The Effect of Phacoemulsification Energy on the Redox State of Cultured Human Corneal Endothelial Cells

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Objective: To evaluate the effects of phacoemulsification energy on the redox state and mitochondrial distribution of cultured human endothelial cells.

Methods: Human corneal endothelial cells from fresh banked human donor tissue not suitable for transplantation were harvested and cultured. Cellular autofluorescence images were obtained using an inverted microscope. The redox fluorometric ratio, which can be related to oxidative stress, was calculated as the net value of fluorescence from the 4,6-diamidino-2-phenylindole channel divided by the net value of fluorescence from the fluorescein isothiocyanate–conjugated channel after subtraction of background. For determining the mitochondrial distribution patterns, the cell area was divided by drawing a line halfway between the nuclear and cell membranes. The average fluorescence in the central area was divided by the average fluorescence in the peripheral area. This ratio was compared.

Results: Human corneal endothelial cells exposed to increasing phacoemulsification times and increasing ultrasonic energy levels displayed dose-dependent decreases in measured redox ratios. Lower redox ratios in response to phacoemulsification did not associate with decreases in cell size or altered patterns of mitochondrial localization.

Conclusion: Redox fluorometry may serve as a useful indicator for the in vitro study of human corneal endothelial cell physiological response to ultrasonic stressors and potentially other nonoxidative stressors.

Clinical Relevance: Redox fluorometry in combination with human corneal endothelial cell morphometric measurements has potential to serve as an indicator of human corneal endothelial cell injury resulting secondary to ultrasound phacoemulsification.

CULTURE OF HCECs

Endothelial cells were cultured according to previously published methods. Corneal endothelial cells from fresh banked human donor tissue not suitable for transplantation (Central Florida Lions Eye and Tissue Bank, Tampa, Florida, and Tissue Banks International, Baltimore, Maryland) were harvested attached to the Descemet membrane and cultured in OptiMed-1 (Gibco, Invitrogen Corp, Carlsbad, California) containing 8% fetal bovine serum, the complex was centrifuged and washed in Hank balanced salt solution (Mediatech, Inc, Herndon, Virginia). Next, the endothelial cells and Descemet membrane complex were incubated for 1 hour in 0.02% EDTA solution, stirred vigorously with a flame-polished pipette to disrupt cell junctions, centrifuged for 5 minutes at 3000 g, and seeded onto culture plates coated with FNC coating mix (Athena Enzyme Systems, Baltimore) for 5 minutes at 3000 g-force, and seeded onto culture plates coated with FNC coating mix (Athena Enzyme Systems, Baltimore) to reduce photobleaching. Negative control staining was performed in parallel with the omission of primary antibodies. The CF:PF ratio was compared.

IMMUNOFLUORESCENCE STAINING

The HCECs, cultured in 8-well chamber slides (Laboratory-Tek II chamber slide; PGC Scientific Corp, Gaithersburg, Maryland), were washed with phosphate-buffered saline (PBS) and fixed for 20 minutes with 4% paraformaldehyde solution. Cells were permeabilized with 0.1% Triton X-100 for 10 minutes and blocked with 5% donkey serum for 1 hour at room temperature. After washing, cells were incubated with rabbit polyclonal antibody to the cell membrane (1:100) and bovine type I collagen (33 mg/mL). The cells were cultured in OptiMed-1 media supplemented with 8% fetal bovine serum, calcium chloride (200 mg/L), chondroitin sulfate (0.08%), ascorbic acid (20 µg/mL), pituitary extract (100 µg/mL), epidermal growth factor (5 ng/mL), nerve growth factor (20 ng/mL), gentamicin (1:200), penicillin (1:100), streptomycin (1:100), and amphotericin (1:100) under 10% carbon dioxide. Medium was changed every 2 days. At confluence, the cells were split 1 to 3, and passage 4 cells were used for experiments. The experiments were performed at 70% confluence.

PHACOEMULSIFICATION

The Alcon Legacy unit was used (Alcon Surgical, Fort Worth, Texas). A phacoemulsification probe with a 30° round, 1.1-mm TurboSonics ABS Tip (Alcon Surgical) was introduced into culture dishes, taking care to avoid touching the cells at a predetermined and constant distance from the dish culture surface of 2 mm, which was carefully maintained by sustaining the position of the handpiece under the operating microscope after marking the probe. The cells in 2-mL media without serum were treated with 30% US phacoemulsification for 0, 3, 5, and 5 seconds and treated with 0%, 30%, 50%, and 70% US power for 3 seconds. The cells selected for analysis were taken from the same area of the culture plate such that the distance between the probe and the cells examined was constant.

AUTOFLUORESCENCE MICROSCOPY

Cellular autofluorescence images were obtained using an inverted microscope (Axiovert 200M). The microscope was equipped with a mercury lamp (HB 103) and a cooled charge-coupled device camera (AxioImag MR5; Zeiss) for taking images. A DAPI filter set (excitation, G365; emission bandpass, 445/50) was used to detect intrinsic reduced pyridine nucleotides and an FITC filter set (excitation bandpass, 450-490; emission bandpass, 515-565) was used to detect oxidized flavoproteins. To minimize photobleaching and light stimulation, the illumination source was turned off during fluorescence imaging.

The all images were processed and analyzed using AxioVision 4.5 software (Zeiss). Prior to autofluorescence microscopy imaging, all cells were equilibrated in balanced salt solution (Alcon) and then imaged at room temperature under room air.

REDOX STATE CALCULATION

After outlining the cell border, the average intensities of cellular and background fluorescences were automatically calculated by the AxioVision 4.5 software. Twenty cells were randomly selected in each group and analyzed. The net value of cellular fluorescence was obtained by subtracting background intensity from cellular intensity. The redox ratio, which is inversely proportional to the cellular metabolic rate, was determined as the net value of fluorescence from the DAPI channel divided by the net value of fluorescence from the FITC channel (Figure 1).

MITOCHONDRIAL DISTRIBUTION

The cell area was divided by drawing a line halfway between the nuclear and cell membranes. The net value of cellular fluorescence in each of the 2 zones was obtained by subtracting background intensity from cellular intensity. The average fluorescence in the central area (CF) was divided by the average fluorescence in the peripheral area (PF). The CF:PF ratios in the range of more than 1.70 were defined as having a perinuclear mitochondrial arrangement (Figure 2A), while ratios of less than 1.70 defined the homogenous mitochondrial pattern (Figure 2B). The CF:PF ratio was compared.

CELL VIABILITY ASSAY

Cell viability was assessed by cell counting with trypsin blue (Gibco, Invitrogen Corp) staining. Four dishes per each group were used. After the cells were trypsinized (0.025% Trypsin-EDTA), the cells were suspended in media. To determine the number of live cells, cells were stained with 0.4% trypan blue, and the unstained live cells and stained dead cells were counted with a hemocytometer. The average cell counts of culture plates were recorded.
STATISTICAL ANALYSIS

Data are expressed as mean (SD). Comparisons between controls and treated groups were performed using the Mann-Whitney test and $\chi^2$ test. A $P$ value $<.05$ was considered statistically significant.

RESULTS

IMMUNOFLOUORESCENT STAINING OF HCECs

Corneal endothelial phenotype was verified by the intense positive staining with type VIII collagen $\alpha$ 2 monoclonal antibodies (Figure 3A). The bright red signal within cytoplasm indicates collagen VIII $\alpha$ 2 synthesis. Immunocytochemical study of ZO-1, a protein associated with tight junctions, demonstrated its presence in cultured cells (Figure 3B).

REDOX RATIO

Redox ratios decreased with longer phacoemulsification time and increased power. Redox ratios of cells at 5 seconds and 7 seconds with 30% US power were lower compared with normal control (Figure 4) ($P = .002$ and $<.001$, respectively, Mann-Whitney test). Cells with 50% and 70% US power at 3 seconds showed lower redox ratios compared with normal control (Figure 5) (Table) ($P < .001$ and $<.001$, respectively, Mann-Whitney test).

MITOCHONDRIAL DISTRIBUTION

The percentage of cells with a perinuclear mitochondrial distribution was not different between groups.

CELL VIABILITY ASSAY

The percentage of nonviable, stained cells increased with longer phacoemulsification time (Figure 2A) ($P = .01$, .01, and .01, Mann-Whitney test) and stronger power (Figure 2B) ($P = .01$, .01, and .01, Mann-Whitney test).
known to be formed during phacoemulsification and to hydroxyl radical and other free radical species. These are of extracellular reactive oxygen species (ROS), such as energy can reduce the redox ratio in cells is by generation of mitochondrial distribution determination. It is more difficult to perceive additional modes of injury in this complex HCEC stressor.

In the present study, redox ratios were significantly reduced in a dose-dependent manner in response to increasing phacoemulsification times as well as increasing phacoemulsification power, each reflecting an indicator of increasing total energy transfer to the HCEC.

Predictably, the percentage of nonviable, stained cells increased with both longer phacoemulsification times and higher phacoemulsification powers. Redox ratios were well correlated with the percentage of nonviable, stained cells. However, in the present study, cell size (area) was not significantly altered by either greater phacoemulsification time or increased ultrasonic power. Moreover, phacoemulsification did not have a significant effect on mitochondrial distribution patterns. While US phacoemulsification of HCECs results in shifts in the redox ratio that implicate ROS, the injury pattern is distinct from that seen following extracellular exposure of HCECs to the chemical oxidant tBHP. Concentrations of tBHP sufficient to cause both injury and apoptosis do result in similar redox ratio shifts but are also associated with characteristic changes in cell morphology such as decreased cell size (area) and a perinuclear pattern of mitochondrial distribution. Therefore, the redox fluorometry data presented herein support a role for oxidative injury as a component of phacoemulsification injury as well as implicate additional modes of injury in this complex HCEC stressor.

Finally, we used balanced salt solution as the medium when obtaining autofluorescence images. BSS Plus (Alcon) is supplemented with glutathione, which is thought to prevent oxidative stress placed on the cells.23-24 Further study is necessary to investigate the effect of BSS Plus on the redox state in HCECs during phacoemulsification.

In this study, cultured cells, and not whole organs, were used. Monolayer cultured cells were necessary to obtain live cell images for redox ratio calculation and mitochondrial distribution determination. It is more difficult to perform such imaging in endothelial cells through hazier banked corneas. The purpose of this study was to compare different phacoemulsification parameters in vitro and to develop in vitro model conditions that allow quanti-
A qualitative assessment of phacoemulsification-induced endothelial cell damage and response to protective measures. Further experimental study as well as clinical correlation will be necessary to establish the clinical relevance of this novel laboratory test system. The ultrasound tip was directed at the cells at 2 mm, because some surgeons prolapse the cataractous lens into or above the iris plane rather than operate in the bag. Two millimeters, the distance of the tip from endothelial cells, was chosen to provide a “worst-case” scenario. These were pilot experiments, and geographic parameters were chosen arbitrarily.

**Figure 4.** Two-dimensional redox fluorometric photomicrographs of human corneal endothelial cells with longer ultrasound (US) phacoemulsification time or stronger US phacoemulsification power.

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<th>US power, %</th>
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As shown in Figure 3A, unlike in vivo, cultured HCECs under these environmental conditions were not hexagonal. Staining for ZO-1 also revealed a different distribution compared with the in vivo state. Many different methods to evaluate mitochondrial distribution, including transmission electron microscopy\(^{24-26}\) and Mitotracker staining,\(^{24,27,28}\) have been used. Although transmission electron microscopy can be helpful in mitochondrial evaluation, it was not necessary in this study and can be technically difficult and prone to artifact. In this study, standard trypan blue exclusion staining was used as a cell viability test. Other cell viability tests, which may or may not be more accurate, including fluorometric assays and the MTT (3-[4,5-dimethylthizol-2-yl] 2,5-diphenyltetrazolium bromide) assay, were not tested.\(^{29-34}\)

In conclusion, redox fluorometry in combination with HCEC morphometric measurements has potential to serve as an indicator of HCEC injury resulting secondary to US phacoemulsification. Differences in injury response between chemical oxidant injury and US phacoemulsification injury may provide fundamental insights into the mechanisms of injury involved. Redox fluorometry is at present an in vitro, inexpensive, rapid, and reproducible means to quantitatively assess HCEC injury following US phacoemulsification and may prove a useful means by which to examine, modify, and optimize current and evolving phacoemulsification tools and techniques. The method may prove of particular value in the preclinical identification and screening of potential HCEC protective agents applicable to the prevention and treatment of corneal edema following cataract surgery.

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