Cataracts are the leading cause of vision loss in humans, and this is also likely true for dogs. Surgical removal via phacoemulsification is currently the accepted standard of care for resolution of cataracts. Posterior capsule opacification is the most common complication following cataract surgery, and it reportedly occurs in up to 100% of canine patients. Although the pathogenesis of PCO is not completely understood, cataract surgery likely initiates a wound-healing response that stimulates remaining LECs. Through posterior migration, proliferation, and differentiation of residual LECs, PCO can progressively obstruct the visual axis and result in secondary loss of vision. Strategies to prevent PCO have largely been aimed at altering surgical and mechanical factors (eg, changes to the biomaterial and design of a surgically implanted intraocular lens) and more diligent polishing of the lens capsule during surgery. In a recent study, investigators found a nonsignificant reduction of PCO in dogs following implantation of an acrylic lens in combination with use of NSAIDs; all dogs of that study developed PCO. Pharmacological attempts to prevent PCO via targeted destruction of LECs represent an important area for PCO research. Several agents have been investigated for prevention of PCO, including chemotherapeutic agents, gene treatments, immunosuppressive drugs, and anti-inflammatory drugs. Unfortunately, none of these methods has proven successful in a clinical setting.

Effects of grape seed extract, lutein, and fish oil on responses of canine lens epithelial cells in vitro

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OBJECTIVE
To determine the effects of grape seed extract (GSE), lutein, and fish oil containing omega-3 fatty acids on oxidative stress, migration, proliferation, and viability of lens epithelial cells (LECs).

SAMPLE
Lens capsules or cultured LECs obtained from canine cadavers.

PROCEDURES
An antioxidant reductive capacity assay was used to determine reducing capability of each substance. The LECs were cultured and incubated with various substances, including N-acetyl cysteine (NAC), when appropriate, and dimethyl sulfoxide (DMSO) as positive and vehicle control substances, respectively. A dichlorofluorescein assay was used to evaluate reactive oxygen species (ROS) production, and a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay was used to determine cell viability. Ex vivo posterior capsule opacification (PCO) was used to evaluate LEC migration and proliferation.

RESULTS
Antioxidant reductive effects of GSE surpassed those of NAC, lutein, and fish oil containing omega-3 fatty acids. The GSE reduced ROS production in LECs, compared with the DMSO vehicle control, whereas lutein was pro-oxidative. All test substances reduced cell viability. Ex vivo PCO was not altered by GSE, was decreased by lutein, and was increased by fish oil containing omega-3 fatty acids, compared with results for the DMSO vehicle control.

CONCLUSIONS AND CLINICAL RELEVANCE
Only GSE had significant antioxidant capabilities and reduced ROS production; however, no effect on ex vivo PCO was detected. Fish oil containing omega-3 fatty acids increased ex vivo PCO. No conclusions could be made regarding antioxidant effects of these substances on LECs. These findings suggested that the substances will not decrease PCO.
Reactive oxygen species may be one of the factors that plays a role in LEC behavior following cataract surgery. Frequently, ROS are considered mediators of cell damage. Although research has focused on developing methods to reduce the damaging effects of ROS, it has been suggested that production of ROS at sublethal concentrations can be beneficial or even necessary for cell survival and function. Use of ROS as signaling metabolites is known as redox signaling. Of particular importance, stimulation of low-level ROS production by growth factors promotes migration and proliferation in a variety of cells, including LECs. The ROS can act as signal intermediates for several growth factors, including transforming growth factor-β and epidermal growth factor, both of which have been recognized as growth factors important in PO. Furthermore, expression of platelet-derived growth factor in the lens results in ROS generation; in turn, ROS regulate diverse downstream signaling pathways within LECs, which stimulates the LECs to undergo migration. Studies involving the use of human LECs have revealed that proliferation and mitogenic signaling can be ROS-dependent events. Although these findings suggest that ROS-mediated signaling pathways are important to lenticular development and physiologic function, the role of low-level ROS production in relation to PCO currently is unknown.

Use of antioxidants has been promoted to slow aging and prevent disease. Several antioxidant products are commercially available for humans and companion animals. Antioxidant products for dogs are often sold as oral formulations that are marketed for promoting general health, ocular health, immune function, or liver health. Antioxidant products to promote general health are generally regarded as safe. Specific literature to support the safety of GSE, lutein, and omega-3 fatty acids in dogs is sparse and to the authors’ knowledge, no reports of major adverse effects have been reported.

A novel approach for reducing PCO is the administration of antioxidants during the perioperative period to reduce redox signaling. Use of antioxidants to prevent oxidative damage to cells is not uncommon. Traditionally, the positive effects of antioxidants are thought to be a result of a reduction of cell damage mediated by high concentrations of ROS. Ocular examples for this approach include the ability of GSE, omega-3 fatty acids, and lutein to exert beneficial effects related to cataractous changes, oxidative damage to retinal pigmented epithelial cells, and macular degeneration, respectively. However, in contrast to the traditional role whereby ROS induce cellular damage, evidence exists that sublethal concentrations of ROS play an important role in growth factor–stimulated proliferation and migration of LECs. Therefore, we postulated that use of antioxidants to mitigate lens redox signaling would reduce LEC proliferation and migration and could subsequently prevent or delay PCO. This supposition is supported by the fact that lutein inhibits proliferation and migration of bovine LECs in vitro.

It has been suggested that antioxidants can alter in vitro LEC behavior. Therefore, use of antioxidants would appear to be a reasonable potential therapeutic approach for PCO prevention. The purpose of the study reported here was to evaluate the effects of GSE, lutein, and fish oil containing omega-3 fatty acids on oxidative stress, migration, proliferation, and viability of LECs. The objective was to determine whether these commonly available products could prevent or modify ex vivo PCO in canine tissues. We hypothesized that the products would act as antioxidants and decrease in vitro PCO by reducing redox-stimulated proliferation and migration of canine LECs.

**Materials and Methods**

**Sample**

Grossly normal eyes were collected from cadavers of dogs that had been euthanized for reasons unrelated to the study reported here. Dogs were euthanized by administration of an overdose of sodium pentobarbital, and globes were collected within 1 to 2 hours after the dogs were euthanized. Globes were rinsed with iodine solution and stored for up to 24 hours in PBS solution at 4°C until time of cell harvest. All protocols were approved by The Ohio State University Institutional Animal Care and Use Committee.

**Canine LEC cultures**

Canine LECs were harvested. Globes were manually held in position with forcesps, and the cornea and iris were removed. Once the anterior lens surface was fully exposed, a continuous curvilinear capsulorrhexis was performed by creating a small capsular opening with capsulotomy scissors. Continuous circular tearing of the capsule with capsulorrhexis forcesps was used to remove approximately 60% of the axial portion of the lens capsule. That portion of the lens capsule and its associated LECs was placed in a microcentrifuge tube containing 0.25% trypsin and stored for up to 24 hours in PBS solution at 4°C until time of cell harvest. All protocols were approved by The Ohio State University Institutional Animal Care and Use Committee.

**Preparation of test substances**

Products containing GSE, lutein, and fish oil containing omega-3 fatty acids were obtained. The concentrations used were based on a commercially available veterinary dietary product. Each product was...
derived from natural sources; thus, the purity of all tested products was unknown. However, the manufacturer indicated that purity of the GSE was 95%, the lutein was derived from marigolds and comprised 20% of the product, and the fish oil contained 55% eicosapentaenoic acid and docosahexaenoic acid. Fish oil containing omega-3 fatty acids and GSE were dissolved in DMSO and diluted with culture medium (DMEM containing 1% antimicrobial) to achieve working concentrations of 50 mg/L (fish oil containing omega-3 fatty acids) and 25 mg/L (GSE). Lutein was insoluble in DMSO; therefore, it was dissolved in 100% ethanol and diluted with culture media to create a working solution with a concentration of 15 mg/L. Culture medium containing 0.09% DMSO was used as a vehicle control. When appropriate, NAC was used as a positive antioxidant control substance and was prepared in deionized water and diluted with DMEM to achieve a 1mM solution.

**Antioxidant reductive capacity assay**

A commercially available antioxidant reductive capacity assay was used to determine the relative antioxidant ability of the various substances. The assay measured the ability of aqueous samples to reduce Cu²⁺ to Cu⁺; the Cu⁺ then reacted with bathocuproine to form a complex with a colorimetric absorbance between 480 and 490 nm. Therefore, measurement of samples before and after the addition of bathocuproine created a net difference that was directly proportional to the reductive capacity. Net absorbance values were compared with values on a standard curve generated by use of a water-soluble analog of vitamin E. A solution (2μM water-soluble analog of vitamin E) provided with the assay was serially diluted to create 6 standards with concentrations ranging from 2 to 0.063μM. Each GSE, lutein, and fish oil containing omega-3 fatty acids product (n = 8) and standard was diluted (dilution, 1:40) by use of the dilution buffer (which contained bathocuproine) that was provided with the assay. Diluted solutions then were added to a microplate. Absorbance of each sample or standard was measured with an automated microplate reader. The Cu⁺⁺ solution was added to each well and allowed to incubate for 3 minutes at room temperature (21°C). Stop solution provided with the assay was then added to each well, and the absorbance was measured again. The difference in absorbance for each well was calculated and used to create a standard curve. Results were reported as mean number of copper-reducing equivalents.

**Dichlorofluorescein assay for detection of ROS generation**

The LECs were incubated with trypsin, and harvested cells were manually counted by use of a standard hemacytometer and light microscope. Cultured canine LECs were plated in a 96-well laminin-coated microplate at a concentration of approximately 10,000 cells/well and incubated with DMEM at 37°C overnight. Dichlorofluorescein reagent was diluted (final concentration, 5μM) with PBS solution in accordance with the manufacturer’s instructions and added to each well. Cells were incubated with the dichlorofluorescein reagent at 37°C for 20 minutes. The dichlorofluorescein reagent was removed, and cells were washed gently with PBS solution. To induce ROS, cells were exposed to UV light (600 J/m²). Culture medium was removed and replaced with PBS solution, and cells were then placed under a UV source (emittance range, 290 to 320 nm). Immediately after exposure to UV light was completed, cells were incubated (100 μL/substance) with fresh DMEM containing DMSO (0.09%; vehicle control), GSE (25 mg/L), fish oil containing omega-3 fatty acids (50 mg/L), lutein (15 mg/L), or NAC (1μM) at 37°C for 6 hours (n = 8). Fluorescence was then measured by use of the automated microplate reader, with ROS production directly proportional to the measured fluorescence. Results were reported as median and IQR percentage of fluorescence.

**MTT assay of cell proliferation and viability**

A commercially available MTT assay kit was used. Cultured canine LECs were plated in a 96-well laminin-coated microplate; 100 μL of DMEM was added to each well, and plates were incubated at 37°C and 5% CO₂ overnight. The following day, the medium was aspirated and replaced (100 μL/substance) with DMEM containing DMSO (0.09%; vehicle control), GSE (25 and 12.5 mg/L), fish oil containing omega-3 fatty acids (50 and 10 mg/L), lutein (15 and 7.5 mg/L), or mitomycin C (0.002 mg/L; n = 7). Cells were incubated for 24 hours. The medium was then replaced with fresh DMEM, and 12μM MTT was added to each well; wells were incubated for 4 hours at 37°C. Then, 50 μL of DMSO was added to each well, and wells were incubated for 10 minutes at 37°C. Absorbance was measured by use of the automated microplate reader. Results were reported as median and IQR absorbance.

**Ex vivo PCO assessment**

Mock cataract surgery was performed on canine globes, as described elsewhere. Enucleated globes were manually held in position with forceps, and the cornea was removed by creating a circumferential grooved incision in the sclera approximately 2 mm caudal to the limbus with a No. 10 scalpel blade. The incision was completed with tenotomy scissors, and the cornea was removed. Iris scissors were then used to remove the iris by initially transecting radially from the pupil to the base and then transecting circumferentially at the base. Once the anterior lens surface was fully exposed, a continuous curvilinear capsulorrhexis was performed as described previously for cell culture. Lens fibers were removed with a lens loop, and capsules were washed by use of gentle irrigation with PBS solution and a 22-gauge cannula.

772 AJVR • Vol 79 • No. 7 • July 2018
The lens capsule was freed from the zonular attachments with capsulotomy scissors and placed in a culture dish with DMEM containing DMSO (0.09%; vehicle control), GSE (25 mg/L), fish oil containing omega-3 fatty acids (10 mg/L), or lutein (15 mg/L; n = 6). Medium was changed every 2 days throughout the incubation period. Inverted phase-contrast microscopy was used to estimate the percentage of LEC confluence on the posterior capsule and obtain photomicrographs on days 0 (day of initial preparation), 2, 4, 6, 8, 10, 12, 14, and 15. After the 15-day evaluation period was completed, capsules were placed in formalin, sectioned, and stained with H&E stain for histologic evaluation and determination of cell counts. Owing to experimental error, 1 lens capsule from each of the incubations with GSE and fish oil containing omega-3 fatty acids was removed from the ex vivo PCO evaluation and histologic cell assessment. The results for capsule coverage were reported as median percentage of confluence over time. Results for histologic cell counts were reported as median and IQR total cell number of 6 histologic sections/capsule.

Data analysis
An ANOVA with a Tukey posttest was used to determine differences in antioxidant reductive capacity (differences in mean number of copper-reducing equivalents among substances). Because of unequal variances, data for the dichlorofluorescein assay, MTT assay, and ex vivo PCO were considered nonparametric; therefore, they were compared by use of the Kruskal-Wallis test with a Conover post hoc analysis for multiple comparisons. For ex vivo PCO, median percentage of posterior capsular confluence over time was reported, and differences among substances were compared for values obtained on day 15 (end of study).

Statistical analyses were performed with commercially available software. Values of \( P < 0.05 \) were considered significant.

Results

Antioxidant reductive capacity assay
The reductive capacity of fish oil containing omega-3 fatty acids and lutein was negligible (Figure 1). However, GSE surpassed the reductive capacity of NAC (positive control substance). The reductive capacity of GSE differed significantly (\( P < 0.001 \)) from that for fish oil containing omega-3 fatty acids or lutein.

Dichlorofluorescein assay for detection of ROS generation
A dichlorofluorescein assay was used to determine ROS production in LECs incubated with the test products and exposed or not exposed to UV light. For LECs that were not exposed to UV light, lutein significantly increased ROS production (median fluorescence, 69.5%; IQR, 53.0% to 84.5%) but NAC significantly decreased ROS production (median fluorescence, 20.0%; IQR, 16.5% to 24.0%), compared with the DMSO vehicle control LECs (median fluorescence, 44.0%; IQR, 34.5% to 49.5%). The ROS production for LECs that were not exposed to UV light and incubated with fish oil containing omega-3 fatty acids or GSE did not differ significantly, compared with ROS production for the DMSO vehicle control LECs. As expected, UV exposure induced ROS production in all LECs regardless of incubation substance. When evaluating the effects of incubation with various substances...
on UV-exposed LECs, GSE (median fluorescence, 51.0%; IQR, 42.5% to 57.5%) or NAC (median fluorescence, 47%; IQR, 37.0% to 55.5%) significantly reduced ROS production, compared with ROS production for UV-exposed DMSO vehicle control LECs (median fluorescence, 71.5%; IQR, 60.5% to 107.5%). Following UV exposure, LECs incubated with fish oil containing omega-3 fatty acids and lutein (median fluorescence, 82.5% [IQR, 58.5% to 92.0%] and 78% [IQR, 57.5% to 105.0%], respectively) did not have a significant reduction in ROS production, compared with ROS production of UV-exposed DMSO vehicle control LECs (Figure 2).

**MTT assay of cell proliferation and viability**

Cells incubated with GSE, lutein, or fish oil containing omega-3 fatty acids all had changes in cell morphology and a lower cell density, compared with results for the vehicle control LECs (Figure 3). Because morphological changes were detected, an MTT assay was performed to evaluate LEC viability and proliferative activity. After LECs were incubated with various substances for 24 hours, GSE, lutein, and fish oil containing omega-3 fatty acids at all concentrations reduced cell viability and proliferation; however, this change was significantly different only when comparing results for GSE (10 mg/mL), lutein (7.5 mg/mL), or lutein (15 mg/mL) with results for the DMSO vehicle control (Figure 4).

**Ex vivo PCO**

In other studies, our research group has established that use of our ex vivo PCO methods accurately represents cellular changes during naturally occurring PCO. Therefore, as expected, there was proliferation and migration of LECs in all capsules over time in the present study. At day 15 (end of study), the median percentage for posterior capsule coverage was 100%, 50%, 10%, and 50% for fish oil containing omega-3 fatty acids, GSE, lutein, and the DMSO vehicle control, respectively. When compared with capsule coverage for the vehicle control substance, capsule coverage was significantly higher for fish oil containing omega-3 fatty acids, significantly lower for lutein, and not different for GSE (Figure 5). Capsules were fixed in formalin on day 15, and histologic cell counts were performed. Capsules incubated with fish oil containing omega-3 fatty acids contained a median of 473.0 cells (IQR, 350.1 to 520.7 cells), whereas the median number of cells was significantly fewer for capsules incubated with lutein (64.6 cells; IQR, 10.0 to 84.0 cells), GSE (308.7 cells; IQR, 132.0 to 572.9 cells), or the DMSO vehicle control (230.2 cells; IQR, 202.2 to 258.8 cells).
compared with results for the vehicle control capsules, there was a significant difference from results for capsules incubated with fish oil containing omega-3 fatty acids or lutein but not results for capsules incubated with GSE (Figure 6). These cell counts compared favorably with the percentage of posterior capsular coverage determined by use of inverted microscopy.

**Discussion**

We hypothesized that 3 common commercially available products that contained GSE, lutein, and fish oil containing omega-3 fatty acids would act as antioxidants and decrease in vitro PCO by reducing redox-stimulated LEC proliferation and migration. The potential effectiveness of the test products was evaluated. Overall reductive capacity was determined initially in the absence of LECs; only GSE functioned as an antioxidant. These data corroborated well with results for the dichlorofluorescein assay, whereby it was determined that GSE was the only test product capable of reducing ROS production in LECs exposed to UV light. Grape seed extract possesses substantial reducing power. Investigators of 1 study\(^{45}\) found that GSE could scavenge oxygen radicals better than could vitamins C or E. Furthermore, GSE can reduce oxidative stress in both human and canine LEC cultures.\(^{35,36}\)

In contrast, lutein and fish oil containing omega-3 fatty acids had negligible reducing power in the present study. This finding may be explained by inadequate quantities of lutein or fish oil containing omega-3 fatty acids in the tested products. The pro-oxidative effect of lutein detected with the dichlorofluorescein assay was unexpected. Although carotenoids often act as antioxidants, they can have a pro-oxidative effect with various concentrations, oxygen tensions, and biological tissues (e.g., through interactions with other cellular antioxidants).\(^{46}\) The reason for this shift from antioxidant to pro-oxidant, although not fully understood, could explain the findings for the study reported here.

Morphological changes of the LECs incubated with the test products prompted us to evaluate cell viability. This was an unexpected finding given that dosages were similar to those used in other studies.\(^{35,36,39,40}\) Results for the MTT assay confirmed that cell viability was decreased by all the tested products, compared with viability for the vehicle control substance. Antioxidants can cause lipid peroxidation and subsequent cellular damage by affecting the redox cycling of iron.\(^{47}\) This phenomenon could explain the decreased cell viability caused by the addition of the products in the study reported here. Additionally, although concentrations of the substances used in the present study were based on information in the literature, no dosing or toxicity curves have been evaluated to determine optimal nontoxic antioxidant concentrations.

An established ex vivo canine lens capsule method was used to determine possible effects of the test products on PCO. Although GSE had no effect, fish oil containing omega-3 fatty acids accelerated ex vivo PCO, and lutein reduced posterior capsule coverage. The decrease in PCO for lutein was unlikely to have been related to a hypothesized decrease in redox signaling because lutein was unable to reduce ROS production. This result could have been explained by ROS-induced apoptosis attributable to lutein’s pro-
oxidative effect because a large body of evidence indicates that oxidative stress can induce apoptosis in a lens.\textsuperscript{48–52} In comparison, the increase in ex vivo PCO for fish oil containing omega-3 fatty acids could have been explained by redox signaling. As indicated by results of the dichlorofluorescein assay, fish oil containing omega-3 fatty acids slightly increased ROS production. This small generation of ROS may have occurred for the capsule method and provided sufficient ROS production to promote redox signaling and subsequent LEC migration and proliferation. An alternate possibility would have been an unknown positive interaction between the LECs and capsule attributable to the presence of fish oil. The lens capsule is an important regulator of LEC adhesion, migration, and proliferation and can function as a reservoir.\textsuperscript{53–56} It is possible the presence of the lens capsule in the ex vivo method may have played a role in the fish oil–induced PCO if the presence of fish oil containing omega-3 fatty acids promoted supportive changes in the capsule matrix components. Overall, it was likely that the tested products did not have the ability to reduce PCO in a clinically relevant manner. When compared with results for the DMSO vehicle control LECs, even with a slowed rate of ex vivo PCO for lutein, there was a continuous increase of LEC proliferation and migration; this cellular behavior would likely have continued with time.

The present study had several limitations. First, the test substances were not considered pure and likely contained other unknown substances. Thus, precise concentrations for each product could not be determined, and the cellular effects specific to the products could not be conclusively determined. The quality of the provided products also was questionable. All test products were obtained from the manufacturer of a veterinary dietary product\textsuperscript{42}; 2 of the test products did not have substantial reducing capabilities. Our hypothesis was that the antioxidant effects on LEC behavior would be through a reduction of ROS important for cellular functions (ie, redox signaling). Because of the inability of 2 of the test products to reduce ROS, the hypothesis could not be fully tested or proven. Owing to the fact that the lutein product was insoluble in DMSO, ethanol was used as the solvent. The vehicle control substance used throughout the experiments was DMSO alone; this could have potential implications on how the data were analyzed and was a study flaw. However, the final concentration of both DMSO and ethanol as solvent was < 0.1%. On the basis of results of previous studies,\textsuperscript{35,44,57–60} it was unlikely that the solvent substantially altered study outcomes.

Another limitation was the lack of information regarding a safe or effective dose range for the products with regard to LECs. The GSE and lutein concentrations were similar to those used in other studies\textsuperscript{35,36,39,40} conducted to evaluate the ability of these products to reduce oxidative stress in LECs. To the authors’ knowledge, no studies have been conducted to evaluate the effects of fish oil or omega-3 fatty acids on LECs. Lutein can inhibit migration and proliferation of bovine LECs, although the exact mechanism was not investigated.\textsuperscript{59} Our hypothesis that lutein and fish oil containing omega-3 fatty acids would decrease LEC migration through regulation of redox signaling could not be proven in the present study because the tested products lacked reducing capabilities.

Future research should include establishing an appropriate dose range with high-quality antioxidants to exclude the variability of contaminants and reduce cellular toxicosis. To the authors’ knowledge, there is no information on ocular pharmacokinetics of these substances. Carotenoids (ie, lutein zeaxanthin) are found in multiple ocular tissues such as the retina and lens and undoubtedly come from the diet, which indicates an ability to reach the eyes.\textsuperscript{60} However, in vivo detection of these substances in the aqueous humor after oral or topical administration is vital to determine whether the substances can pass ocular barriers and become available to the LECs. Without this information, further studies with these products may not be of value.

In the study reported here, we sought to determine whether GSE, lutein, and fish oil containing omega-3 fatty acids could alter the behavior of LECs in vitro. Of these products, only GSE acted as an antioxidant at the concentrations tested. All the test products had a negative effect on the viability of cultured LECs. Finally, by use of an ex vivo method, PCO was slowed by lutein, increased by fish oil containing omega-3 fatty acids, and unaltered by GSE, compared with results for the DMSO vehicle control. No conclusions should be drawn regarding the ability of antioxidants to alter PCO because the overall antioxidant power of the products used in this study was weak. These findings did not support the use of the tested products for reduction of PCO in canine patients.

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Footnotes

a. Gibco, Carlsbad, Calif.
b. Beckton-Dickinson, Franklin Lakes, NJ.
c. Provided by Animal Necessity, New York, NY.
d. Ocu-GLO, Animal HealthQuest Solutions, Bellingham, Wash.
f. Trolox, Sigma-Aldrich Inc, St Louis, Mo.
g. Infinite 200, Tecan, Mannedorf, Switzerland.
h. Molecular Probes Inc, Eugene, Ore.
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