Oxidative stress in the outer retina (OR = distance from external limiting membrane to the retinal pigment epithelium–choroid boundary) is a condition of unregulated and continuous production of free radicals (e.g., superoxide and hydroxyl radicals). Experimental studies implicate OR oxidative stress/damage, as measured ex vivo, as pathogenic in many incurable blinding diseases, such as age-related macular degeneration, diabetic retinopathy, and retinitis pigmentosa, as well as in visual performance declines during “healthy” aging.1–7 However, it is not possible to measure OR oxidative stress in patients and so therapies that appear promising in preclinical studies have not been adequately tested clinically. At present, physicians must make educated guesses regarding antioxidant (AO) dose, timing, drug combinations, and whether the selected treatment strategy indeed reduces oxidative stress in the target tissue. Also, clinical studies often rely on a one-antioxidant-solves-all approach that may be too simple or started too late to be effective in changing disease outcomes. Not surprisingly, many clinical trials find unclear medical benefits from “guessedmated” AO dosing. It is not clear whether the role of oxidative stress in aging and disease largely uncertain. Poor outcomes in AO clinical trials are insufficient evidence for ruling out a pathogenic role of oxidative stress in patients.

We have been addressing this problem with the development of various QUEnch-assiSTed (QUEST) MRI protocols that involve AO correction (i.e., a “quench”) of retinal function or excessive free radical production.8 Agreement between QUEST magnetic resonance imaging (MRI) approaches and gold standard methods has underscored their usefulness as noninvasive indices of OR oxidative stress.1,8–12 Recently, we have found that d-cis-diltiazem, an Food and Drug Administration (FDA)-approved drug, induces a temporary and nondamaging production of excessive reactive oxygen species in the OR of C57BL/6j mice in vivo as measured by QUEST MRI and by a gold standard ex vivo assay.9,13,14

We, and others, have found in various mice strains that when light stimulation causes a significant increase in hydration of the OR (measured by proton density MRI), the distance (i.e., expansion) between external limiting membrane (ELM) and retinal pigment epithelium (RPE) increases as measured in vivo by optical coherence tomography (OCT), and water mobility in this region increases as measured by diffusion MRI in agreement with previous microelectrode studies of an impermanent probe.1,15–26 Furthermore, in 2- to 3-month-old diabetic mice (i.e., before vascular histopathology), light-stimulated OR expansion as measured by MRI is impaired but could be restored by AO, supporting the conclusions of OR oxidative stress.
Retinal Oxidative Stress Measured by QUEST OCT

![Diagram](image)

**Figure 1.** Timeline of drug administration and dark versus light exposure.

stress early in the course of diabetes. However, it has remained unclear if the light-stimulated expansion of the OR measured by OCT is similarly affected by oxidative stress.

In this study, we applied a QUEST strategy with ultrahigh-resolution OCT imaging to test the hypothesis that correcting oxidative stress-suppressed light-evoked expansion of OR with AOs is a novel way to encode oxidative stress information into the OCT image.

**Materials and Methods**

All animals were treated in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research, and Institutional Animal and Care Use Committee authorization. Animals were housed and maintained in a 14 hour:10 hour light-dark cycle laboratory lighting, unless otherwise noted.

**Groups**

Two- to 3-month-old C57BL/6j (Jackson Labs, Bar Harbor, ME, USA) mice were studied as outlined in Figure 1. After overnight dark adaptation, animals were injected under dim-red light with either d-cis-diltiazem dissolved in saline (n = 9, bolus subcutaneous, 30 mg/kg; D-2521, >99% purity, Sigma-Aldrich Corp., St. Louis, MO, USA) or saline three times (i.e., instead of d-cis-diltiazem and the two AO injections, n = 4) as a control group; un.injected mice were also used as a control group (n = 6). On each eye, OCT imaging was first performed in darkness ~1 hour after the d-cis-diltiazem or saline injection. After imaging in dark, mice were exposed to room light (~500 lux) for ~1 hour, and second OCT images were captured for each eye under room light. The d-cis-diltiazem-treated mice were given AOs (n = 5): 1 mg/kg methylene blue (MB, intraperitoneal injection), dissolved in saline before overnight dark adaptation (~20 hours before d-cis-diltiazem, Fig. 1) and 50 mg/kg α-lipoic acid (ALA, i.p., dissolved in saline and pH adjusted to ~7.4) after the first OCT imaging (i.e., ~1 hour before the second OCT examination in light, Fig. 1). To test effects of AOs on dark-adapted OR thickness, a subgroup of animal (n = 4) received only MB and ALA, but no d-cis-diltiazem, and were kept in dark throughout the imaging examination.

**Ultrahigh-Resolution OCT**

Light exposure elicits an increase in OR thickness as measured by OCT in mice and humans; the procedure has been previously published. Briefly, after anesthetizing mice with ketamine (100 mg/kg) and xylazine (6 mg/kg), retina OCT images were captured with Envisu UHR2200 (Bioptigen, Durham, NC, USA), with OCT beam bandwidth of 160 nm, and theoretic axial resolution of 1.6 μm in tissue. The mouse eye was positioned with the optic nerve head (ONH) in the center of the OCT scan.
FIGURE 2. Representative OCT images of inferior retina in dark (D) and light (L) for an uninjected control mouse (control), a d-cis-diltiazem (DIL)-injected mouse, and a d-cis-diltiazem + MB/ALA (DIL+AO) mouse; images from thrice-saline-injected controls and dark only + AO mice appear similar to controls and DIL images, respectively (data not shown); similar results are seen in superior retina (data not shown). Layer assignments: nerve fiber layer (NFL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), ELM: rod inner segment layer (IS); rod outer segment layer (OS); RPE, and anterior choroid boundary (choroid). Vertical double arrowhead bar between the ELM and retina-choroid border indicates the OR region measured.

RESULTS

Overall Repeatability Within Subjects

The ICC estimated from our data was 0.347 (95% CI: 0.186, 0.509), which differed from 0 and reflects a significant correlation within subjects. As such, OR thickness showed repeatability within subjects.

OR Thickness and Photoresponse in Controls

Shown in Figure 2 are examples of OCT images obtained under darkness and after 1-hour exposure to light for a mouse eye at the inferior retinal region. The OR photoresponse in control mice can be appreciated upon visual inspection of the OCT data. Quantitative analysis showed that light-exposed uninjected or thrice-saline-injected controls had OR thicknesses of 57.7 ± 0.7 μm (mean ± SEM) and 59.7 ± 0.8 μm, respectively (Fig. 3); these were not different from each other (P > 0.05). In the dark, these controls had OR thicknesses of 54.2 ± 0.7 μm and 54.5 ± 0.8 μm, respectively; no statistically significant difference was found between these values (P > 0.05). OR thickness in dark-only mice given only AOs was 54.8 ± 0.8 μm and was not different (P > 0.05) from that in dark-adapted control mice.

In uninjected or thrice-saline-injected controls, 1-hour light exposure produced an elongation in OR thickness of 3.5 ± 0.5 μm or 5.2 ± 0.6 μm, respectively (Fig. 3). Both of these OR photoresponses were different (P < 0.0001) from 0 (but not from each other [P = 0.2]) (Fig. 3). Mice that were kept in the dark and given AOs did not change (P = 0.64) OR thickness (−0.3 ± 0.6 μm) from 0 (Fig. 4).

OR Thickness and Photoresponse After d-cis-Diltiazem and AOs

In a d-cis-diltiazem-treated mouse, light OR thickness appeared similar to that in the dark (Fig. 2), suggesting an impaired OR photoresponse. Light-exposed d-cis-diltiazem-injected mice had an OR thickness of 53.0 ± 0.5 μm, which was different (P < 0.0001) from both uninjected and thrice-saline-injected control values (Figs. 2, 3). In dark-adapted d-cis-diltiazem-treated mice, OR thickness was 53.6 ± 0.5 μm, which was not different from the other dark OR thicknesses.

d-cis-Diltiazem prevented the light-induced extension of OR thickness (Fig. 4). Averaged light-induced OR thickness change was −0.6 ± 0.4 μm, which was not different (P = 0.14) from 0 and was smaller (P < 0.0001) than those observed in control animals (Fig. 4).

OR Thickness and Photoresponse After d-cis-Diltiazem and AOs

Treatment with AOs visibly restored the photoresponse in an inferior image of a d-cis-diltiazem + AO-injected mouse (Fig. 2). In light-exposed mice given both d-cis-diltiazem and AOs, the OR thickness was 55.9 ± 0.7 μm, which is not different (P = 0.4) from that in uninjected controls but was different (P = 0.01) from thrice-saline-injected control values (Figs. 2, 3). In dark-adapted d-cis-diltiazem and AO-treated mice, OR thickness was 52.6 ± 0.7 μm, which was not different from the other dark OR thicknesses.

d-cis-Diltiazem mice treated with AOs showed a light-evoked OR expansion of 3.3 ± 0.6 μm, which is different (P < 0.0001) from 0 but not from that in uninjected (P = 1.0) and thrice-saline-injected control mice (P = 0.2; Fig. 4).

DISCUSSION

In this study, we demonstrated for the first time that OR oxidative stress can be detected with OCT. MB is an alternative electron transporter that effectively suppresses generation of superoxide from a variety of sources, and ALA is a potent free radical neutralizer. Having established feasibility of QUEST OCT with MB and ALA, we anticipate that future studies will investigate other AO approaches and how their dosing and timing can be optimized in animal models and in humans.

The present results further support earlier observations that a robust aspect of OR physiology (i.e., its light-evoked expansion) is measurable by imaging the OR microstructure with OCT. Unlike OCT images of fully (5–6 hours) light-adapted mouse retina that exhibit a prominent hyporeflective band between photoreceptor tip and RPE bands, at only 1 hour of light exposure the hyporeflective band is often visually undetectable on OCT images (Fig. 2); the hyporeflective band is very likely linked to fluid accumulation in the subretinal space. Although local changes in refractive index could, in theory, alter the optical path in the retina and induce apparent changes in OR...
thickness, native laminar structure corresponds well with OCT images for mouse retina (unpublished observation), suggesting that local variations in refractive index are relatively small; similar light-evoked OR changes were measured with OCT, diffusion MRI, and hydration-sensitive MRI measurements and support this conclusion.\(^\text{21}\) In addition, the data herein highlight that oxidative stress impairs the normal OR photoresponse and can be corrected by acute AO treatment (QUEST protocol) as a noninvasive way to detect OR oxidative stress.\(^\text{1}\) In our previous study, we have found that diabetes impaired the OR photoresponse owing to oxidative stress as measured by QUEST diffusion MRI.\(^\text{1,11}\) Also, we have found that OR oxidative stress is a feature of the rd10 mouse model of retinitis pigmentosa, and it has recently been reported that this model shows an impaired OR photoresponse.\(^\text{2,24}\) These data suggest that the impaired OCT photoresponse in the rd10 mouse is due to oxidative stress. Together, these considerations raise the strong possibility that QUEST OCT will be useful for mapping oxidative stress and treatment efficacy in various OR diseases.

AO defenses in the retina are typically measured in preclinical studies following a provocatation with a strong oxidizing agent, such as sodium iodate or paraquat, drugs that also produce substantial neurodegeneration.\(^\text{20,51-54}\) Needless to say, these drugs will not be useful clinically for evaluating antioxidant defenses. In contrast, our previous and present results support the use of the FDA-approved calcium channel blocker and cardioprotectant d-cis-diltiazem for producing a temporary OR oxidative stress in healthy dark-adapted retina without neurotoxicity.\(^\text{9}\) For example, B6 mice, which experience greater photoreceptor oxidative stress and histopathology than 12986/Ev mice following low-dose sodium iodate, also show a greater d-cis-diltiazem OR oxidative stress than 12986/Ev mice, as measured by QUEST MRI.\(^\text{9,29,34,35}\) One potential mechanism by which d-cis-diltiazem may generate oxidative stress is via outer retinal cytochrome P450; more work is needed to test this hypothesis.\(^\text{9}\) Nonetheless, the present data raise the possibility that the combination of QUEST OCT and d-cis-diltiazem will be a useful approach to study AO defenses in the prodromal stage of aging or diseased retina.

Previously we have shown that 1 hour post d-cis-diltiazem treatment in dark-adapted B6 mice, OR oxidative stress occurs in inferior retina as measured by QUEST MRI.\(^\text{3}\) However, in this study, we found evidence that both inferior and superior OR had oxidative stress (data in Figs. 3, 4). In addition to spatial resolution and thus is better able to detect subtle microstructural changes than the lower-resolution MRI. On the other hand, QUEST MRI interrogates all retinal layers for oxidative stress, whereas QUEST OCT is limited to the outer segment and RPE layers.

In summary, the main finding here was a demonstration for the first time of a new functionality for OCT: the ability to measure localized oxidative stress in vivo. The present study carefully built on our previous findings that light-evoked expansion of the OR is an essential physiology measurable by OCT, and that light-evoked expansion of the OR is sensitive to oxidative stress as measured by MRI.\(^\text{1,36}\) However, MRI is not as widely available as OCT, and the OCT photoresponse has not previously been shown to be a useful index for measuring oxidative stress. The present results are important because they address a longstanding problem, namely, that conventional assays are unable to noninvasively measure OR oxidative stress in vivo and potentially in patients to personalize antioxidant treatment options. We also reported that combining QUEST OCT with an acute administration of d-cis-diltiazem is a unique way to noninvasively evaluate OR AO defenses. Finally, we found that the OR thickness in the light alone can be used to detect OR oxidative stress, an observation that may help facilitate translation of QUEST OCT into a clinical setting. QUEST OCT appears to be a promising new clinically relevant tool for early diagnosis and individualized AO treatment in sight-threatening diseases with an OR oxidative stress etiology.
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