Effect of grape polyphenols on oxidative stress in canine lens epithelial cells

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Objective—To evaluate whether the effects of oxidative stress could be attenuated in cultures of canine lens epithelial cells (LECs) by incubation with grape seed proanthocyanidin extract (GSE), resveratrol (RES), or a combination of both (GSE+RES).

Sample Population—Primary cultures of canine LECs.

Procedures—LECs were exposed to 100µM tertiary butyl-hydroperoxide (TBHP) with or without GSE, RES, or GSE+RES. The dichlorofluorescein assay was used to detect production of reactive oxygen species (ROS), and immunoblot analysis was used to evaluate the expression of stress-induced cell-signaling markers (ie, the mitogen-activated protein kinase [MAPK] and phosphoinositide-3 kinase [PI3K] pathways).

Results—GSE and GSE+RES significantly reduced ROS production after a 30-minute exposure to TBHP. Only GSE significantly reduced ROS production after a 120-minute exposure to TBHP. Incubation with GSE reduced TBHP-induced activity of the MAPK and PI3K pathways.

Conclusions and Clinical Relevance—GSE inhibited key components associated with cataractogenesis, ROS production, and stress-induced cell signaling. On the basis of the data reported here, there is strong evidence that GSE could potentially protect LECs from the damaging effects of oxidative stress. (Am J Vet Res 2008;69:94–100)

Oxidative stress has been defined as a state in which the overall production of ROS exceeds the overall antioxidant defenses. The term ROS describes components that cause oxidative stress, including oxygen radicals, superoxide anions, hydroxyl radicals, hydroxyl anions, and hydrogen peroxide. Production of ROS can result in lipid peroxidation, protein oxidation, and DNA damage. Oxidative stress has been linked to chronic diseases in humans, including diabetes mellitus, Alzheimer’s disease, Parkinson’s disease, age-related macular degeneration, proliferative vitreoretinopathy, and cataracts.

Lenses are one of the most oxidative stress in the form of UV radiation as well as other environmental and endogenous factors. To combat these problems, lenses are equipped with several means for resisting oxidative damage, which include ascorbic acid, ferritin, thioredoxin, catalase, and the glutathione reductase system. However, these endogenous antioxidants decrease with age and cataract formation.

Proanthocyanidins are natural compounds found in high concentrations in fruits, vegetables, wine, tea, nuts, seeds, flowers, and bark. Proanthocyanidins have a wide range of biological activities, such as the capability to scavenge oxidants and free radicals, anti-inflammatory and antimicrobial properties, inhibition of growth of cancer cells, prevention of oxidation of low-density lipoproteins, cardioprotection, and inhibition of viral replication. In vitro experiments in which investigators used GSE revealed that GSE is a more potent free-radical scavenger, compared with the scavenging ability of vitamin C or vitamin E. Furthermore, GSE can prevent cataract formation in rats predisposed to hereditary cataracts. In that study, investigators determined that a diet supplemented with GSE...
significantly decreased the grade of cataract, compared with results for those consuming a typical, nonsupplemented diet. However, to our knowledge, no studies have been conducted to evaluate the mechanisms by which GSE attenuates cataractous changes.

Resveratrol (3,4’,5-trihydroxy-trans-stilbene) is a polyphenolic compound found in grapes, peanuts, and mulberries. It has antimycotic, antiproliferative, antiviral, antioxidant, and anti-inflammatory properties. In human retinal pigmented epithelial and HeLa cells, RES can inhibit the activation of MAPK signaling molecules, such as ERK 1/2, JNK, and p38, which control cell migration, proliferation, and differentiation. Also, RES can prevent selenite-induced cataracts in rodents.

The study reported here was designed to determine the effects of GSE, RES, or GSE+RES on oxidative stress, which is associated with cataract formation in canine LECs. If oxidative stress could be attenuated by these compounds, then perhaps cataract formation could be prevented or slowed by dietary supplementation with these compounds. Delaying cataract formation would be beneficial because it has been estimated that a delay of 10 years for cataract formation in humans would decrease sight-threatening cataracts by 45%. Therefore, even a slight delay in the progression of cataract formation could provide increases in visual acuity.

Materials and Methods

Sample population—Primary cultures of canine LECs were used in the study. Samples were obtained from 10 eyes of dogs during each of 10 visits to the local animal shelter (approx 100 eyes total) immediately after dogs were euthanized by administration of an overdose of a barbiturate.

Culture of canine LECs—Globes were obtained by enucleation from dogs with clinically normal eyes and in good general health that were euthanized at a local animal shelter for population control purposes. The animals were humanely euthanized, and the globes were collected within 1 hour after euthanasia. Eyes were immediately placed in 2% betaine solution and then rinsed and immersed in 1X PBS solution (pH, 7.2) until dissection, which was performed within 2 hours after enucleation. Lenses were excised as previously described. Briefly, each anterior lens capsule with adherent LECs was dissected, placed in trypsin (0.23% trypsin and 1X EDTA), and incubated at 37°C for 5 minutes. After incubation, the solution and lens capsule were centrifuged for 3 minutes at 0.3 X g. Fluid was decanted, and complete medium (Dulbecco minimal essential medium containing 10% fetal bovine serum and 1% antimicrobial) was then added. The solution and lens capsule were centrifuged again. The solution, including the lens capsule, was transferred into a 25-mm laminin-coated tissue culture flask and incubated in a humidified incubator at 37°C and 5% carbon dioxide.

The LECs were grown until 80% to 90% confluence; then the LECs were incubated in trypsin, centrifuged to form a pellet, resuspended, counted, and placed into appropriate plates at an appropriate concentration, depending on the experiment being performed.

Ability of grape polyphenols to scavenge ROS—The LECs were placed into each well (1.5 X 10⁶ cells/well) of a 96-well plate that had been coated with laminin–human fibronectin. Plates were incubated overnight in complete media in a humidified incubator at 37°C and 5% carbon dioxide. The LECs were then placed in incomplete medium (Dulbecco minimal essential medium containing 1% antimicrobial) that contained vehicle (1% dimethyl sulfoxide), GSE (20 mg/L), RES (100mM), GSE+RES (20 mg/L), or 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (vitamin E analogue; 500mM) for 1 hour in a humidified incubator at 37°C and 5% carbon dioxide. Activin GSE was a highly soluble, high-grade proanthocyanidin extract from 100% pure grape seeds (Vitis vinifera) and dried roots of Polygonum cuspidatum. The GSE was extracted in a water-only extraction process that resulted in a 100% pure (dry-weight basis) grape seed extract that contained 6% free flavanol monomers. It comprised 80% to 95% phenols and contained < 5,000 µg of trans-RES/g. Grape seed extract has been deemed by the US FDA to be generally regarded as safe for use in food and beverages and as a dietary supplement. The GSE+RES (also called IH636) was a highly soluble, high-grade proanthocyanidin extract from 100% pure grape seeds (V vinifera) and dried roots of P cuspidatum. The GSE+RES was extracted by a water-only extraction process that resulted in a 100% pure (dry-weight basis) extract that contained 6% free flavanol monomers. It comprised at least 80% phenols and contained > 5,000 µg of trans-RES/g.

Initial doses were chosen on the basis of published literature. Then, a range of concentrations for the cells was evaluated in our study to determine toxic effects of the compounds, which was defined as the highest dose that did not cause cell death or changes in cell morphologic characteristics or behavior. Medium was removed and replaced with incomplete medium containing 20µM 2’,7’-dichlorodihydrofluorescein diacetate, and LECs were then incubated for another hour. Medium was again removed, and LECs were washed once with sterile 1X PBS solution. The LECs were then placed back into incomplete medium with or without 100µM TBHP. Immediately after the addition of TBHP, the 96-well plate was transferred into a fluorescent plate reader, which maintained an ambient temperature of 37°C and recorded values at an excitation of 485 nm and an emission of 528 nm at 3-minute intervals for 2 hours. The dichlorofluorescein assay was used to measure the ability of GSE, RES, GSE+RES, and vitamin E analogue to inhibit the production of intracellular ROS. This experiment was performed 3 times, with each experiment yielding similar results.

Effects of grape polyphenols on oxidative stress–induced cell signaling—Western immunoblotting was used to evaluate changes in markers of oxidative stress, including p-ERK 1/2, p-JNK, p-SAPK/JNK, and p-Akt 1/2. The experimental design was identical to that used for evaluation by use of the dichlorofluorescein assay except that, after incubation with or without 100µM TBHP for 30 minutes in a humidified incubator at 37°C and 5% carbon dioxide, wells were washed once with sterile 1X PBS solution, and plates were then frozen im-
mediated at a −80°C freezer until protein was extracted for western blot analysis. These experiments were repeated 3 times, with each experiment yielding similar results.

**Western blot analysis**—Protein was extracted with 1X lysis buffer in accordance with the manufacturer’s instructions. Western blot analysis was performed as described elsewhere. Primary antibodies were diluted in 1X Tris-buffered saline solution–Tween-20 containing 5% bovine serum albumin as follows: p-ERK 1/2, ERK 1/2, p-p38, p38, p-SAPK/JNK, and SAPK/JNK were diluted at 1:1,000; p-Akt 1/2/3 and Akt 1/2 were diluted at 1:500; and β-actin was diluted at 1:5,000. Anti–β-actin antibody was used as the loading control sample. Image analysis software was used to obtain densitometry readings for all western blots.

**Statistical analysis**—All statistical analyses were performed with commercially available software. All data were analyzed by use of a 1-way ANOVA with a Tukey post test. Values of *P* < 0.05 were deemed significant. All graphs were generated by commercially available software and provided the mean and SEM.

**Results**

**Ability of grape polyphenols to scavenge ROS**—Results for all treatment groups were compared with results for the TBHP-treated group. After incubation for 1 hour with the test compounds, GSE significantly (*P* = 0.01) reduced ROS production by 37.1%. After incubation for 30 minutes with TBHP, RES reduced ROS production by 13.4%, which was not a significant decrease. The GSE+RES significantly (*P* = 0.01) reduced ROS production by 29.2%, and incubation with vitamin E analogue caused a significant (*P* < 0.001) reduction of 68.9% in ROS production after incubation for 30 minutes (Figure 1). After treatment with TBHP for 120 minutes, GSE, RES, and GSE+RES all caused a decrease in ROS production. The GSE significantly reduced ROS production by 34.1%, whereas RES and GSE+RES reduced ROS production, but not significantly, by 12.2% and 19.4%, respectively. Vitamin E analogue significantly (*P* < 0.001) reduced ROS production by 72.1%.

**Effects of grape polyphenols on oxidative stress–induced cell signaling**—Oxidative stress–induced cell signaling was evaluated by western immunoblotting for activated p38, ERK 1/2, SAPK/JNK, and Akt 1/2. Results for each antibody were adjusted to results for β-actin, and results for all treatment groups were compared with those for the TBHP-treated group. The TBHP-induced activity of p38 was reduced by 72.6% with GSE treatment and 5.4% with GSE+RES treatment. Both RES...
and vitamin E analogue increased p38 activity by 22.3% and 2.1%, respectively (Figure 2). All treatment groups reduced TBHP-induced ERK 1/2 activity, with GSE reducing it by 65.7%, RES reducing it by 8.7%, GSE+RES reducing it by 75.4%, and vitamin E analogue reducing it by 56.2% (Figure 3).

The TBHP-induced activation of the SAPK/JNK pathway was reduced in all of the treatment groups. Treatment with GSE significantly (P = 0.01) reduced activation of the pathway by 77.0%, whereas GSE+RES significantly (P < 0.05) reduced it by 85.1%, and vitamin E analogue significantly reduced it by 67.0% (Figure 4). Treatment with RES reduced activation of the SAPK/JNK pathway by 36.1%, which was not a significant reduction. Treatment with GSE reduced TBHP-induced Akt 1/2 activity by 51.5%, and RES decreased Akt 1/2 activity by 5.8%; however, GSE+RES and vitamin E analogue increased Akt 1/2 activity by 10.3% and 30.5%, respectively (Figure 5). The GSE was the only compound tested that decreased TBHP-induced activity of p38, ERK 1/2, SAPK/JNK, and Akt 1/2.

**Discussion**

Dietary components such as polyphenols, vitamins, organic extracts, and proanthocyanidins possess a wide range of biological activities with the potential to prevent, inhibit, or delay the progression of chronic disease.\(^1\)\(^,\)\(^4\)\(^,\)\(^11\) A major mechanism by which many of these dietary components cause effects is through reducing oxidative stress and associated stress-activated cellular-signaling pathways. Dietary modulation of MAPK and PI3K pathways has emerged as a potential target of dietary antioxidants.\(^5\)\(^,\)\(^6\) Members of these 2 pathways, as well as p38, ERK 1/2, SAPK/JNK, and Akt, are involved in the regulation of cellular differentiation, migration, and proliferation as well as cell survival.\(^22\)\(^-\)\(^26\) Oxidative stress–induced cell signaling, cell migration, and inflammation are associated with cataract formation.\(^1\)\(^-\)\(^4\)\(^,\)\(^7\)\(^,\)\(^8\)

It has been found that GSE and RES attenuate processes associated with cataractogenesis as well as cause slowing or inhibition of cataract formation in rodents.\(^4\)\(^,\)\(^10\)\(^,\)\(^27\)\(^,\)\(^28\) Furthermore, GSE and RES have been found to be nontoxic in studies involving rodents and dogs; most cases of grape or raisin toxicosis are believed to be caused by a compound contained in the skins of the fruits and associated with massive consumption (10 to 57 g/kg).\(^13\)\(^,\)\(^16\)\(^,\)\(^22\) Therefore, the study reported here was conducted to evaluate the effects of GSE and RES on factors that contribute to cataract formation.
including ROS production and oxidative stress–induced cell signaling.

Treatment of LECs with GSE and GSE+RES significantly decreased TBHP-induced intracellular ROS production. However, RES treatment alone did not significantly decrease TBHP-induced ROS production, which suggested a primary role of GSE in scavenging of ROS. A major contributing factor to the apparent superiority of GES over RES with regard to the ability to scavenge ROS could be attributable to the molecular structure of GSE. The GSEs consist of high–molecular-weight oligomers and polymers, including gallic acid, catechin, and epicatechin. Investigators in another study suggested that the GSE fractions with the highest percentage of gallic acid were the most effective at reducing proliferation of cancer cells. Further research is necessary to determine the role of gallic acid in modulating oxidative stress in lens epithelium. Regardless, the ability of GSE to significantly suppress intracellular ROS production is evidence that GSE is a potent antioxidant. To our knowledge, this is the first time that this compound has been tested in canine LECs and found to have these properties.

The ability of RES and GSE, separately and in combination, to modulate oxidative stress–induced cell signaling in canine LECs was evaluated. Treatment of cells with GSE alone effectively attenuated oxidative stress–induced cell signaling. The GSE was the only test compound that had considerable inhibitory effects on the activity of p38 and Akt. These findings may elucidate 1 or more of the specific mechanisms through which GSE exerts antcataractogenic effects. Activation through phosphorylation of p38 is required for activation of activator protein-1, a trans-acting factor necessary for induction of tumor necrosis factor and interleukin-1, which are inflammatory cytokines. Expression of tumor necrosis factor was increased in 70% of specimens obtained during anterior capsulotomy from human cataract patients. Inflammation can cause cataracts and vice versa. As such, inhibition of p38 and its downstream inflammatory mediators may be able to attenuate cataract progression. Therefore, GSE inactivation of p38 is a possible route through which it could inhibit inflammation and, potentially, cataractogenesis. Activator protein-1 can also be involved in regulation of matrix metalloproteinase-9 and migration in cancer cells in an Akt-, ERK-, and JNK-dependent manner. In addition, GSE decreases protein expression for activator protein-1 in a dose–dependent manner in a human cancer cell line. This provides additional potential mechanisms by which GSE could inhibit oxidative stress and control inflammatory cytokines. Further research is necessary to determine whether GSE acts through inhibition of activator protein-1 and whether this could serve as a new target to prevent cataract formation and associated uveitis. In this study, GSE was the most potent inhibitor of Akt. In another study conducted by our laboratory group, we found that Akt is overexpressed in cataractous LECs, and inhibition of Akt by GSE may be another mechanism by which GSE prevents cataractogenesis in rodents.

Resveratrol was unable to modulate activation of MAPK or Akt signaling after oxidative stress, which suggested that RES may inhibit cataractous changes via other cell-signaling mechanisms. It has been hypothesized that RES can inhibit cell migration as a result of changes in the cytoskeleton. More specifically, RES causes epithelial cells to become unpolarized and extend large numbers of unorganized peripheral filopodia that are not associated with focal adhesions. In support of these findings, data from our laboratory indicate that RES can inhibit cell migration. Although data from the study reported here were not able to provide the reason or reasons that RES may delay cataractogenesis, it is possible that RES is more effective at controlling LEC migration than reducing oxidative stress in lenses.

Vitamin E analogue was used as a control treatment in all experiments because it is an established and accepted antioxidant. Vitamin E, or α-tocopherol, is a fat-soluble vitamin and an excellent antioxidant that protects cells against lipid peroxidation. It also inhibits UVB radiation–induced activation of p38 and JNK in immortalized human LECs. Numerous epidemiologic studies have found that vitamin E varies in its effectiveness in preventing cataracts in humans. Compared with the results for the vitamin E analogue, the results for GSE indicated that GSE is a good inhibitor of intracellular ROS formation. However, GSE and GSE+RES were better at inhibiting TBHP-induced induction of ERK1/2 and SAPK/JNK. Furthermore, GSE alone was

Figure 5—Representative western blot from 3 experiments to determine the ability of grape polyphenols to decrease THBP induction of p-Akt 1/2 and Akt 1/2 in canine LECs (A) and mean ± SEM densitometry values from all 3 western blots, which were adjusted on the basis of β-actin content for loading control purposes. *P < 0.01 and **P < 0.05 from the value for incubation with TBHP alone. See Figures 1 and 2 for remainder of key.
the most potent inhibitor of Akt 1/2, compared with the effect of the vitamin E analogue. Clearly, there are various mechanisms by which oxidative stress is inhibited. The study reported here provided evidence that GSE may be of use to prevent or delay cataract formation in canine LECs. Treatment with GSE significantly decreased TBHP-induced intracellular ROS production and oxidant-induced cell-signaling pathways associated with cataractogenesis. These findings provide justification for further testing of GSE as a dietary supplement to inhibit or delay cataract formation in dogs in vivo. Ideally, a balanced diet with various antioxidant supplements that resist oxidative stress via several mechanisms would potentially ameliorate the various mechanisms by which oxidative stress causes damage to LECs.

References