Protective effect of idebenone on ethambutol-induced optic neuropathy in R28 cells and mice

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Above all things, I wish to thank my loving wife, Soobin Lee, and my daughter, Damin Han, the foundation of my life.

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<ABSTRACT>

Protective effect of idebenone on ethambutol-induced optic neuropathy in R28 cells and mice

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Ethambutol is an anti-tuberculosis medication that induces retinal ganglion cell injury in the visual system. Oxidative stress and mitochondrial dysfunction are widely accepted as possible pathogenic mechanisms of ethambutol-induced optic neuropathy. Idebenone, a Coenzyme Q-10 derivate, is a potent free radical scavenger with demonstrated neuroprotective effect in experimental models of Leber's hereditary optic neuropathy, which shares common clinical features with ethambutol-induced optic neuropathy. The present study was designed to investigate whether idebenone could provide protective effect against ethambutol-induced optic neuropathy in R28 cell line and mice. The cultured R28 cell lines were pretreated with different dose of idebenone for 24h, followed by the challenge with 3mM ethambutol for 24h. Exposure of ethambutol caused the loss of cell viability and the ATP content with dose-dependent manner in R28 cell. Idebenone pretreatment had protective effect on cell survival and ATP contents in ethambutol induced neuronal death of R28 cells. However, we demonstrated that ethambutol exposure did not significantly elevate intracellular reactive oxygen species generation and mitochondria-derived superoxide production, and it had no effect on mitochondrial membrane potential. However, zinc accumulation in lysosome and microtubule associated protein 1 light chain 3 were increased with

ethambutol treatment, which were efficiently attenuated by idebenone pretreatment. In mouse model, 200mg/kg ethambutol was injected intraperitoneally alternate day for 6 weeks and 200mg/kg idebenone was introduced with oral gavage. Idebenone had a slight protective effect of visual function in mouse model, which was tested by optomotor test. However, it failed to demonstrate a beneficial effect on ganglion cell structure by optical coherence tomography, and photopic negative response was not differ among groups. In conclusion, these results suggest that idebenone has a mild protective effect against ethambutol-induced neurotoxicity, and these beneficial effects of idebenone may be attributable to its roles on lysosomal zinc accumulation, not directly in preventing reactive oxygen species.

Key words: ethambutol-induced optic neuropathy, idebenone, neuroprotection

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I. INTRODUCTION

Ethambutol (EMB) is one of the first-line drugs used in the treatment of tuberculosis and non-tuberculous mycobacterial disease. EMB inhibits arabinosyl transferase essential for the biosynthesis of the arabinogalactan wall of *Mycobacterium tuberculosis*.[1] The mode of action was reported to chelate metals such as iron and copper, and it also inhibit RNA synthesis in mycobacterium.[2,3]

Mitochondria produces most of energy required to cellular metabolism through oxidative phosphorylation. Mitochondria has its own independent genome and RNA synthesis machinery that show substantial similarity to bacterial ones. Due to such a similarity, anti-microbacterial drugs involved in bacterial RNA synthesis inhibition also interfere eukaryotic ribosomal RNA function in mitochondria. Therefore, drugs such as EMB, chloramphenicol, linezolid and erythromycin not only inhibit 70s ribosomal RNA functions, but also disrupt mitochondrial protein synthesis.[4,5] Therefore, long-term use of these drugs results in defective ATP production in human mitochondria. Moreover, EMB also disrupt oxidative phosphorylation and mitochondrial function by iron-containing complex I and copper-containing complex IV via metal-chelating effect.[6] EMB toxicity is known as dose- and time-dependent. The World Head Organization estimates that there are about 9.2 million new

cases of tuberculosis each year. About half of these patients take EMB for treatment of *Mycobacterium tuberculosis* or *Mycobacterium avium*. Lee et al reported that 1.5% of patients who are taking EMB experienced optic neuropathy attributable to EMB,[7] so the estimated annual incidence of EMB-induced optic neuropathy is 75,000 cases per year worldwide.

Although uncommon at standard doses, EMB-induced optic neuropathy is the most serious side effect. The clinical presentation is characterized by slowly progressive bilateral central visual loss, dyschromatopsia, central or cecocentral scotoma. Patients frequently reported their symptom as cloudy vision or a central haze. Clinical features such as central visual field loss, dyschromatopsia and temporal pallor of optic nerve can be explained by selective damage to parvocellular retinal ganglion cell, which is similar with other mitochondrial optic neuropathy such as Leber hereditary optic neuropathy (LHON) or dominant optic atrophy (DOA). Several cases were reported that EMB may be a triggering risk factor for the occurrence of LHON and DOA.[8,9] EMB-induced optic neuropathy may share common pathogenic mechanisms with other mitochondrial optic neuropathies. It has been reported that parvocellular retinal ganglion cell is more susceptible than magnocellular retinal ganglion cell due to high energy demand and their small caliber axon.[10] Unfortunately, no proven treatment is available other than stopping the drug. Although one thirds of patients recover visual acuity and visual field defect after stopping EMB, the remainder of patients suffered from irreversible visual loss.[7]

The exact mechanism of EMB-induced optic neuropathy is yet to be elucidated. A histopathology study showed that axonal swelling in optic chiasm was observed in albino rats model.[11] EMB induces a decrease in copper levels in the monkeys and rats,[12] and copper deficiency produces mitochondrial insufficiency by inhibiting cytochrome-c oxidase activity.[13] Previous study suggested that EMB are toxic to retinal ganglion cells via stimulation of

glutamate through N-methyl-D-aspartate (NMDA) receptor.[14] EMB results in increases of mitochondrial calcium and mitochondrial membrane potential, which perturbs mitochondrial function. However, Yoon et al demonstrated that glutamate antagonist, antioxidant or cycloheximide did not attenuate EMB neuro-toxicity, contrary to the previous study.[15] They concluded that EMB-induced cytotoxicity in rat retinal cell culture was not likely mediated by excitotoxicity, oxidative stress, or apoptosis mechanism, and chelation of intracellular zinc completely abrogated the cytotoxic effects of EMB. Their subsequent study showed that zinc and lysosomal membrane permeabilization play significant roles in EMB-induced retinal cell death.[16] Tsai et al reported that EMB may exert toxic effects in retinal pigment epithelium via protein kinase C (PKC) pathway, and inhibitor of PKCδ prevent the EMB-induced phenotypic changes.[17] EMB treatment might impair the fusion of autophagosomes and lysosomes, and thus inhibit autophagic flux.[18]

Co-enzyme Q10 is a lipophilic molecular which shuttles electrons from complex I and II and other flavoprotein dehydgenases to complex III of the mitochondrial respiratory chain. It also functions as a lipid-soluble antioxidant, scavenges reactive oxygen species (ROS), and is involved in multiple aspects of cellular metabolism.[19] Unfortunately, bioavailability of Coenzyme Q10 is relatively poor due to its hydrophobic nature.[20] Idebenone (2,3-dimethoxy-5-methyl-6-(10-hydroxydecyl)-1,4-benzoquinone) is a structural analog of co-enzyme Q10 with a benzoquinone nucleus and a hydroxydecyl side chain.[21] Due to its small size and increased water solubility, idebenone has greater bioavailability than co-enzyme Q10 with similar antioxidant properties.[22] There are encouraging evidences supporting beneficial effects of idebenone in LHON and DOA.[23-26] Idebenone inhibits glutamate release by suppression of voltage-dependent calcium channel, stabilizes mitochondrial membrane potential, and has a cytoprotection in glutathione-depleted cell cultures. These findings provide a strong rationale for

investigating protective role of idebenone on EMB-induced optic neuropathy by scavenging ROS and stabilizing mitochondrial membrane potential.

Here we describe the pharmacological effect of idebenone in a retinal precursor cell line and mouse model of EMB-induced optic neuropathy and demonstrated the protective effect of idebenone on the ganglion cell function and visual performance.

II. MATERIALS AND METHODS

1. In vitro experiments

A. Reagents

Silibinin, GF 109203X, rottlerin, catalase, MTT, propidium iodide was purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA). DCFH-DA and DiOC6(3) were obtained from Molecular Probes (Eugene, OR, USA). Antibodies were obtained from Cell Signaling Technology Inc. (Beverly, MA, USA). Media and cell culture supplements were purchased from Invitrogen (Carlsbad, CA, USA). Tissue culture plastic ware was from Falcon (BD Biosciences, Bedford, MA, USA). Idebenone was generously donated from Santhera Pharmaceuticals (Liestal, Swizterland). Ethambutol, metabolic substrates, and miscellaneous chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

B. R28 cell line

The R28 cells were purchased from Kerafast (Kerafast Inc., Boston, MA, USA). Cultures representing passages 50 to 60 were used for experiments. The R28 cell line originated from a mixed population of retinal cells (designated E1A-NR.3) immortalized with Psi2 to 12S- EIA replication-defective retroviral vector. The R28 clonal population was obtained by three rounds of cloning by limiting dilution while selecting for clones with neuronal morphologies. It has been shown that R28 cells grown with laminin and cAMP have a neuronlike

phenotype. All cells were passaged and maintained in low glucose formulation DMEM culture medium (Glutamax; Invitrogen, Waltham, MA, USA) and 1 mM pyruvate, plus 10% fetal bovine serum (FBS), penicillin, and streptomycin. The final glucose concentration in the 10% FBS-supplemented medium was approximately 5.3 mM. For all experiments, the R28 cells were plated onto tissue culture plastic coated with 0.01% Poly-L-Ornithin(Sigma Aldrich, St. Louis, MO, USA) and 1.0 g/cm2 laminin for approximately 24 hours before treatment.

C. Cell viability assays

Cell viability was measured using the Cell Counting Kit-8 (CCK-8) according to the manufacturer's instruction (Dojindo Molecular Technologies, Kumamoto, Japan). In brief, after the indicated treatment, R28 cells cultured in each well of 96-well plates were incubated with 10ul of CCK-8 solution for 3h at 37℃. The absorbance was detected with a microplate reader (Tecan, Mannedorf, Switzerland) at 450 nm.

D. Determination of ATP contents

ATP contents were detected using CellTiter-Glo® Luminescent cell viability assay kit (Promega, Madison, WI, USA) according to the manufacturer's instruments. Briefly, R28 cells were cultured in 100ul 10 % FBS-DMEM in opaque white 96-well plate for 24 hours. After the indicated treatment, an aliquot of 100 ul reaction buffer containing 1mM dithiothreitol, 0.5mM luciferin and 12.5 ug/ml luciferase was directly added in a well and gently mixed. The luminescence was detected using luminometer.

E. Measurement of reactive oxygen species (ROS)

The intracellular generation of total ROS/superoxide was measured using Cellular ROS/superoxidedetection assay kit (Abcam, Cambridge, MA, USA) in

the living cells according to the manufacturer's instruments. In briefly, fluorescence microscopy; the cells were cultured and treated on Poly-D-Lysine/Laminin cellware 12 mm round coverslip (Corning, NY, USA). The media was changed to fresh just before ROS/superoxide experiment. They were loaded with the ROS/superoxide detection mix with the addition of the vehicle and ROS inducer (pyocyanin as positive control) for 60 minutes at 37℃. The detection mix was carefully removed and washed twice. The glass slip was immediately overlaid on a glass slide. The fluorescent products generated by the two dyes for ROS and superoxide were visualized using wide-field fluorescence equipment with standard green (Ex/Em = 490/525 nm) and orange (Ex/Em = 550/620 nm). Fluorescence microplate assay; the cells 1 $x10^4$/200ul/well in 96-well white/clear bottom plate were prepared for Fluorescence microplate assay. The cells were incubated in 100ul/well of ROS/superoxide detection solution for 60 minutes at 37℃. The intensity was measured using a fluorescence microplate-reader with standard green (Ex = 488 nm, Em = 520 nm) and rhodamine (Ex = 550 nm, Em = 610 nm). Flow cytometry; the cells were seeded and treated on 6-well plate (40 $x10^4$/well). After treatment, the cells were harvested with 0.125% trypsin for 10 minutes at 37℃, centrifuged at 400x g for 5 minutes, and then the supernatant was removed. After washing and decanting with PBS twice, the cells were resuspended in mix of fresh media and the detection reagent. The cell suspension was incubated under normal culture condition for 30 minutes at 37℃. Flow cytometry analysis was taken with the cell suspension without washing.

F. Mitochondrial superoxide detection

The mitochondrial superoxide level was measured using MitoSox Red (Invitrogen, Waltham, MA, USA), mitochondria targeted fluorescent probe for highly selective detection of mitochondrial superoxide. Briefly, after the indicated treatments R28 cells cultured in 96-well plates (1 x 10^4 cells per well)

were incubated with culture medium containing 5uM MitoSox Red for 10 min at 37℃ in dark followed by staining with DAPI for nucleus staining.

G. Measurement of caspase-3 activity

Caspase-3 was detected using CellEvent™ Caspase3/7 Green readyProbes® Reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Briefly, the dye reagent binds DNA, generating a bright green fluorescence upon activation of caspase-3/7 in apoptotic cells. One drop of the reagent/0.5 ml medium/well was added to the cultured cells on a glass slip in a 24-well plate and incubated for 30 minutes. Then after treated with 4 % parafolmaldehyde for 20 minutes, the cells were stained by DAPI which is cell nuclear dye.

H. Measurement of AIF nuclear translocation

Cells were cultured on Poly-D-Lysine/Laminin cellware 12 mm round coverslip (Corning, NY, USA). After washing with PBS, the cells were fixed with 4 % paraformaldehyde in PBS for 20 minutes at room temperature, and permeabilized with 0.5 % Triton-X 100 in 10 mM sodium citrate for 10 minutes. After washing twice with PBS, cells were blocked with 2 % BSA in PBS for 60 minutes. Cells were incubated with anti-rabbit polyclonal AIF antibody (10ug/ml, Abcam, Cambridge, MA, USA) overnight at 4℃. After washing twice with PBS, the cells were incubated with anti-rabbit AlexaFluor 488 (Life technologies, Waltham, MA, USA) conjugated antibody for 60 minutes room temperature. The cells were washed three times and then embedded on glass slide with UltraCruz™ mounting medium containing 1.5ug/ml or DAPI (Santa Cruz biotechnology, Dallas, TX, USA), staining nuclei. Images were acquired on a Zeiss LSM510 META confocal microscope at room temperature using Zeiss LSM510 v.3.2 software.

2. *In vivo* experiments

Male, 6- to 8-week-old, specific-pathogen-free C57BL/6 mice were purchased from Orient Bio Laboratories (Seongnam, Gyeongido, South Korea) and maintained in individually ventilated cages (Ebeco, Castrop-Rauxel, Germany) under biosafety level III conditions. EMB treatment began and was treated with intraperitoneal injection (200mg/kg) alternate day for 6 weeks until the mice were sacrificed. Mice in the experimental group received idebenone (200mg/kg) by oral gavage with intraperitoneal EMB. Control group was only received corn oil by oral gavage alternate day for 6 weeks.

A. Immunohistochemical staining on paraffin-embedded retina sections

Mouse eyes were harvested after acquisition of ERG and OCT, stained with standard hematoxylin and eosin, and processed for histopathologic examination. The retina tissue section slides were deparaffinized in two changes of Neo-Clear® (Xylene substitute, Merck KGaA, HX69828943) for 3 minutes each. The slides were transferred to the mix of Neo-Clear® and 100 % Ethanol (1:1) for 3 minutes. The slides were transferred to 100 % Ethanol, for two changes for 3 minutes each, and then placed once through 95 %, 70 %, and 50 % Ethanol respectively for 3 minutes each in order to dehydrate. The slides were rinsed with running tap water for 10 minutes. The slides were boiled in Tris-EDTA buffer (10mM Tris Base, 1mM EDTA solution, 0.05 % Tween 20, pH9.0) for 20 minutes and then rinsed with running tap water for 10 minutes again. After rinsing with 0.025 % Triton X-100 in the TBS (50 mM Tris-Cl, 150mM NaCl, pH7.6) for 5 minutes twice, the sample tissue slices were blocked in 10 % normal serum with 1 % BSA in TBS for 2 hours at room temperature. The primary antibody diluted in TBS with 1 % BSA was applied and kept in humidified chamber at 4℃ in dark overnight. The slides were rinsed twice with TBS-0.025 % Trito X-100, applied the fluorescein second antibody diluted in TBS with 1 % BSA, and incubated for one hour at room temperature

in a dark. Finally, the slides were embedded with UltraCruz™ mounting medium containing 1.5ug/ml or DAPI (Santa Cruz biotechnology, sc-24941), staining nuclei. Cells were immunostained with the following antibodies: anti-LC3-II (1:500) (Sigma-Aldrich, L7543). Twenty-four hours later, cells were incubated with AlexaFuor 488 anti-rabbit IgG (Life Technologies; 1:500) or Alexa 546 anti-mouse IgG (Life Technologies; 1:500) for 1h. Cells were embedded with UltraCruz™ mounting medium containing 1.5ug/ml or DAPI (Santa Cruz biotechnology, sc-24941), and images were acquired on a Zeiss LSM510 META confocal microscope at room temperature using Zeiss LSM510 v.3.2 software.

B. Optomotor testing

The optomotor response measured as previously described using an OptoMotry system (CerebralMechanics Inc., Canada).[27] The device consists of a testing chamber created with four screens facing into a square. Mice were placed on a platform in the center of the square. A virtual cylinder comprised of a vertical sine wave grating was projected in 3D coordinate space and rotated around mice. A video camera, located above the mice, provided real time behavioral response on another screen. Mice were allowed to move freely on the platform, and the spatial frequency of the grating was maintained at the mice's viewing position be recentering the cylinder on the mice's head. The cylinder was rotated at a constant speed (12 degree/s).

The experimenter judged whether the mice made tracking motions with reflexive head and neck movement following the stimulus. Mice were assessed for tracking behavior for 5 seconds, and then a gray stimulus appeared, to reduce the possibility of adapting to the stimulus. Spatial frequency thresholds were calculated by systemically increasing the spatial frequency of the grating at 100% contrast until the animals no longer responded. This threshold was considered the maximum visual acuity.

C. Electroretinogram

Mice were adapted to darkness overnight and prepared for recording under a dim red light. Animals were anesthetized with an intraperitoneal injection of a mixture of ketamine (77 mg/kg) and xylazine (4.6 mg/kg), and maintained on a heating pad at stable temperature. Pupils were dilated by applying a topical drop of 0.5%% tropicamide and 0.5% phenylephrine (Santen Pharmaceuticals, Osaka, Japan). A topical drop of methylcellulose gel was instilled in each eye before situating the corneal electrode. Furthermore, a drop of 0.9% saline was applied occasionally to the cornea to prevent dehydration and to allow electrical contact with the recording electrode. Two 25-gauge platinum needles inserted under the scalp, behind the eyes, served as the reference electrodes, with a ground electrode located in the tail. Stimulus presentation and data acquisition were provided by the Phoenix Ganzfeld ERG (Phoenix Research Labs, Pleasanton, CA, USA).

D. Optical coherence tomography

Mice were anesthetized and their pupils dilated as described above, and the fundus was imaged using the Micron IV retinal imaging system (Phoenix Research Labs, Pleasanton, CA, USA). Retinal thickness was measured manually from each B-scan OCT image, approximately 200, 400, and 600 μm away from the both edge of the optic disc.

III. RESULTS

1. Idebenone protects R28 cell against ethambutol-induced neurotoxicity

Exposure of R28 cells for 24h to 1-8 mM EMB induced a substantial vacuolar change and neuroral loss (Figure 1). The effects of idebenone on cell viability of R28 cells were investigated by using the CCK-8 assay. There were no toxic effects of idebenone up to 5uM on the cell viability (data not shown). Then, R28

cells were exposed to ethambutol at range of concentration from 0.5mM to 8 mM for 24h to determine a working dose for subsequent cell death and cytoprotection assay. The dose response of ethambutol treatments on R28 cell ATP content and viability are shown in Fig.2. After incubating with ethambutol for 24h, cell viabilities were significantly decreased in 1-8mM treatment groups compared with control group. The less toxic concentration, 3mM of EMB was selected for experiments testing idebenone-mediated neuroprotection.

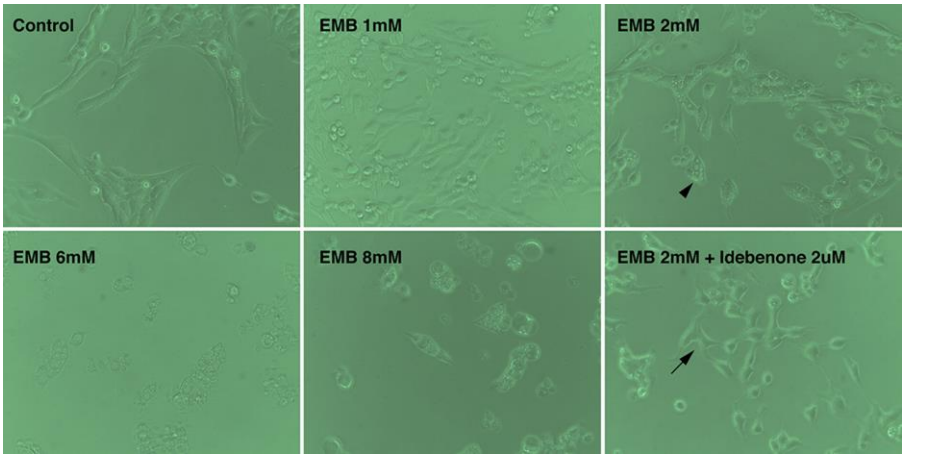

Figure 1. EMB-induced vacuole formation in cultured R28 cells. R28 cells were untreated (control) or treated with 1, 2, 6, 8 mM ethambutol for 24 hours. Vacuolar changes were observed as dose dependent manner with ethambutol treatment. At the 6 mM and 8 mM of ethambutol, cell death with significant vacuolar changes were evident. Idebenone 2uM was pretreated for 1 day before ethambutol 2mM treatment. The vacuolar formation was attenuated with idebenone pre-treatment.

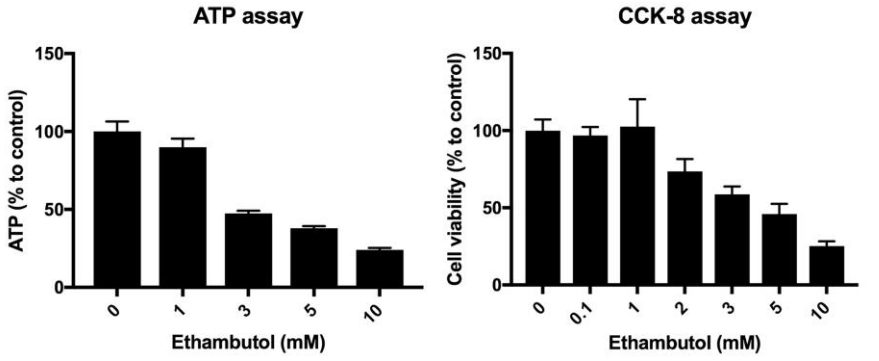

Figure 2. EMB suppress ATP synthesis and cell viability of R28 cells. R28 cells were untreated and treated with 0.5-10.0 mM EMB for 24h. (A) ATP synthesis was suppressed with dose-dependent of EMB. (B) Cell viability were measured with cell counting kit-8 assay. Cell viability of R28 cells were proportionally decreased with EMB concentration.

To investigate whether idebenone could protect R28 cells from injury induced by ethambutol insult, cultured R28 cells were pretreated with idebenone at different concentration (10nM, 100nM, 500nM, 1uM, and 2uM) for 16h before ethambutol addition. The cell viability of R28 cells was measured 24h after ethambutol insult. It was found that idebenone inhibited the decrease of cell viability induced by ethambutol insult up to 2 μM concentration (Figure 3). At the 8μM of idebenone, a detrimental effect of cell viability was found.

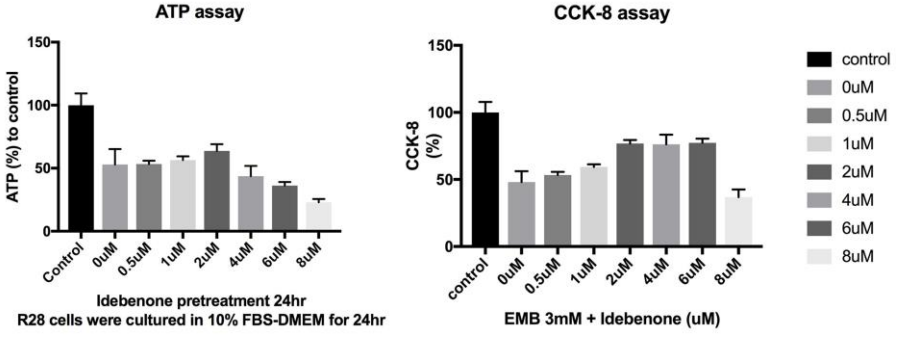

Figure 3. Idebenone protects on survival of R28 cell in vitro. (A) Idebenone attenuated ethambutol-induced reduction in ATP content. The whole cell ATP content was measured using an ATP Determination Kit. R28 cells were incubated with vehicle (DMEM containing 0.1% DMSO) or idebenone at 1, 0.5, 1, 2, 4, 6, and 8uM for 24h. Treatment with ethambutol caused 50% reduction in ATP content in R-28 cells. Idebenone pre-treatment for 1 days rescued ATP content at 2 uM concentration. (B) Dose-dependent protective effect of idebenone on ethambutol-induced loss of cell viability. Idebenone was applied to R28 cells at 0.5, 1, 2, 4, 6, and 8uM. After incubation for 24h. the cultures were then challenged with 3mM ethambutol for 12h in the continued presence of idebenone. The cell viability of cultured R28 cells was determined by the CCK-8 assay. Results were presented as a percentage of the control cell survival (set to 100%). Cell viability was increased with dose-dependent manner with idebenone treatment, but toxic effect of idebenone was observed at 8uM.

2. Ethambutol did not induce oxidative stress in R28 cells

To determine whether idebenone attenuates ethambutol-induced neurotoxcity in R28 cells by suppressing oxidative stress or not, the intracellular ROS level was assessed by DCF fluorescence assay. Idebenone pretreatment did not prevent the ethambutol-induced intracellular ROS and superoxide accumulation ($P>0.05$, Figure 4).

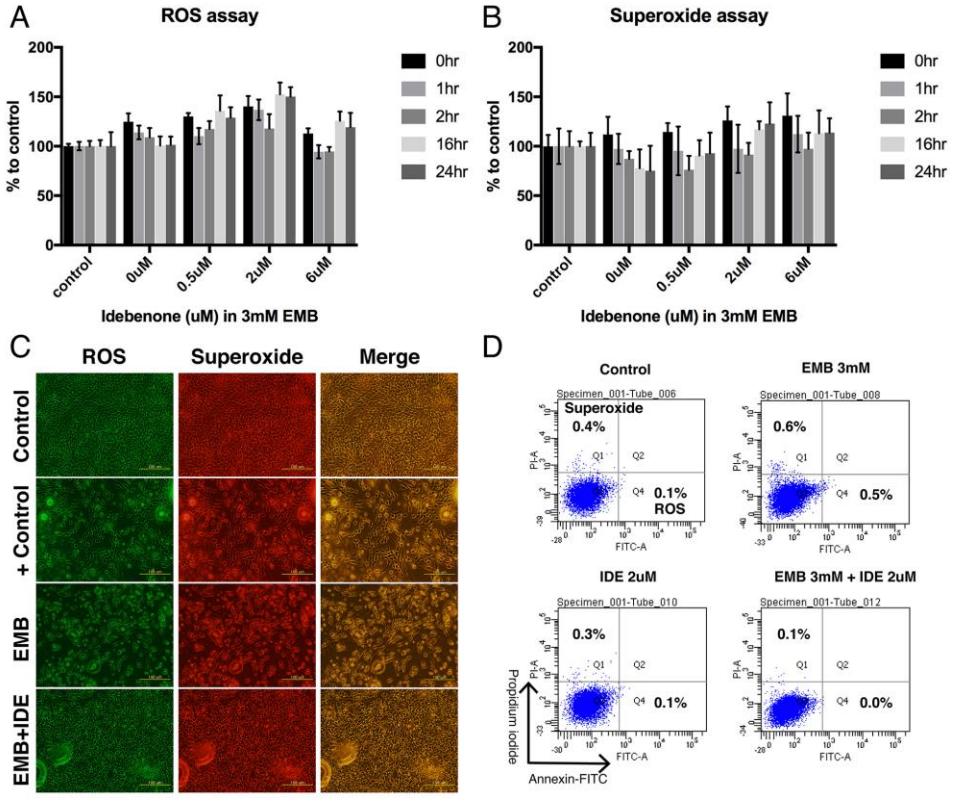

Figure 4. Analysis of ROS levels in EMB-treated and idebenone co-treated R28 cells. (A, B) ROS and superoxide assay at different time point after idebenone treatment were measured. There was no reduction of ROS and superoxide after idebenone treated R28 cells. (C) Fluorescence microscopy ROS assay for detection ROS (green) and superoxide (red). Positive control exhibited green and red fluorescence, but R28 cells treated with EMB did not induce fluorescence. (D) Annexin FITC/PI double-staining assay of R28 cells. Flow cytometric analysis of EMB-induced ROS and superoxide generation. EMB did not induce high levels of ROS and superoxide generation. The results are expressed as the means. Reactive oxygen species are Annexin +/PI – cells and superoxide are Annexin -/PI + cells.

Mitochondria are the major source of superoxide which is the main component

of oxidative stress. To investigate the contribution of mitochondria to EMB-induced ROS generation, mitochondria-derived superoxide generation was measured using MitoSOX Red. As shown in Figure 5, treatment of R28 cells with 2mM EMB for 24h did not increased MitoSOX Red fluorescence significantly. There was no difference on MitoSOX red immunoreactivity in idebenone co-treated R28 cells.

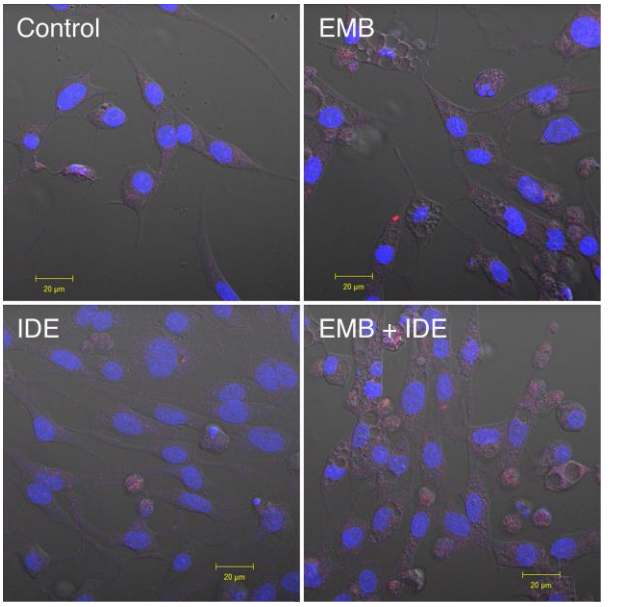

Figure 5. Idebenone did not prevents the ethambutol-induced mitochondrial oxidative stress. The intracellular mitochondrial ROS levels were measured by the DCF fluorescence method. The mitochondrial superoxide level was measured using MitoSox Red. Representative confocal images of R28 cells stained with MitoSox Red and DAPI staining.

3. Ethambutol did not activate caspase-dependent apoptosis nor apoptosis inducing factor translocation into nucleus

We examined whether EMB activates caspase-dependent apoptosis or non-caspase dependent pathway via AIF translocation. As shown in Figure 6,

caspase-3 immunostaining was observed only in death cells, most of R28 cells with vacuolar changes did not stained with caspase-3. Therefore, it is assumed that EMB did not initially activate caspase-cascade, and it could be late effect because only lysis cells were stained with capase-3. The translocation of AIF into nucleus was not observed (Figure 7). The redistribution of AIF into nucleus was not shown, and it means that EMB-induced neurotoxicity is not associated with caspase-independent AIF pathway.

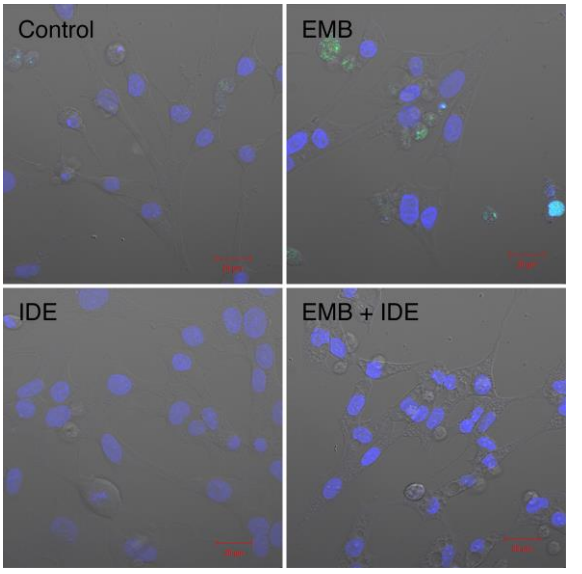

Figure 6. Confocal microscopic image with caspase-3 FITC staining. The cells were stained with Caspase-3 FITC for 30 min at 37 and then treated with 4% paraformaldehyde. EMB (3mM) did not activate caspase-3 in vacuolated cells. Caspase-3 staining was only seen in death cells with lysis.

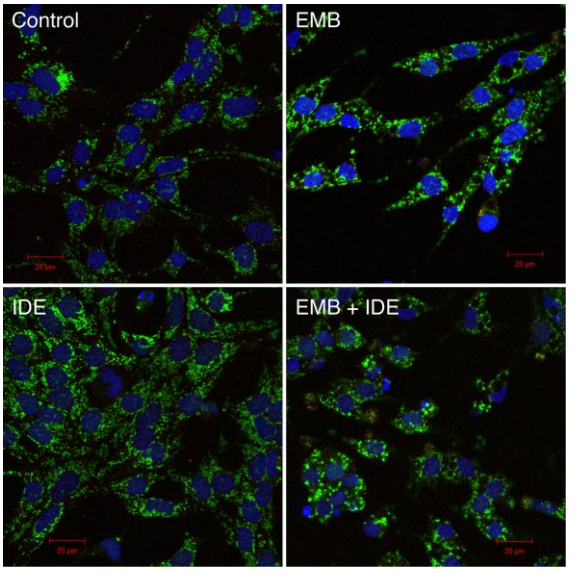

Figure 7. EMB did not induce cell death via apoptosis inducing factor redistribution into the nuclei. The experiments were repeated three times with similar results.

4. Ethambutol activates autophagic flux and loss of acidification in lysosome by zinc accumulation

The addition of idebenone to ethambutol-treated retinal precursor cells markedly suppressed induction or activation of microtubule-associated protein1 light chain 3 (LC3-II) and zinc accumulation (Figure 8). Idebenone reduced the level of lysosomal zinc, partially attenuated cell death induced by EMB. We examined whether significant levels of LC3-II and p62 were expressed in the murine retina and specifically in the RGC cells which are most affected in EMB-induced optic neuropathy. When untreated mouse retina sections were stained for LC3-II expression, no immunoreactivity was found in RGC cell layer and the photoreceptor layer. At the same time, high level immunoreactivity for LC3-II and p62 were observed in EMB-treated mouse, that is attenuated by idebenone co-treatment (Figure 9). The presence of strong LC3-II and p62 immunoreactivity in RGCs is agreement with the proposed

mode of action where EMB induces autophagosome accumulation and autophagy by inhibiting of lysosomal enzyme.

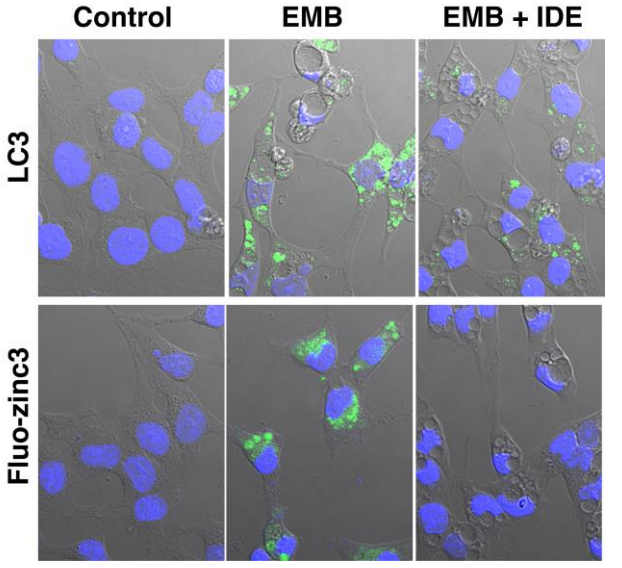

Figure 8. Idebenone attenuates zinc accumulation in lysosome. In EMB-treated R28 cells, LC3-II and fluo-zin 3 immunoreactivity were increased in vacuoles. Idebenone partially reduced LC3-II activation, and zinc accumulation in lysosome was significantly reduced in idebenone co-treated R28 cells.

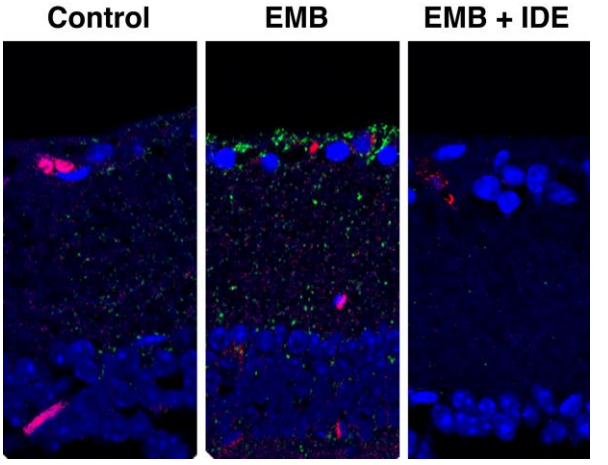

Figure 9. Staining of LC3-II and p62 in retinal section. LC3-II positivity of retinal ganglion cell in EMB-treated mice. LC3-II positivity was reduced in idebenone co-treated mice.

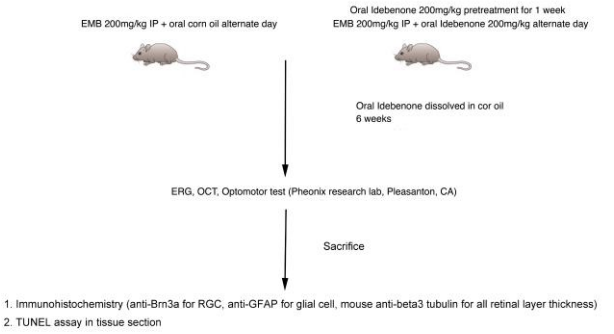

Figure 10. In vivo model of ethambutol-induced optic neuropathy. Scheme of the experimental design. C57BL/6 mice were pre-treated with idebenone or corn-oil for 1 week before ethambutol was injected intraperitoneally for 6 weeks alternate day. After 6 weeks, optomotor test, electroretinogram, and optical coherence tomography were measured before obtaining retinal section.

5. Idebenone protects against EMB-induces RGC death and retinal damage in vivo

Figure 10 showed experimental design of mouse model of EMB-induced optic neuropathy. Since RGC loss is associated with visual acuity impairment, we investigated the functional effect of idebenone pre-treatment on visual acuity in mice with intraperitoneal injection of EMB using the optomotor head-tracking test (Figure 11). The mean spatial frequency was 0.388 ± 0.02 cycle/degree in controls, 0.345 ± 0.03 cycle/degree in EMB group, 0.368 ± 0.03 cycle/degree in IDE group (P=0.004). In multiple comparison, there was statistical difference between control and EMB group (P=0.003). However, it was not statistically significant in functional vision between EMB group and EMB-IDE group (P=0.103).

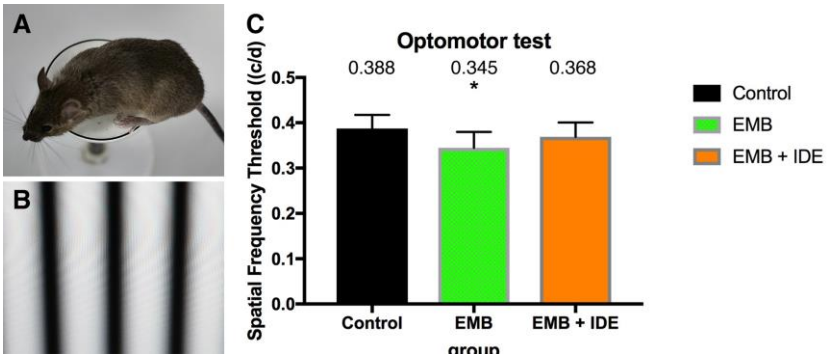

Figure 11. Idebenone restores EMB-induced loss of vision. Visual acuity was evaluated by counting the number of head movements with optokinetic 6 weeks after intraperitoneal injection of EMB (200mg/kg) alternate day. Idebenone group were treated with idebenone (200mg/kg) by oral gavage. n = 10 in each group. The optomotor response was significantly reduced in EMB group compared to controls (P = 0.003). The mean spatial frequency was 0.368 ± 0.033 in EMB-idebenone group and 0.345 ± 0.031 in EMB group, which is not statistically significant (P = 0.103).

As photopic negative response (PhNR) represent as retinal ganglion cells function, we specifically measured PhNR in each group. The mean PhNR amplitues were 52.6 ± 5.7 μV in controls, 32.1 ± 2.1 μV in EMB group, and 38.4 ± 13.9 μV in EMB-IDE group (Figure 12). There was no statistical difference of PhNR between groups (P>0.05).

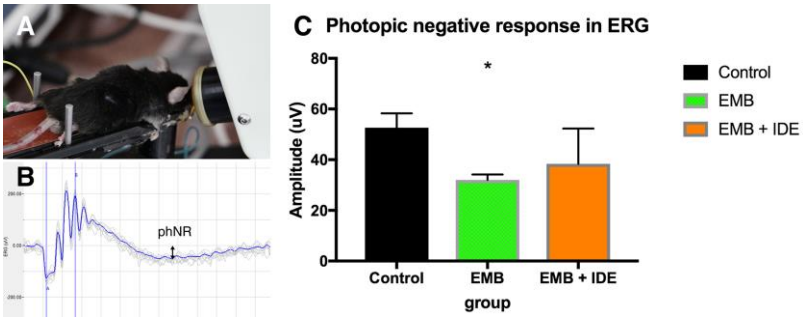

Figure 12. Idebenone rescues partially ganglion cell function in EMB-induced loss of vision. Electrophysiology testing was done 6 weeks after intraperitoneal injection of EMB (200mg/kg) alternate day. Idebenone group were treated with idebenone (200mg/kg) by oral gavage. n = 10 in each group. (A) mouse was placed on the platform to measure electroretinogram in right eye. (B) The photopic negative response (PhNR) was measured from the baseline to the trough of the negative response following the b-wave. The PhNR amplitudes were assessed at the flash intensity 10 cd.s.m^{-2}. The change of PhNR was not statistically significant (P = 0.068).

Optical coherence tomography revealed no statistical difference of retinal nerve fiber layer and ganglion cell layer between controls, EMB, and idebenone co-treated EMB group (Figure. 13). Mean retinal nerve fiber layer + ganglion cell inner plexiform layer thickness was 74.4 ± 6.8 μm in controls, 71.6 ± 8.4 μm in EMB group, and 76.5 ± 5.8 μm in EMB-IDE group (P>0.05).

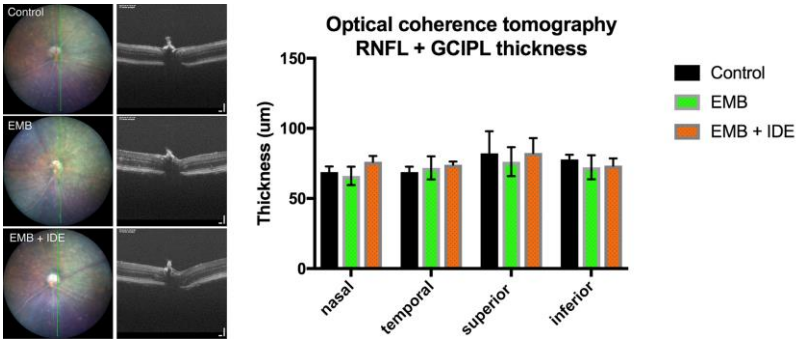

Figure 13. Structural analysis by optical coherence tomography. Retinal nerve fiber layer and ganglion cell inner plexiform layer were measured at the location of 200, 400, and 600 μm from the center of optic nerve head in four quadrants. No difference in RNFL and GCIPL thicknesses was noted in eyes with control, EMB-treated, and EMB-idebenone co-treated. n = 10 in each group.

In histologic section, the mean number of ganglion cells per millimeter were 93.7 ± 7.6 in control, 79.6 ± 6.8 in EMB, 92.6 ± 10.0 in EMB + IDE group, respectively. However, ganglion cell inner plexiform layer thickness did not differ among groups.

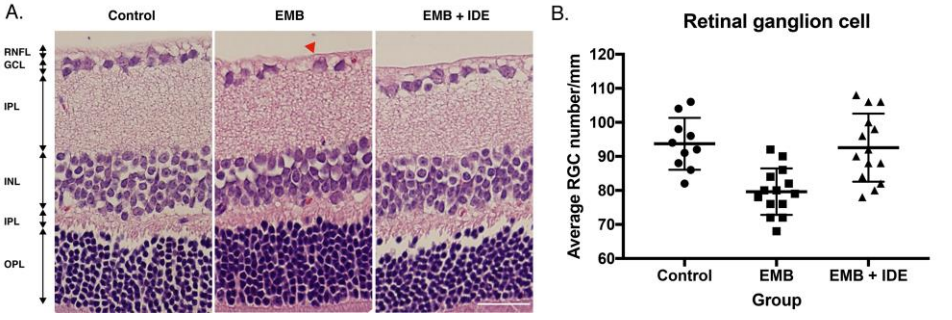

Figure 14. Idebenone protects against ethambutol-induced decrease in ganglion cell. (A) Histologic section of retina with H&E staining. The number of ganglion cells were decreased with ethambutol treatment (red arrowhead). (Scale bar = 25 μm). (B) Average retinal ganglion cell count was decreased with ethambutol treatment (p=0.001), which was attenuated by idebenone co-treatment in mouse model (p=0.001, EMB vs EMB + IDE).

IV. DISCUSSION

Previous study showed that EMB activates PKCδ signaling, mediate caspase-3 activity and inhibits the PI3K/Akt/mTOR pathways, which results in impaired autophagic flux and apoptosis of RGCs. In this study, we demonstrated that idebenone prevent zinc accumulation in lysosome, which lead to preserve lysosome function. Idebenone treatment increase cell viability and ATP contents in in vitro model of EMB-induced optic neuropathy. Preserved enzymatic function of lysosome can degrade the autophagosome induced by EMB-induced neurotoxicity. However, the protective effect of idebenone was not robust, and vision restoration was not consistently observed in mouse model of EMB-induced optic neuropathy. We observed protective effect of idebenone in

immortalized cell line, but this protective effect was not statistically significant in mouse model of EMB-induced optic neuropathy. Therefore, prospective randomized human clinical trials should be conducted to confirm the efficacy of idebenone as a therapeutic drug in EMB-induced optic neuropathy.

Poly (ADP-ribose) polymerase-1 (PARP-1) protects the genome by functioning in the DNA damage surveillance network. PARP-1 is also a mediator of cell death after ischemia –reperfusion injury, glutamate excitotoxicity, and various inflammatory processes. PARP-1 activation is required for translocation of apoptosis-inducing factor (AIF) from the mitochondria to the nucleus and that AIF is necessary for PARP-1-dependent cell death.[28] Previous study suggested that the visual loss associated with EMB may be mediated through an excitotoxic pathway and glutamate antagonists may be useful in limiting the side effect.[14] In EMB-induced neurotoxicity, we did not observed AIF translocation into nucleus. Therefore, cell death mechanism of EMB was thought to be not associated with AIF-dependent apoptosis.

Normal lysosomal acidification is essential for activating lysosomal hydrolytic enzymes and fusion of autophagosome-lysosomal complex.[29] Accumulation of zinc in lysosome impair the activity of hydrolytic enzyme and disrupt fusion of autophagosome-lysosome. Previous study demonstrated that EMB impaired downstream autophagosome-lysosome fusion.[30] Our study also showed that EMB induced LC3-II and zinc accumulation in lysosome, which was attenuated by idebenone pretreatment. This provides rationale of idebenone treatment on ethambutol-induced optic neuropathy. Histological examination of the mouse retina showed retinal ganglion cell number was reduced with ethambutol treatment, and retinal ganglion cell number was preserved with idebenone treatment. However, functional assessment failed to show significant protective effect. However, the more structural integrity of retinal ganglion cell was remained, the more likely visual function will improve.

A hallmark of autophagy is the autophagosome, a double membrane, cytosolic vacuole formed around the protein and organelles that are subsequently degraded by fusion of the autophagosome with a lysosome.[31] LC3 and sequestosome 1 (p62/SQSTM1) are among the most widely used markers to monitor autophagy.[32] We found that LC3 immunoreactivity was elevated after EMB treatment in retinal ganglion cells. In turns, EMB also induces zinc accumulation in the lysosomal complex. Metal-chelating effect of EMB may be related to zinc sequestration from cytosol to lysosome, thus it elevates pH in lysosome and prevents autophagosome-lysosome fusion. Previously, Wang and Sadun proposed possible mechanism of drug-related mitochondrial optic neuropathy.[4] The agents such as EMB or linezolid induces ROS and reduction in ATP synthesis, and it causes reduction of mitochondrial membrane potentials. As consequence, cytochrome c was released by opening of mitochondrial permeability transition pore. Cytochrome c then binds to apoptosis activating factor-1 (APAF-1), which activates procaspase-9, triggering the caspase cascade and apoptosis. Levin also found that increased levels of superoxide after intravitreal injection of EMB in rats.[33] EMB resulted in a robust fluorescence of superoxide compared to controls in cultured rat retinal ganglion cells. However, we found that EMB treatment did not induce ROS and caspase activation in R28 retinal precursor cells. It has been known that idebenone acts as a free-redical scavenger and bypasser of mitochondrial respiratory chain complex I. Although EMB-induced optic neuropathy has similar clinical features with LHON, pathogenic mechanism of EMB-induced optic neuropathy is related to zinc accumulation in lysosome, not by elevation of ROS.

The route of administration could have hindered the therapeutic effect of idebenone. Oral administration of idebenone results in rapid first-pass metabolism by the liver and severely reduced and short-lived plasma concentration.[34] In mice, a single dose of idebenone at 60 mg/kg delivered by gavage resulted in a maximum concentration peak of 9ng/ml (26.6 nM) and

37.4 ng/ml (110.5nM) in the vitreous and aqueous humor, respectively, but falling to levels below the limit of quantification after 30 min and 120 min, respectively.[35] Many experiments have used idebenone concentrations at 10 μM (even to 100 μM in some cases), and it is where many authors have apparently showed that protective effect of idebenone in cellular functions.[22,35,36] Previous study have shown that 10 nM was the minimum concentration of idebenone required to restore retinal ganglion cell function in rotenone-induced LHON model.[35] However, idebenone is known to inhibit Complex I activity at high concentration,[37] and even cause the opening of the permeability transition pore complex.[38] In R28 cells, the protective effect of idebenone has diminished at higher (> 6μM) concentration, and even detrimental effect on cell survival at 6~8 μM concentration. Idebenone can also bind to the hydrophobic quinone-binding site of Complex I and promote superoxide formation.[39] Recent study showed that idebenone inhibit activity of a calcium-activated chloride channel, which is expressed in axon terminals of bipolar cells.[40] Therefore, it may be critical to determine therapeutic dosage of idebenone to treat EMB-induced optic neuropathy.

Idebenone is approved in the EU as a treatment option for LHON. It has been reported that the aberrant mitochondrial effect from *OPA1* mutation are more complex than in LHON, with alternative roles for *OPA1* including the regulation of thermogenesis and beta-oxidation,[41] the mediation of cell apoptosis through inhibiting cytochrome c release.[42] The mechanism of EMB-induced neurotoxicity was not clear yet, EMB induced zinc accumulated in lysosome, and elevated their luminal pH. Accordingly, EMB blocks autophagic flux via inhibiting autophagosome-lysosome fusion. Previous study found that EMB induces vacuoles in cultured retinal cells, and the formation of these vacuoles was markedly decreased in the absence of intracellular free zinc.[15] EMB is a strong metal-chelating agent, and the mechanism of action may be related to inactivation of zinc and copper in the pathogenic organisms.[43]

This study has several limitations. First, our experiment used R28 retinal precursor cell line in vitro model. Immortalized cell line is a genetically engineered cell line, which has a permanent proliferation. Transformed ocular cell lines such as ARPE-19, GTM13, NTM5, HLEB3, 661W and others, have been used extensively and will continue to be useful resources to better understand ocular pathology.[44] However, the process of transformation invariably leads to dedifferentiation of the cells when using such cell lines. Accordingly, transformed cell lines may lose characteristic properties of their cells of origin in the eye. Second, the acute neurotoxicity induced by EMB may be different from EMB-induced optic neuropathy in human. EMB-induced vacuole formation was apparent within 1 hour after challenge of EMB in cultured retinal cells.[16] In human, long-duration of treatment is needed to develop EMB-induced optic neuropathy as minimum of 2 months. It is assumed that chronic process of EMB-induced optic neuropathy is different from acute toxicity of EMB in retinal immortalized cells. Third, data obtained from mouse may or may not be pertinent to human disease as these animals often have different responses from humans to inflammation and other type of insults.[45] For examples, substances with promising results in providing neuroprotection in rats after cerebrovascular insults failed to provide the same protection in humans. Moreover, bioenergetics process of mitochondria in rodent is different than primates, and rodents have no parvocelluar retinal ganglion cells which is a main target of EMB-induced optic neuropathy. Therefore, it may be more appropriate to test the hypothesis in nonhuman primates.[46]

V. CONCLUSION

In conclusion, we found that idebenone has some protective effect in EMB-induced neurotoxicity via decreasing lysosomal zinc accumulation, not by decreasing reactive oxygen species. We observed that idebenone also increases cell survival and ATP contents in R28 retinal precursor cells compared to the controls. However, In mouse model of EMB-induced optic neuropathy, the

protective effect of idebenone was not statistically significant to restore electrophysiological changes. However, functional vision was slightly increased with idebenone treatment. Thus, our results suggest that possibility of using idebenone to treat EMB-induced optic neuropathy.

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APPENDICES

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ABSTRACT(IN KOREAN)

에탐부톨 시신경병증의 R28 cell과 마우스 모델에서 이데베논의 보호 효과

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에탐부톨 시신경병증은 결핵시 복용하는 에탐부톨의 장기복용에 의해 발생하는 부작용으로 전세계적으로 매년 약 10만명의 새로운 환자가 발생하고 있는 질환이다. 에탐부톨 시신경병의 기전은 산화스트레스와 미토콘드리아의 기능 이상에 의해 생긴다고 알려져 있으며 임상양상은 미토콘드리아 DNA의 점돌연변이에 의해 발생하는 레버씨 유전성 시신경병증과 매우 유사하다. 이데베논은 코엔자임 Q10 유사체로서 레버씨 유전성 시신경병증에서 효과가 최근 임상실험에서 입증이 되어 본 연구는 에탐부톨 시신경병증에서 이데베논이 시신경 보호효과가 있을지 알아보고자 한다. 마우스의 망막신경세포로 부터 유래된 R28 세포주에 3mM의 에탐부톨을 처리하였을때 24시간 후 소포성 세포변화가 명확히 관찰이 되었으며, 이 소포성 세포변화는 이데베논 처리에 의해 감소함이 관찰이 되었다. 세포생사판별시험과 ATP양을 측정하였을때도 이데베논이 에탐부톨에 의한 독성을 줄일 수 있음을 관찰하였다. 그에 따라, 그 기전을 밝히고자 활성산소를 측정하였는데 에탐부톨만 처리한 군에서도 활성산소는 크게 증가하지 않았으며, 미토콘드리아 막전위의 변화도 없었다. Fluo-Zin3로 측정한 세포내 아연이 에탐부톨에 의해 생성된 소포내로 축적이 되는 것을 확인할 수 있었으며 lysotracker로 측정한 부위에서 이러한 소포가 라이소좀임을 확인하였다. 이러한 라이소좀으로 아연이 축적되는 과정을 이데베논이 감소시킴을 확인하였다. 마우스모델에서는 6주간 에탐부톨을 복강내 주사를 하여 확립하였으며, 실험군에는 이데베논을 oral gavage로 200mg/kg로

투여하였다. 6주 실험한 마우스의 시력변화를 측정하는 optomotor test에서 통계학적으로 유의하지는 않았으나 이데베논을 투여한 군에서는 약간의 시력호전이 있었다. 망막신경절 세포의 기능을 측정하는 망막전위도 검사의 photopic negative response에서도 역시 뚜렷한 유의한 차이는 아니지만 약간의 호전이 관찰이 되었다. 망막의 구조적인 변화는 큰 차이를 보이지 않았다. 본 연구의 결과를 미루어 보건대, 이데베논은 에탐부톨 시신경병증에서 아연의 소포체 내로의 축적을 방해하여, autophagosome lysosome complex의 결합에 의한 autophagic flux에 대한 보호효과를 보이며, 활성화 산소에 대한 효과는 없는 것으로 나타났으며, 이러한 기전으로 에탐부톨 시신경병증에 작은 시신경 보호 효과를 보인 것으로 생각한다.

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