retinal precursor cells and RGCs of optic nerve crush (ONC) mice. In this study, hydrogen peroxide (H_2O_2) was used to generate free radicals in order to induce

oxidative stress on R28 cells and ONC was performed to induce degeneration of RGCs in mice. ONC is a well-established model of axonal injury that results in secondary RGC apoptotic cell death resembling glaucoma.[9,10]

II. MATERIALS AND METHODS

1. In Vitro

   A. Cell culture

   R28 cells are rat embryonic precursor neurosensory retinal cells, originating from a postnatal day 6 rat retinal culture immortalized with the 12S E1A (NP-040507) gene of the adenovirus in a replication-incompetent viral vector.[11] R28 cells express genes characteristic of neurons,[12] and have functional neuronal properties.[13] R28 cells were purchased from KeraFAST (Boston, MA, USA). R28 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Gibco, Carlsbad, CA, USA) and 100 μg/mL streptomycin (Gibco, Carlsbad, CA, USA). Cells were passaged every 2 to 3 days and incubated at 37℃ in 5% CO_2 to reach 80% confluence, and then they were transferred to a serum-free environment for 24 hours to reach a sessile nonproliferative state.

B. Oxidative stress and maltol treatment

Oxidative stress was caused by the addition of H_2O_2 (Sigma-Aldrich, St. Louis, MO, USA) to the culture media. R28 cells were exposed to several concentrations of H_2O_2 (0.0 0.2, 0.4, 0.6, 0.8, 1.0, and 1.5 mM) for 48 hours. After 24 hours of exposure to 1.0 mM H_2O_2, 60% cell cytotoxicity was reached so a 24 hour exposure to 1.0 mM H_2O_2 was selected for oxidative stress conditions. Oxidative stressed R28 cells were then incubated with 0.0, 0.1, 0.5, 1.0, and 1.5 mM maltol (Sigma-Aldrich, St. Louis, MO, USA) for 24 hours.

C. Lactate dehydrogenase assay

Cell cytotoxicity was quantified by the measurement of LDH released into the culture media from damaged cells according to the manufacturer's instructions (CytoTox 96® Non-Radioactive Cytotoxicity Assay Kit; Promega Corporation, Madison, WI, USA). The level of LDH release was normalized to the total LDH content following cell lysis in a medium. The absorbance was determined at 490 nm using a Multimode Microplate Reader (Berthold Technologies, Bad Wildbad, Germany). LDH release was expressed as a percentage of the maximum LDH released after cell lysis.

D. Terminal deoxynucleotidyltransferase mediated dUTP nick-end labeling (TUNEL) assay

To analyze the occurrence of DNA fragmentation, the TUNEL assay (BD Biosciences Pharmingen San Diego, California, USA) was used. R28 cells were plated in 6-well tissue culture plates at a cell density of 2.0×10^5 cells per well. The cells were treated for 16 hours with the compounds. The cells were removed by trypsinization, washed twice with phosphate-buffered saline (PBS) and fixed for 1 hour in 1% paraformaldehyde. The cells were washed twice with PBS and perrmeabilized for 48 hours in 70% ethanol at −20°C. Transferred bromolated deoxyuridine triphosphate nucleotides (Br-dUTP) to the free 3'-OH of cleaved DNA by terminal deoxynucleotide transferase (TdT) were detected by the anti-BrdU-fluorescein isothyocyanate (FITC) antibody under fluorescence microscopy. Subsequently the cells were labeled with FITC-dUTP and propidium iodide (PI) as described in the manufacturer's manual (BD Biosciences Pharmingen San Diego, California, USA).

E. Western blot analysis

For extraction of whole cellular proteins, cells were washed twice with ice-cold phosphate buffered saline and then lysed with cell lysis buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% Na-deoxycholate,

150 mM NaCl, 1 mM EDTA, 10 mM Na3VO4, 50 mM NaF, 1 mM PMSF, 1 μg/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin) on ice for 30 minutes. Lysates were sonicated, and the cell homogenates were centrifuged at 15,000 g for 10 minutes (4℃). After centrifugation, supernatants were electrophoresed in 10% acrylamide gels and transferred onto nitrocellulose membranes. The proteins were probed overnight with antibodies against nuclear factor-kappa B (NF-κB), extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38, phosphorylated NF-κB (p-NF-κB), phosphorylated ERK p44/42 (p-ERK), phosphorylated JNK (p-JNK), phosphorylated p38 (p-p38) (Cell Signaling Technology, Inc., Danvers, MA, USA) and β-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). The immunoreactive bands were detected with horseradish peroxidase-conjugated secondary antibodies and visualized by enhanced chemiluminescence.

2. In Vivo

A. Animals

Adult, 8 to 10-week-old male C57BL/6 mice (Charles River Laboratory, Wilmington, MA, USA) were used in accordance with the

ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. The research protocol was approved by Institutional Animal Care and Use Committee of the Yonsei University College of Medicine. A total of 45 adult male C57BL/6 mice were randomly divided into three experimental groups: ONC injury, ONC with post-treatment of maltol intraperitoneal (IP) injection (100 mg/kg) for 5 consecutive days, and ONC with pre-treatment of maltol IP (100 mg/kg) for 5 consecutive days.

B. Mouse model of ONC

Controlled optic nerve crush (CONC) was performed as previously described.[14] In brief, mice were anesthetized with an intraperitoneal injection of a mixture of zolazepam/tiletamine (30 mg/kg; Zoletil 50®, Virbac, France) and xylazine (10 mg/kg; Rompun®, Bayer HealthCare, Germany) and applied by 1% procaine hydrochloride topically. The left optic nerve of mouse was then crushed for 10 seconds using self-closing forceps (Roboz RS-5027, Roboz Surgical Instrument Co., MD, USA). The right eye of each animal served as a control. After this procedure, antibiotic ointment was applied to the surgical site. In the postoperative period, mice exhibited normal eating and drinking behavior. In mice under anesthesia produced by an intraperitoneal injection of a mixture of zolazepam/tiletamine (30 mg/kg; Zoletil 50®,

Virbac, France) and xylazine (10 mg/kg; Rompun®, Bayer HealthCare, Germany), each eye was enucleated after 1 day (n=15), 3 days (n=15), and 7 days (n=15).

C. Histological analysis

The enucleated eyeballs were immersed overnight in 4% paraformaldehyde at 4℃ and embedded in paraffin; Six paraffin-embedded sections with 4-μm thickness were cut parallel to the maximum circumference of the eyeball through the optic disc. These sections were stained with hematoxylin and eosin (H&E). Three sections from each eye being used for the morphometric analysis. Light microscope images were photographed, using a digital camera (COOLPIX 4500; Nikon, Tokyo, Japan), and the cell counts in the ganglion cell layer (GCL) at a distance between 375 μm and 625 μm from the optic disc, were measured on the photographs in a masked manner by a single observer and then averaged to give a single value. Data from three sections, selected randomly from the six sections, were averaged for eye and three eyes at each time point and each condition were used to evaluate the cell count in the GCL.

D. TUNEL assay

The enucleated eyeballs were immersed overnight in 4%

paraformaldehyde at 4℃ and embedded in paraffin; 4-μm thickness sections were cut parallel to the maximum circumference of the eyeball through the optic disc. Using the Apo-BrdU In Situ DNA Fragmentation Assay Kit (BD Biosciences Pharmingen San Diego, California, USA), apoptotic cells were detected by the anti-BrdU- FITC antibody under a fluorescence microscopy. Nuclear counterstaining was performed using PI.

E. Immunohistochemistry

The eyes were enucleated, fixed in 4% paraformaldehyde overnight at 4℃, immersed in a 20% sucrose solution, and embedded in an optimal cutting temperature compound (Sakura Finetechnical, Tokyo, Japan). 4-μm thickness sections were mounted on the slides and incubated with blocking buffer (10% goat serum, 0.5% gelatin, 3% BSA, and 0.2% Tween 20 in PBS). Next, they were incubated with rabbit polyclonal anti p-NF-κB, p-ERK, p-JNK, and p-p38 antibody overnight at 4℃. The sections were incubated with Alexa 488 secondary antibody (1:200; Invitrogen) for 1 hr. Photographs of the retina were taken using fluorescence illumination (Leica TSE SPE; Leica Microsystems, Wetzlar, Germany).

3. Statistical analysis

Data are expressed as the mean ± standard deviation (SD) of at least three different experiments performed from separate cell/animal preparations, and at least triplicate determinations were performed in each experiment. A 1-way analysis of variance (ANOVA), with Dunnett's posttest for multiple comparisons was used to compare the difference among multiple groups. Unpaired Student's t-test was used for the comparison between two groups using SPSS 17.0 (SPSS, Chicago, IL, USA). A value of p less than 0.05 was considered statistically significant.

III. RESULTS

1. Oxidative stress-induced cytotoxicity in R28 cells

R28 cells were exposed to 0.0, 0.2, 0.4, 0.6, 0.8, 1.0, and 1.5 mM H_2O_2 for 24 hours and exposed to 1.0 mM H_2O_2 for 1, 3, 6, 16, 24 and 48 hours. Cytotoxicity was quantified by measurement of released LDH to culture media from injured cells. As shown in Fig. 1, H_2O_2 increased LDH release in a dose- and time-dependent manner. When R28 cells were exposed to 1.0 mM H_2O_2 for 24 hours, the cytotoxicity was 60.69 ± 5.71%. The 24 hours of exposure to 1.0 mM H_2O_2 was selected as the oxidative stress conditions for further experiments.

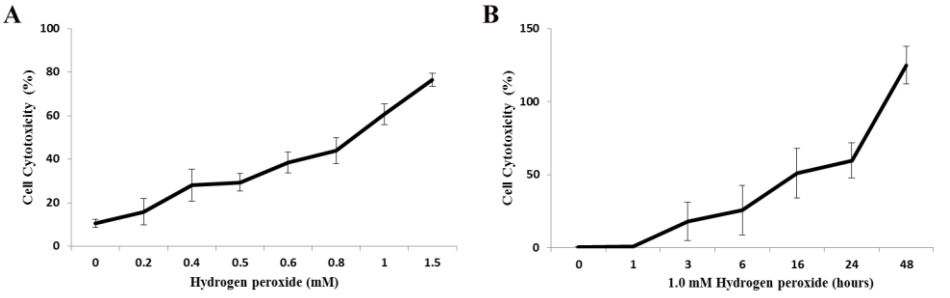

Figure 1. Hydrogen peroxide (H_2O_2) induced cytotoxicity in R28 cells. (A) R28 cells were exposed to H_2O_2 ranging from 0.0 to 1.5 mM for 24 hours. (B) R28 cells were exposed to 1.0 mM H_2O_2 for up to 48 hours. Cell cytotoxicity was quantified by an LDH assay. H_2O_2 increased LDH release in a dose- and time-dependent manner. When R28 cells were exposed to 1.0 mM H_2O_2 for 24 hours, the cytotoxicity was 60.69 ± 5.71%. Data are expressed as the mean ± SD.

2. Effect of maltol on oxidative stressed R28 cells

R28 cells were co-cultured with 1.0 mM H_2O_2 and different concentrations of maltol ranging from 0.1 to 1.5 mM for 24 hours. Co-culture with maltol attenuated H_2O_2-induced cytotoxicity in a dose-dependent manner (Fig. 2). When compared to 1.0 mM H_2O_2 treated cells, the protective effect of 0.1 mM maltol was not statistically different, but 0.5, 1.0 and 1.5 mM maltol significantly decreased LDH leakage from injured cells. LDH leakage markedly increased with the addition of 1.0 mM H_2O_2 (59.25 ± 2.81%) when compared to a control (10.48 ± 1.80%; $P < 0.05$). However, 1.0 mM maltol treatment significantly attenuated these cytotoxic effects (19.86 ± 1.11%). To investigate the underlying mechanism of the neuroprotective effects of maltol, the co-culture condition of 1.0 mM H_2O_2 and 1.0 mM maltol was selected for following experiments.

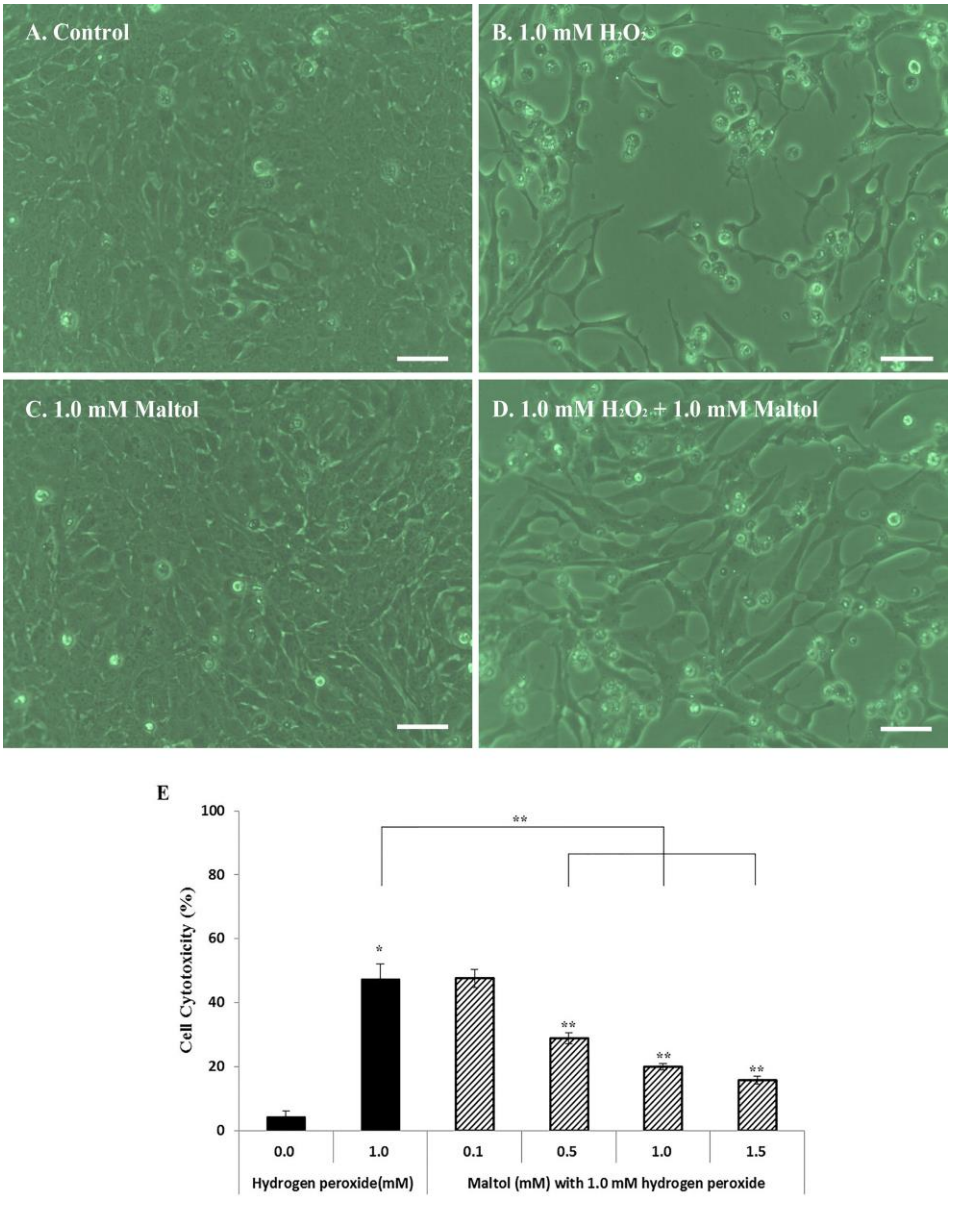

Figure 2. The protective effect of maltol treatment. (A-D) Bright field microscopy. (A) Control. (B) R28 cells exposed to 1.0 mM H_2O_2. (C) R28 cells exposed to 1.0 mM maltol. (D) R28 cells co-cultured with 1.0 mM H_2O_2 and 1.0

mM maltol. (E) R28 cells were co-cultured with 1.0 mM H_2O_2 and different concentrations of maltol ranging from 0.1 to 1.5 mM for 24 hours. Cell cytotoxicity was quantified by an LDH assay. Maltol decreased LDH leakage from injured cells in a dose-dependent manner. Scale bar in panels A,B,C, and D = 50 μm. Data are expressed as mean ± SD. (The asterisks denote that data are significantly different from the untreated control; * 1.0 mM H_2O_2-treated cells; ** $p < 0.05$)

3. Maltol attenuates oxidative stress-induced apoptosis of R28 cells

In Fig. 3, the TUNEL assay for broken DNA from apoptotic cells revealed a FITC (green fluorescence)-positive signal. Propidium iodide counter-staining demonstrated living cells (red fluorescence). After co-culture with 1.0 mM maltol, R28 cells became more resistant to H_2O_2-induced oxidative stress injury. The ratio of TUNEL positive apoptotic cells to the total number of cells increased with the addition of 1.0 mM H_2O_2 (16.33 ± 2.31%) when compared to a control (1.72 ± 0.31 %; $P < 0.05$). However, the ratio decreased with co-culture of 1.0 mM maltol (2.64 ± 0.52 %). Maltol treatment markedly reduced the loss of R28 cells possibly through a decrease in apoptosis.

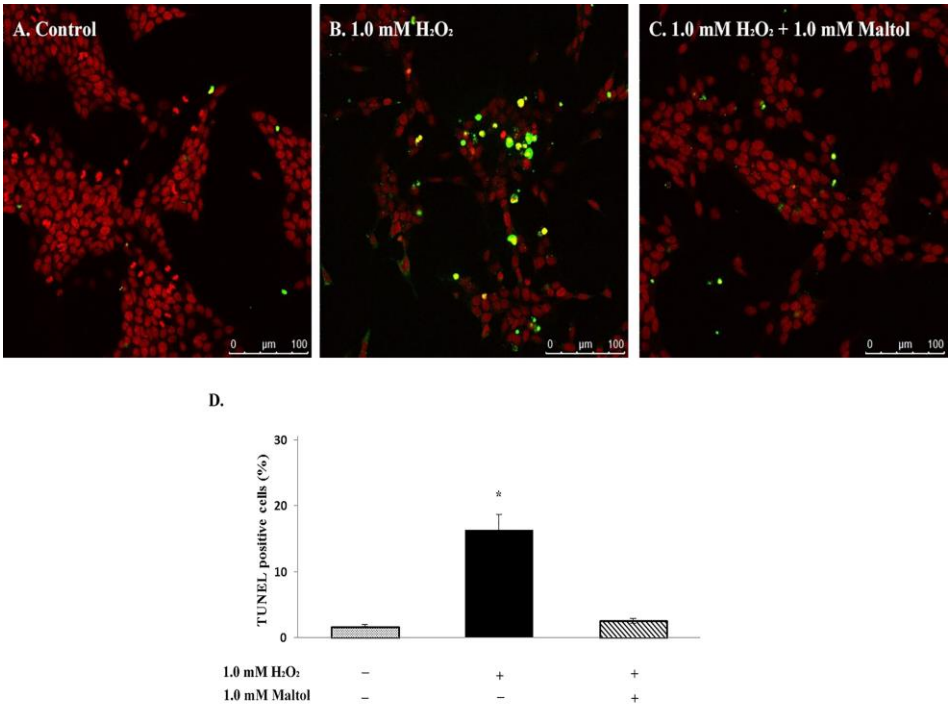

Figure 3. Anti-apoptotic effect of maltol treatment. (A-C) Representative photographs of terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL), in which green fluorescence indicates apoptotic cells and red fluorescence indicates living cells. (A) Control. (B) 1.0 mM H_2O_2. (C) 1.0 mM H_2O_2 with 1.0 mM maltol. (D) Quantification of TUNEL-positive cells; data collected from 10 fields for each group; experiments were repeated three times. Data are expressed as mean ± SD of the ratio of apoptotic cells to the total number of cells. After co-culture with 1.0 mM maltol, oxidative stressed R28 cells became more resistant to hydrogen peroxide injury. (The asterisks denote that data are significantly different from control;* $p < 0.05$)

4. The neuroprotective mechanism of maltol in oxidative stressed R28 cells

To investigate the neuroprotective mechanism of maltol, the expression of NF-κB, ERK, JNK and p38 were evaluated by Western blot analysis. Fig. 4A shows the protein expression of p-NF-κB, p-ERK, p-JNK, p-p38, and β-actin after 48 hours with or without 1.0 mM maltol in R28 cells exposed to 1.0 mM H_2O_2. The expression of p-NF-κB, p-JNK, and p-ERK decreased with co-cpulture of 1.0 mM maltol, especially after 2 hours of H_2O_2 exposure. However, the expression of p-p38 showed no difference in the presence or absence of maltol. To quantify protein expression within 2 hours after exposure to 1.0 mM H_2O_2, the band intensities were measured with the ImageJ program (ImageJ Software; NIH, MD, USA). Fig. 4B shows that the expression levels of p-NF-κB, p-JNK, and p-ERK were 2.16-, 7.7-, and 4.3-times higher than those of a control, and these expression levels were significantly decreased with the addition of 1.0 mM maltol to 1.18-, 2.3-, and 1.8-times higher than those of the control, respectively. However, there was not significant change in p-p38 expression in oxidative stressed R28 cells at 1.7-times higher levels than control without maltol and 1.8-times with maltol.

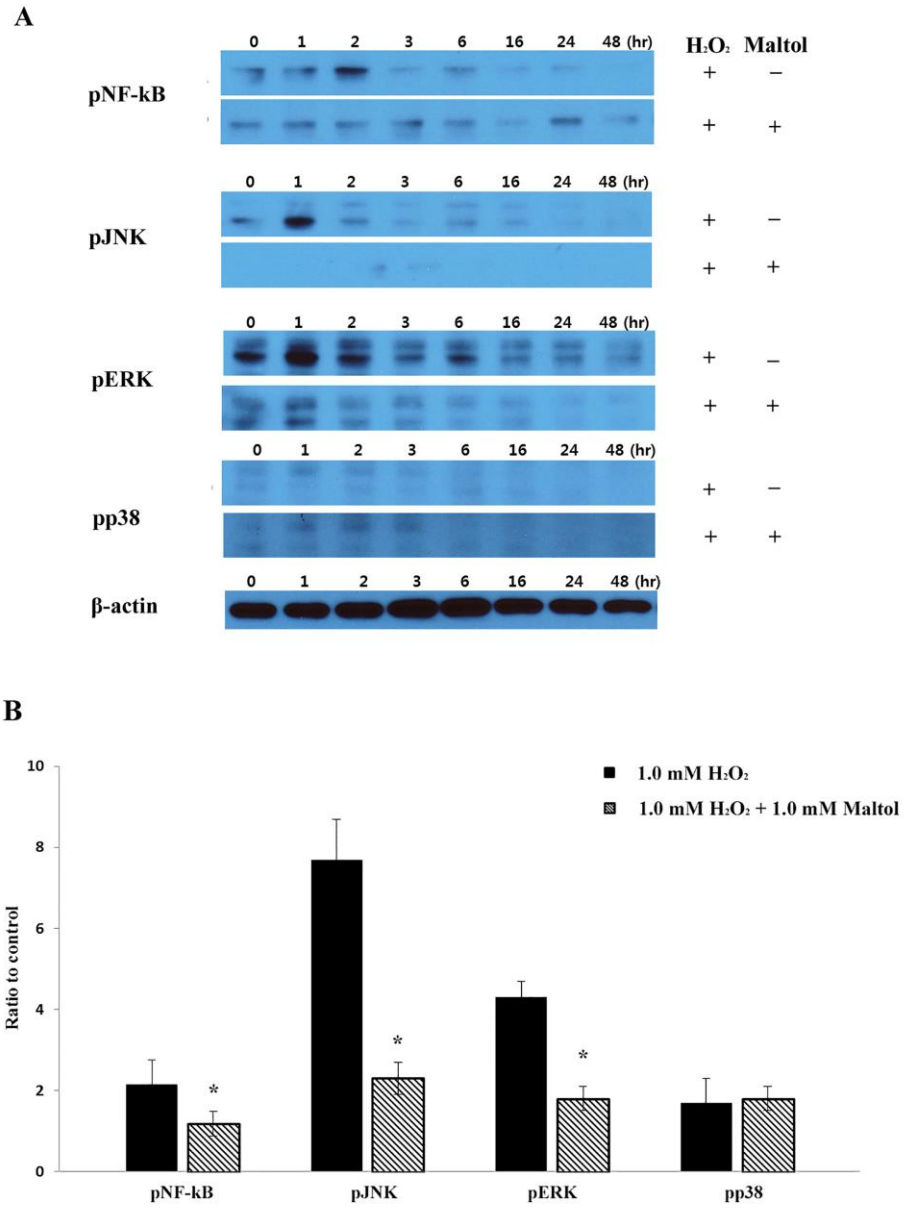

Figure 4. Western blot analysis of nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinases (MAPK) in R28 cells. (A) The expression of

phosphorylated NF-κB (pNF-κB), phosphorylated JNK (pJNK), phosphorylated ERK (pERK), phosphorylated p38 (pp38), and β-actin after 48 hours with or without 1.0 mM maltol in R28 cells exposed to 1.0 mM H_2O_2. (B) The band intensities, relative to a control in R28 cells exposed to 1.0 mM H_2O_2 within 2 hours. The black box indicates exposure to H_2O_2 without maltol treatment and the patterned box indicates exposure to H_2O_2 with maltol treatment. The expression of pNF-κB, pJNK, and pERK was reduced with 1.0 mM maltol treatment. However, there was no significant change of pp38 in the presence or absence of maltol treatment. Data are expressed as mean ± SD. (The asterisks denote that data are significantly different from 1.0 mM H_2O_2-treated cells;* $p < 0.05$)

5. Effect of maltol on RGC damage following ONC in mice

Fig. 5 shows morphologic changes of GCL induced by ONC using H&E stain and Fig. 6 shows quantification of cell numbers in the GCL as an indicator of morphologic damage. Effect of maltol on RGC damage following ONC in mice was evaluated by counting RGCs and expressed as a percentage of control fellow eyes at 1, 3 and 7 days after ONC with or without maltol. Although ONC induced progressive RGC loss in GCL during experimental period, at 1 and 3 days post crush, there was no significant cell loss (98 and 92.5% respectively). However, at 7 days post crush, significant cell loss was noted (68%, $p < 0.05$). Also, in ONC mice with post-treatment of maltol, there was no significant cell loss at 1 and 3 days post crush (98 and 92%, respectively) and at 7 days post crush, significant cell loss was noted (72%). However, in spite of continuous cell loss, cell loss in GCL was not significant in the crushed eyes with pre-treatment of maltol at 1, 3, and 7 days post crush (97, 94 and 80 %, respectively).

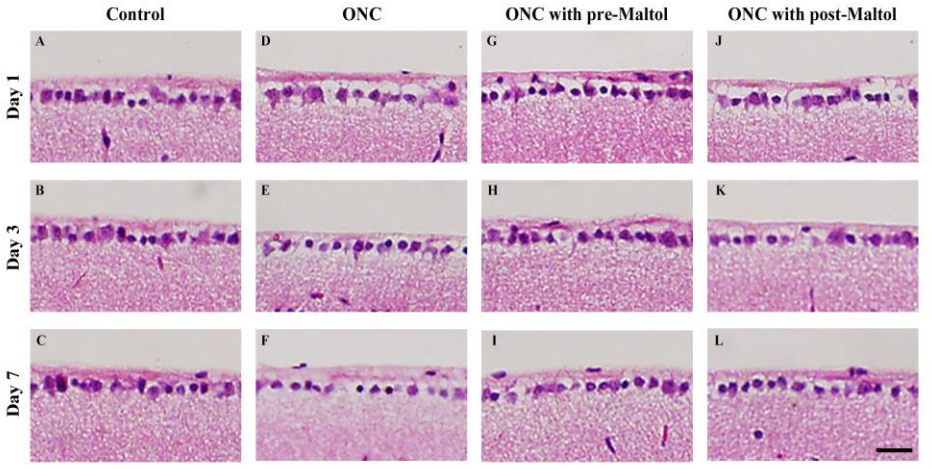

Figure 5. Hematoxylin and eosin stained mouse retina at 1, 3, and 7 days after optic nerve crush (ONC). (A-C) Normal control retinas. (D-F) Retinas of ONC mice (G-I) ONC mice with pre-treatment of intraperitoneal (IP) maltol injection (100 mg/kg) for 5 consecutive days. (J-L) ONC mice with post-treatment of maltol IP injection. Scale bar = 20 μm.

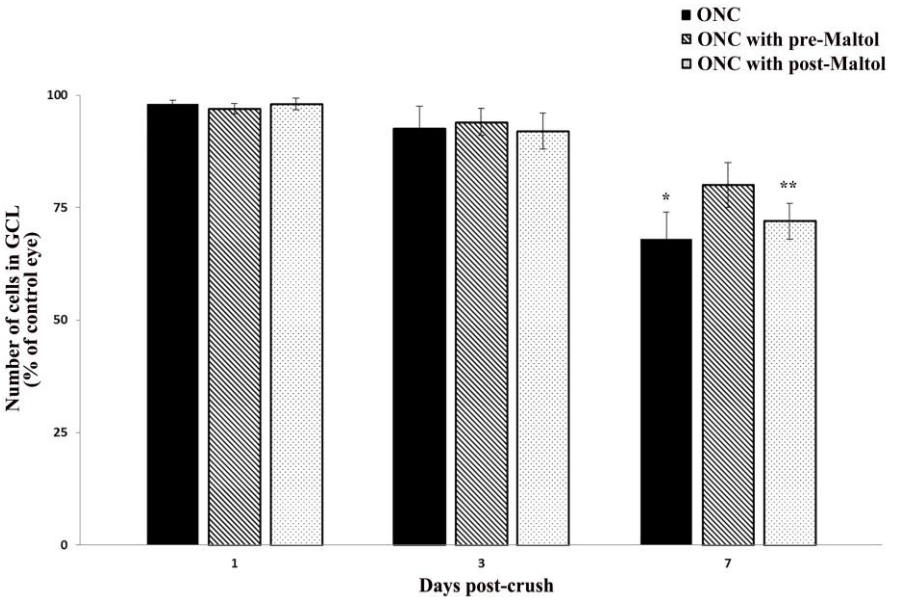

Figure 6. Effect of maltol on retinal ganglion cell (RGC) damage following optic nerve crush (ONC) in mice. Retinal ganglion cell damage was evaluated by counting cell numbers in ganglion cell layer (GCL) and expressed as a percentage of control fellow eyes at 1, 3 and 7 days after ONC without or with pre- or post-treatment of intraperitoneal (IP) maltol injection (100 mg/kg) for 5 consecutive days. At 7 days post crush, cell numbers in GCLs were significantly lower in the crushed eyes without or with post-treatment of maltol. However, in spite of continuous cell loss, there was no significant cell loss in the crushed eye with pre-treatment of maltol. Data are expressed as mean ± SD. (Statistical differences between post crush days within groups were denoted with an asterisk; *, ** $p < 0.05$)

6. Maltol attenuates ONC-induced apoptosis

In Fig. 7, the TUNEL assay for broken DNA from apoptotic cells revealed a FITC (green fluorescence)-positive signal. Propidium iodide counter-staining demonstrated living cells (red fluorescence). TUNEL-positive cells, not present in normal control retina (Fig. 7A), are present in GCL at 3 days after ONC (Fig. 7B). However, TUNEL-positive cells are decreased in GCLs of ONC mice with pre- or post-treatment of maltol (Fig. 7C and 7D). Maltol treatment markedly reduced the loss of RGCs possibly through a decrease in apoptosis.

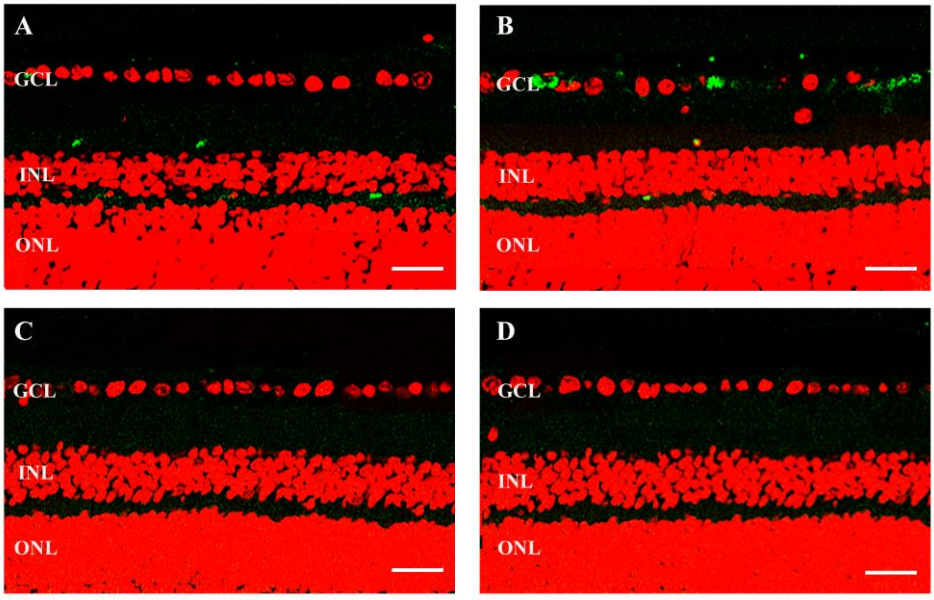

Figure 7. Anti- apoptotic effect of maltol treatment 3 days after optic nerve crush (ONC) without or with pre- or post-treatment of intraperitoneal (IP) maltol injection (100 mg/kg) for 5 consecutive days. Representative photographs of terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL), in which green fluorescence indicates apoptotic cells and red fluorescence indicates living cells. (A) Normal control retina. (B) Reina of ONC mouse. (C) ONC mouse with pre-treatment of maltol. (D) ONC mouse with post-treatment of maltol. After pre-or post- treatment of maltol, RGCs became more resistant against ONC injury. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar = 25 μm.

7. The neuroprotective mechanism of maltol in RGCs of ONC mice

To investigate the neuroprotective mechanism of maltol in RGCs of ONC mice, the immunoreactivity of p-NF-κB, p-ERK, p-JNK, and p-p38 were evaluated. In Fig 8-11, the immunoreactivity of p-NF-κB, p-ERK, p-JNK, and p-p38, not present in normal control retina, are activated in RGCs of ONC mice at 3 days after ONC. However the immunoreactivity of of p-NF-κB, p-JNK, p-ERK and p-p38 are decreased in RGCs of ONC mice with pre- or post- treatment of maltol. The mechanisms underlying the neuroprotection of maltol are associated with NF-kB and mitogen-activated protein kinase (MAPK) pathways.

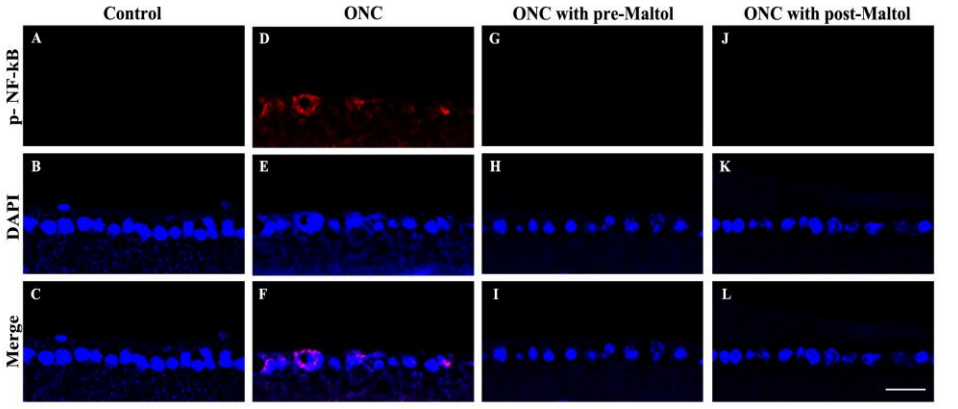

Figure 8. Change of phosphorylated NF-κB (p-NF-κB) immunoreactivity in retinal ganglion cells (RGC) of mice at 3 days after optic nerve crush (ONC) without or with pre- or post-treatment of intraperitoneal (IP) maltol injection (100 mg/kg) for 5 consecutive days. (A-C) Normal control retinas. (D-F) Retinas of ONC mice. (G-I) ONC mice with pre-treatment of maltol. (J-L) ONC mice with post-treatment of maltol. (A, D, G, J) RGCs were labeled with p-NF-κB (red fluorescence). (B, E, H, K) with DAPI (blue). (C, F, I, L) Merge. p-NF-κB immunoreactivity, not present in normal control retina, is activated in RGCs of ONC mice. However, p-NF-κB immunoreactivity is decreased with pre- or post- treatment of maltol. Scale bar = 20 μm.

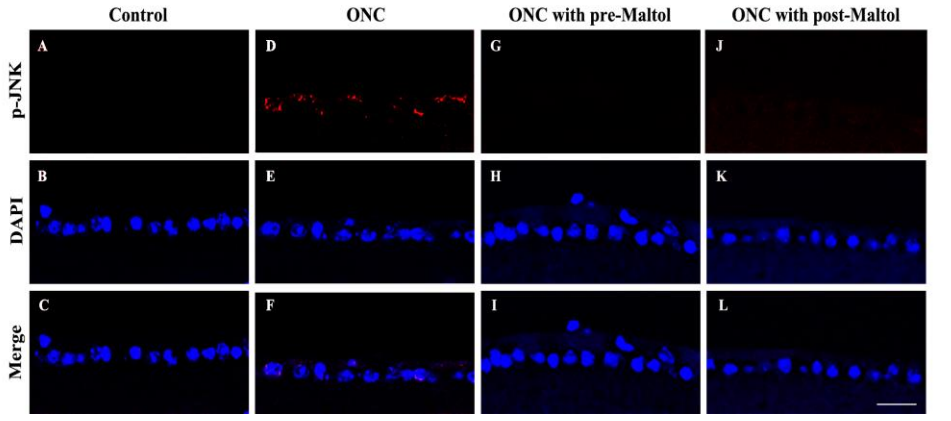

Figure 9. Change of phosphorylated JNK (p-JNK) immunoreactivity in retinal ganglion cells (RGC) of mice at 3 days after optic nerve crush (ONC) without or with pre- or post-treatment of intraperitoneal (IP) maltol injection (100 mg/kg) for 5 consecutive days. (A-C) Normal control retinas. (D-F) Retinas of ONC mice. (G-I) ONC mice with pre-treatment of maltol. (J-L) ONC mice with post-treatment of maltol. (A, D, G, J) RGCs were labeled with p- JNK (red fluorescence). (B, E, H, K) with DAPI (blue). (C, F, I, L) Merge. p-JNK immunoreactivity, not present in normal control retina, is activated in RGCs of ONC mice. However, p-JNK immunoreactivity is decreased with pre- or post-treatment of maltol. Scale bar = 20 μm.

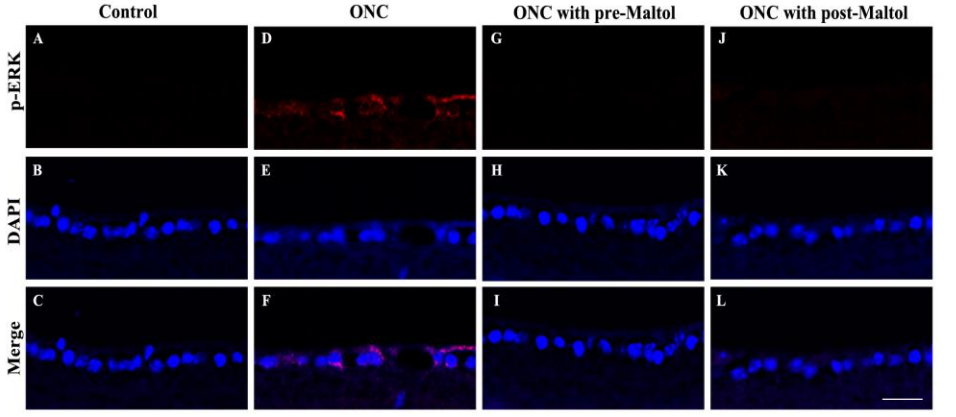

Figure 10. Change of phosphorylated ERK (p-ERK) immunoreactivity in retinal ganglion cells (RGC) of mice at 3 days after optic nerve crush (ONC) without or with pre- or post-treatment of intraperitoneal (IP) maltol injection (100 mg/kg) for 5 consecutive days. (A-C) Normal control retinas. (D-F) Retinas of ONC mice. (G-I) ONC mice with pre-treatment of maltol. (J-L) ONC mice with post-treatment of maltol. (A, D, G, J) RGCs were labeled with p- ERK (red fluorescence). (B, E, H, K) with DAPI (blue). (C, F, I, L) Merge. p-ERK immunoreactivity, not present in normal control retina, is activated in RGCs of ONC mice. However, p ERK immunoreactivity is decreased with pre- or post-treatment of maltol. Scale bar = 20 μm.

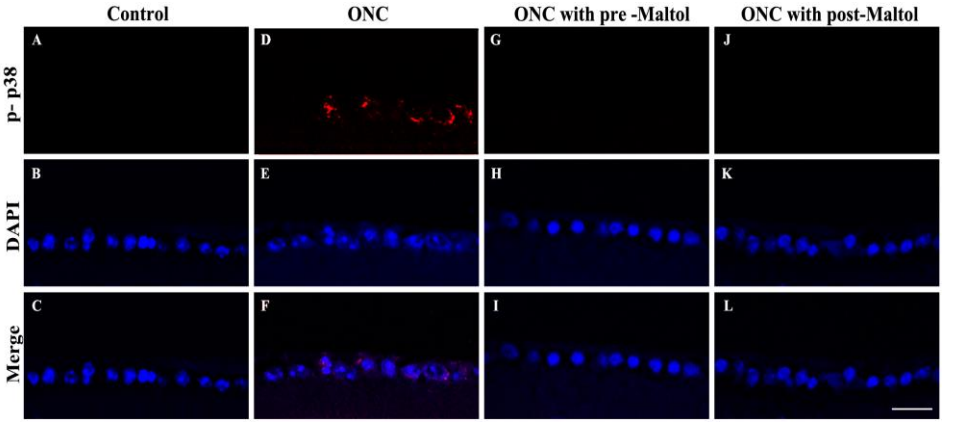

Figure 11. Change of phosphorylated p38 (p-p38) immunoreactivity in retinal ganglion cells (RGC) of mice at 3 days after optic nerve crush (ONC) without or with pre- or post-treatment of intraperitoneal (IP) maltol injection (100 mg/kg) for 5 consecutive days. (A-C) Normal control retinas. (D-F) Retinas of ONC mice. (G-I) ONC mice with pre-treatment of maltol. (J-L) ONC mice with post-treatment of maltol. (A, D, G, J) RGCs were labeled with p- p38 (red fluorescence). (B, E, H, K) with DAPI (blue). (C, F, I, L) Merge. p-p38 immunoreactivity, not present in normal control retina, is activated in RGCs of ONC mice. However, p-p38 immunoreactivity is decreased with pre- or post-treatment of maltol. Scale bar = 20 μm.

IV. DISCUSSION

Maltol has been reported to have various biologic functions such as being a natural scavenger of ROS,[7] and as a mediator of enhanced mitochondrial function, reduced intracellular calcium, and reduced expression of NF-κB.[8] However, its effects and underlying mechanisms of action on experimental glaucoma models have not been previously reported. To our knowledge, this is the first report that represents a mechanism by which maltol has the neuroprotective effect on oxidative stressed R28 cells and RGCs of ONC mice.

In the present study, maltol not only increased cell viability but also attenuated DNA fragmentation both in oxidative stressed R28 cells and in RGCs of ONC mice. In R28 cells, LDH leakage markedly increased with the addition of 1.0 mM H_2O_2 (59.25 ± 2.81%). However, 1.0 mM maltol treatment significantly attenuated these cytotoxic effects (19.86 ± 1.11%). Also, Maltol treatment markedly reduced apoptosis of R28 cells. The ratio of TUNEL positive cells to the total number of cells increased with the addition of 1.0 mM H_2O_2 (16.33 ± 2.31%). However, the ratio decreased with co-culture of 1.0 mM maltol (2.64 ± 0.52 %). This finding is consistent with the results of our *in vivo* study. Although ONC induced progressive cell loss, there was no significant cell loss in GCL of ONC mice with pre-treatment of maltol. At 7 days post crush, RGCs of crushed eyes with pre-treatment of maltol were more resistant against crush-injury than those of crushed eyes without maltol (80% and 68%,

respectively). On the other hand, we did not find significant protective effect of post-treatement of maltol against cell loss induced by ONC. Although post-treatment of maltol also showed anti-apoptotic effect on RGCs in TUNEL method, there was no significant difference between ONC mice with post-treatment of maltol and ONC mice without matlol (72% and 68%, respectively). To elucidate the underlying mechanism of the neuroprotective effect of maltol, we evaluated NF-kB and MAPK signaling pathways.

NF-κB is an important transcription factor that regulates the stimulus-dependent induction of genes critical to the survival and death of neurons.[15,16] Various stimuli such as stress and cytokines can activate NF- κB, and then the activated form of p-NF-κB enters the nucleus and initiates the expression of genes to determine cell survival or death.[17,18] Wu et al. reported that in light-induced photoreceptor degeneration, NF-κB was activated and exhibited neuroprotective and anti-apoptotic functions.[19] However, Grilli et al. reported that in glutamate-induced neurotoxicity, blockade of NF-κB activation was found to be protective.[20] Therefore, NF-κB activation may induce both anti- and proapoptotic effects on different cells or under different pathological conditions.[21] Previously, the activated NF-κB has been reported to participate in glutamate-induced neurotoxicity, N-methyl-D-aspartate (NMDA)-induced retinal neuronal cell death, retinal ischemia, and reperfusion injury.[20-23] In this study, p-NF-κB was increased in oxidative stressed R28 cells by H_2O_2, but was decreased when co-cultured with maltol. This finding is consistent with the

results from previous studies. Yang et al. reported that maltol could reduce NF-κB activated by H_2O_2 and prevent H_2O_2-induced apoptosis in human neuroblastoma cells (SH-SY5Y).[8] Also, in our *in vivo* study, the immunoreactivity of p-NF-κB, not present in normal control retina, was activated in RGCs of the crushed eyes at 3 days after ONC. This result shows that the neuroprotective effect of maltol is mediated by reduction of activated NF-κB.

The MAPK family consists of three main protein kinase families: the ERKs, JNKs, and p38 family of kinases. MAPK pathways have crucial roles in the regulation of cellular activities including proliferation, differentiation, survival, death, and cellular responses to external stresses. Activation of ERKs typically contributes to cell differentiation, proliferation, and survival.[24] On the other hand, JNK and p38 are activated by pro-inflammatory cytokines and environmental stress, and have been suggested to promote apoptosis.[25]

The present study showed that there were significant changes of p-ERK in oxidative stressed R28 cells and RGCs of ONC mice with or without maltol treatment. Previous studies reported that ERK activation is a major factor related to cell survival,[26] and the activations of ERK in the retina rescue the RGCs from apoptosis after an optic nerve injury.[27] However, in the present study, activated p-ERK decreased with maltol treatment in oxidative stressed R28 cells and RGCs of ONC mice. The present findings are in apparent contrast to the above-mentioned studies. However, Luo et al. demonstrated that

inhibition of the ERK pathway promotes RGC survival and axon regeneration, probably via preventing excitotoxic amino acid release or blockade of nonapoptotic processes after optic nerve injury.[27] Additionally, Cagnol et al.[28] reported that prolonged activation of the ERK pathway induces activation of pro-apoptotic caspase-8, and Stanciu et al.[29] reported that ERK activation is involved in glutamate and NMDA receptor-mediated neurotoxicity. The current study does not provide enough evidence to clarify the controversial role of the ERK pathway, because ERK activation could be interpreted as having both anti- and pro-apoptotic effects. Maltol may have a neuroprotective effect through down-regulation of p-ERK as a pro-apoptotic signal, or maltol may sufficiently reduce the oxidative stress on RGCs such that ERK cannot be activated as an anti-apoptotic signal. Although the role of p-ERK has not been clarified, the neuroprotective mechanism of maltol has been found to be associated with the ERK pathway in oxidative stressed R28 cells and RGCs of ONC mice.

In this study, p-JNK was increased in oxidative stressed R28 cells and decreased with maltol addition. Previous studies demonstrated that oxidative stress-induced apoptosis was commonly mediated by the activation of JNK pathways.[30] The present study also showed that H_2O_2 induced activation of JNK and was suppressed by maltol addition. In addtion, the immunoreactivity of p-JNK was increased in RGCs of ONC mice and decrease with maltol treatment. JNK signaling is known to occur in RGCs after many glaucoma relevant injuries, and activated JNK is present in RGCs of human glaucoma patients.[31]

Previous studies using JNK inhibitors have shown that JNK signaling does play a role in RGC death after injury.[32,33] The present study also showed that maltol may block the activation of JNK and rescue RGCs in ONC injury mice. These results suggest that maltol may suppress the pro-apoptotic effect mediated by down-regulation of p-JNK.

In current study, H_2O_2 did not induce a significant change of p-p38 in R28 cells. Accordingly, we cannot evaluate effect of maltol on p-p38 expression in oxidative stressed R28 cells. Although p38 and JNK are often co-activated, there is a specific stimulus to induce an activation of p38 or JNK independently. For example, Ogura M. and Kitamura M. demonstrated that treatment of macrophages with menadione rapidly induced phosphorylation of ERK and p38 MAP kinase, but not JNK.[34] The present study also showed that in oxidative stressed R28 cells, the p38 pathway was less influenced than other MAPK pathways.

On the other hand, in our *in vivo* study, the immunoreactivity of p-p38 in RGCs was activated by crush injury of optic nerve. These different results of *in vitro* and *in vivo* study seem to be induced from the different cell types. R28 retinal precursor cells were used as *in vitro* model. R28 cells have both glial and neuronal characteristics, and have been widely used to elucidate the molecular mechanism of neuronal apoptosis in the retina.[35] However, the RGCs comprise only a very small fraction of retinal cells (one per ten thousand) and R28 cells do not behave like primary RGCs in every aspect. This is a potential limitation

of the present study. However, isolating and culturing primary RGCs are technically difficult and widely used RGC-5 cells, once thought to be a rat retinal ganglion cell line, [36] are now reported to express photoreceptor markers that are also expressed in the unrelated SV40-transformed mouse photoreceptor cell line 661W.[37,38] Use of this cross-contaminated and uncharacterized cell line could result in erroneous scientific conclusions. Therefore, the goal of this study was to use the R28 cell line to determine the neuroprotective effect of maltol with the expectation that the *in vitro* data set obtained from R28 cells can be verified in primary RGCs in a future study. Further, an in vitro cell line that is easy to handle and better represents RGC characteristics should be developed.

Although our study showed that maltol has a neuroprotective effect, maltol has been reported to have weak mutagenic activity and toxic effects mediated through apoptosis.[39] However, at the recommended concentration, maltol does not have any toxic effect. The UN Joint FAO/WHO Export Committee on Food Additives concluded that up to 1 mg/kg per day was an acceptable level of consumption for human beings.[3] For dogs and rats, maltol showed no adverse toxic effect at doses up to and including 200 mg/kg/day for 2 years.[40] Also, Kim et al. reported that maltol had a neuroprotective effect against oxidative stress in the brains of mice challenged with kainic acid at a dose of 100 mg/kg for 5 consecutive days.[7] Previous *in vitro* studies showed that concentrations ranging from 50 to 1000 μM did not significantly affect cell viability, but a concentration of 10 mM decreased cell viability to 75%.[2] Based on these

findings, our *in vitro* studies were conducted at concentrations of maltol ranging from 0.1 to 1.5 mM, and in *in vivo* studies, mice were treated with maltol at a dose of 100 mg/kg/day for 5 consecutive days.

V. CONCLUSION

In the present study, maltol not only increased cell viability but also attenuated DNA fragmentation in oxidative stressed R28 cells and RGCs of ONC mice. The results obtained here show that maltol has neuroprotective effects and its effects may act through the NFκB and MAPK signaling pathways. This study provides evidence that maltol could be an innovative neuroprotective therapeutic agent for glaucoma.

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ABSTRACT(IN KOREAN)

녹내장 실험모델에 대한 말톨의 신경보호 효과

<지도교수 성 공 제>

연세대학교 대학원 의학과

송 유 경

목적: 말톨(maltol; 3-hydroxy-2-methyl-4-pyrone)은 활성 산소 등의 유리기(free radical)를 제거할 수 있는 항산화작용을 가지고 있는 것으로 알려진 천연물질로서, 본 연구에서는 산화스트레스에 의한 신경감각전구세포의 손상에 대한 말톨의 신경보호 효과를 평가하고 이를 녹내장 동물 실험모델을 통하여 확인하고자 하였다.

방법: R28세포(rat embryonic precursor neurosensory retinal cells)를 배양하면서 세포배양액에 과산화수소(0.0 to 1.5 mM)를 첨가하여 산화스트레스를 유도하고 말톨(0.0 to 1.5 mM)을 함께 투여하였을 때 산화스트레스로 인한 R28세포의 손상에 미치는 영향을 LDH 분석과 TUNEL 방법을 이용하여 평가하고, 그 작용기전을 면역형광염색법과 Western blot 분석 등을 이용하여 살펴보았으며, 생쥐의 시신경 손상을 유도한 optic nerve crush model (ONC model) 을 대상으로 말톨의 전처치 또는 후처치가

망막신경절세포에 미치는 영향을 면역형광염색법 및 TUNEL 방법을 이용하여 평가하였다.

결과: LDH 분석을 이용하여 세포독성을 평가하였을 때, 첨가한 과산화수소의 농도에 비례하여 R28세포의 세포사멸이 증가하였으며, 이는 말톨 투여에 의해 현저히 감소하였다. 생쥐의 ONC model에서도 말톨의 복강내 주사(100 mg/kg intraperitoneal injection)는 망막신경절세포의 세포고사를 유의하게 감소시켰으며, 이러한 말톨의 신경보호효과는 nuclear factor-kappa B (NF-kappaB) 및 mitogen-activated protein kinase (MAPK) 등의 신호전달기전과 연관된 것으로 판단된다.

결론: 본 연구를 통하여 말톨이 산화스트레스에 의한 신경감각전구세포의 손상 및 ONC model에서의 망막신경절세포의 손상을 줄이는 신경보호효과가 있음을 발견하였으며, 이는 말톨이 녹내장을 비롯하여 산화스트레스와 관련된 신경질환의 새로운 신경보호치료제로 개발될 수 있는 가능성을 시사한다.

핵심되는 말 : 말톨, 신경보호, 산화스트레스. 신경감각전구세포, 시신경 축삭 모델