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N-Acetylcysteine increases corneal endothelial cell survival in a mouse model of Fuchs endothelial corneal dystrophy

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ABSTRACT

The present study evaluated survival effects of N-acetylcysteine (NAC) on cultured corneal endothelial cells exposed to oxidative and endoplasmic reticulum (ER) stress and in a mouse model of early-onset Fuchs endothelial corneal dystrophy (FECD). Cultured bovine corneal endothelial cell viability against oxidative and ER stress was determined by CellTiter-Glo® luminescent reagent. Two-month-old homozygous knock-in *Col8a2*$^{L450W/L450W}$ mutant (L450W) and C57/Bl6 wild-type (WT) animals were divided into two groups of 15 mice. Group I received 7 mg/mL NAC in drinking water and Group II received control water for 7 months. Endothelial cell density and morphology were evaluated with confocal microscopy. Antioxidant gene (*iNos*) and ER stress/unfolded protein response gene (*Grp78* and *Chop*) mRNA levels and protein expression were measured in corneal endothelium by real time PCR and Western blotting. Cell viability of H_2O_2 and thapsigargin exposed cells pre-treated with NAC was significantly increased compared to untreated controls ($p < 0.01$). Corneal endothelial cell density (CD) was higher ($p = 0.001$) and percent polymegathism was lower ($p = 0.04$) in NAC treated L450W mice than in untreated L450W mice. NAC treated L450W endothelium showed significant upregulation of *iNos*, whereas *Grp78* and *Chop* were downregulated compared to untreated L450W endothelium by real time PCR and Western blotting. NAC increases survival in cultured corneal endothelial cells exposed against ER and oxidative stress. Systemic NAC ingestion increases corneal endothelial cell survival which is associated with increased antioxidant and decreased ER stress markers in a mouse model of early-onset FECD. Our study presents *in vivo* evidence of a novel potential medical treatment for FECD.

Fuchs endothelial corneal dystrophy (FECD) is a degenerative corneal endothelial disease which is characterized by progressive corneal endothelial cell (CEC) loss with concomitant drop-like excrescences (guttae) and thickening of the endothelial basement membrane (Azizi et al., 2011). FECD affects an estimated 5% of the US population and is one of the most common causes for corneal transplantation (Baratz et al., 2010). Corneal endothelial cell apoptosis due to oxidative stress and endoplasmic reticulum (ER) stress may play a central pathogenic role in FECD (Jurkunas et al., 2010; Engler et al., 2010).

N-acetylcysteine (NAC), a thiol-containing antioxidant and radical scavenger, has been used clinically for many years as a reduced glutathione precursor (Arakawa and Ito, 2007). NAC also blocks bovine serum albumin (BSA)-induced ER stress effects such as GRP78 activation, eIF2α phosphorylation, SGLT expression, and α-MG uptake (Lee et al., 2009). Previously, we described two alpha 2 collagen VIII (*Col8a2*) transgenic knock-in mouse models of early-onset Fuchs endothelial corneal dystrophy which show early endothelial cell ER stress/unfolded protein response (UPR) and apoptosis (Jun et al., 2012; Meng et al., 2013). These knock-in mutations specify a glutamine to lysine change at amino acid 455 (Q455K) and a leucine to tryptophan change at amino acid 450 (L450W) (Biswas et al., 2001; Gottsch et al., 2005).

The purpose of this study is to evaluate the effect of NAC as an inhibitor of oxidative and ER stress on corneal endothelial cell survival in cell culture and in the L450W alpha 2 collagen VIII transgenic knock-in mouse model of Fuchs endothelial corneal dystrophy.

Bovine corneal endothelial cells (BCECs) were scraped from the excised corneas of freshly enucleated globes with a surgical blade according to an established protocol (Chifflet et al., 2003), and primary cultures were established by resuspending cells in

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Dulbecco's modification of Eagle's minimum essential medium (DMEM) (Cellgro, Manassas, VA) supplemented with 10% fetal calf serum, antibiotic antimycotic solution (10,000 units of penicillin (base), 10,000 μg of streptomycin (base), and 25 μg of amphotericin B/ml) (Invitrogen, Grand Island, NY) and 50 μg/ml of gentamicin (Invitrogen) at 37 °C and 5% CO_2 in air on a 6 well plate (Cytoone, Orlando, FL). After confluency was reached (usually 7–10 days), the primary cultures were subcultured to 96 wells (Cytoone) in the same medium.

To examine the effects of NAC on BCECs exposed to oxidative and ER stress, corneal endothelial cells were incubated in quadruplicate concentrations (0–10 mM) of N-acetylcysteine (Sigma, St. Louis, MO) in cell culture medium for 48 h. NAC was freshly dissolved in phosphate-buffered saline (PBS; Gibco-BRL, Carlsbad, CA) at room temperature before use. After pretreatment with NAC for 48 h, cell culture medium containing NAC was then replaced with 100 μL cell culture medium without NAC and containing thapsigargin (28 μM) for 24 h or H_2O_2 (0.6 mM) for 4 h. Negative control (no NAC treatment for 48 h and no thapsigargin for 24 h or H_2O_2 for 4 h) was also included to determine baseline cell viability. At the end of incubation in H_20_2, thapsigargin, or control conditions as described above, cell viability was determined by adding 100 μL of CellTiter-Glo® luminescent reagent (Promega, Madison, WI), and the luminescence, produced by the luciferase-catalyzed reaction of luciferin and ATP, was measured using a FLUOstar OPTIMA spectrophotometer (Optima, Tokyo, Japan). The cell viability (%) relative to control was calculated as 100× luminescence in H_2O_2 or thapsigargin conditioned cells/luminescence in negative control cells.

Weaned 2 month old animals were divided into two groups of 30 mice for each treatment. Group I (NAC group) received standard rodent chow orally and 7 mg/mL NAC drinking water (Whitehead et al., 2008) for 7 months. Group II (control group) received standard rodent chow orally and pure drinking water for 7 months. Each group was divided into two subgroups of 15 mice each. These subgroups included mice with the Col8a2 L450W mutation (Meng et al., 2013) or wild-type mice. Animal care and use conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All animal protocols were approved by the Institutional Animal Care and Use Committee of Johns Hopkins University.

All mice were euthanized with isoflurane (Vedco Inc., St. Joseph, MO) verified by checking for the absence of respirations and followed by cervical dislocation. Immediately after euthanasia, corneal endothelial cells were examined by confocal microscope (Nidek Confoscan 3, Fremont, CA). Mice were placed on a customized platform and the head was fixed with the right eye pointing towards the objective. Lubricant gel (Genteal; Novartis, East Hanover, NJ) was used as an immersion fluid, and images of the central corneal endothelium were acquired as previously described (Jun et al., 2012). Corneal endothelial cell imaging and quantitative analysis including cell density and percent polymegathism were performed using the Confoscan 3 software. Cells were manually identified for analysis by a single observer (ECK).

After confocal microscopy examination, eyes were extracted and Descemet membranes (DM) were stripped with a forceps. Total RNA was extracted from murine corneal endothelium on stripped DMs (four eyes per sample, i.e. a single sample (n) included two animals of the same genotype, with 5 samples from 10 animals ($n = 5$) per subgroup analyzed) using TRIzol reagent (Invitrogen) followed by RNeasy column (Qiagen, Hilden, Germany) purification. Thus, 2 mice were included in each sample with $n = 5$ (10 mice) samples for each of four subgroups analyzed, including mutant mice with and without NAC, and wild-type mice with and without NAC. Complementary DNA was generated using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). The resulting complementary DNA (3 μL cDNA) was preamplified with TaqMan® PreAmp MasterMix and mouse specific primers (Applied Biosystems, real time PCR primers for the examined genes are Nos2(iNos); Mm00440502_m1 (assay number), Nfe2l2(Nrf2); Mm00477784_m1, Ptgs2(Cox2); Mm00478374_m1, DDIT3(Chop); Mm01135937_g1, Hspa5(Grp78); Mm00517690_g1, and β-actin; Mm00607939_s1). Real time-polymerase chain reaction (real time PCR) was performed in triplicate using the TaqMan® Gene Expression Master Mix (2×) (Applied Biosystems), preamplified cDNA products (diluted 1:20; 5 μl), nuclease free water, and mouse specific primers in a 20 μL reaction volume. The relative gene expression in *Col8a2*$^{L450W/L450W}$ and WT endothelium was normalized to the housekeeping gene beta-actin (β-actin). A no-template control was included in each quantitative real time PCR experiment to confirm the absence of DNA contamination in the reagents used for amplification. All assays used similar amplification efficiency, and a ΔC_T experimental design was used for relative quantification. Data analysis was performed using StepOne™ software (Version 2.2, Applied Biosystems). Statistical analysis of quantitative real time PCR data between the groups was performed using the ANOVA and DataAssist™ Software v3.0 (Applied Biosystems). A p-value <0.05 was considered statistically significant.

Descemet membrane and endothelial cells were stripped from freshly dissected, 10 month-old mouse corneas and homogenized in Tissue Protein Extract Reagent (Thermo Fisher Scientific, Rockford, IL) with 1% protease inhibitor cocktail (Sigma) and 1% ethylenediaminetetraacetic acid (Sigma). Each sample contained both corneas of the same animal from a total of four subgroups including mutant mice with and without NAC and WT mice with and without NAC. Each subgroup included $n = 5$ mice. The mixture was then microcentrifuged at 48 °C for 10 min at 12,000 rpm. The lysate was removed and the protein concentration was quantified by BCA Protein Assay Kit (Thermo Fisher Scientific). Eight micrograms of protein was mixed with 10 μl of 4× loading dye (Invitrogen) with 2-mercaptoethanol (Sigma) and heated at 65 °C for 5 min.

Samples were loaded onto a 10% Tris–HCl Ready Gel (BioRad, Hercules, CA) and subjected to sodium dodecylsulfate–polyacrylamide gel electrophoresis separation for 1 h at 120 V. Proteins were transferred to a polyvinylidene fluoride membrane (BioRad) and incubated in blocking buffer made of 5% non-fat milk in PBS with 0.1% Tween-20. Membranes were then incubated in primary antibodies: iNOS (1:500, Abcam, Boston, MA), CHOP (1:500, Santa Cruz Biotechnology, Santa Cruz, CA), and GRP78 (1:500, Cell Signaling, Danvers, MA) diluted in blocking buffer for 1 h at room temperature. Subsequently, membranes were washed and incubated in 1:10,000 dilution of anti-rabbit IgG, horseradish peroxidase conjugated antibody (GE Healthcare, Piscataway, NJ) diluted in blocking buffer for 45 min at room temperature. Loading controls were assayed by probing with β-actin (1:1,000, Cell Signaling) as primary antibody after stripping with Restore Stripping Buffer (Thermo Fisher Scientific). Proteins were detected using SuperSignal West Dura (Thermo Fisher Scientific). Densitometry analysis was performed using Image J as previously described (http://www.lukemiller.org/journal/2007/08/quantifying-western-blots-without.html).

Data are presented as mean ± standard deviation (SD). Statistical analysis of cell viability in culture experiments and cell density/polymegathism by confocal microscopy was performed by Mann–Whitney 2-tailed test. Real time PCR and Western blot assay results were also analyzed by Mann–Whitney 2-tailed test. A p-value <0.05 was considered statistically significant.

We devised a short term cell culture model to assess whether NAC could improve viability against oxidative and ER stress in corneal endothelial cells. Cell viability of 1 mM (69.6 ± 3.2%, $p = 0.004$), 3 mM (75.5 ± 4.7%, 0 = 0.001), and 10 mM (67.7 ± 4.1%,