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The Effect of the Dietary Polyphenols Quercetin and Resveratrol on Wound Healing in Bovine Corneal Endothelial Cells in Vitro

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Abstract

The corneal endothelium is a monolayer of cells critical in maintaining a transparent cornea. Its cells are amitotic during adulthood; thus, tissue repair depends primarily on cellular growth and migration. Enhancing repair may improve recovery time from surgical procedures and corneal diseases that damage the endothelium. Inflammation resulting from endothelial injury is characterized by increased concentrations of free radicals in the endothelium and aqueous humor, slowing wound repair. The antioxidant vitamins and antioxidant enzymes neutralize free radicals, but damage can occur when this system is overwhelmed by high concentrations of reactive oxygen species. This project develops an *in vitro* model of endothelial wound repair and investigates the effects of the dietary antioxidants quercetin and resveratrol on wound healing of cultured bovine corneal endothelial cells. Cells from confluent cultures are mechanically ablated with a silica gel-tipped tool. Cultures are then treated with combinations of peroxide and antioxidants and monitored at twenty-four hour increments. Cell migration into the wounded area is documented by phase-contrast microscopy. Significant wound-healing inhibition is present in the presence of 100μM hydrogen peroxide ($p<0.001$). Although no significant effect is observed with the addition of 50μM quercetin or 40μM resveratrol, higher concentrations lead to additional significant wound-healing inhibition.

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Introduction

The Cornea. Occupying the anterior one-sixth of the eye, the cornea is the transparent portion of the corneoscleral coat.[1] It has three cellular layers—an outer epithelium, a refractile substantia propria (stroma), and an inner endothelium. The epithelium serves as a protective barrier for the eye and absorbs nutrients from the tear film. The corneal stroma, which comprises ninety percent of the cornea's volume, is the first refractile organ which light traverses in the eye. The perpendicular rows of collagen fibers in the stroma swell easily in the presence of excess fluid.[1] Because this phenomenon changes the refractive index of the tissue, fluid content of the stroma has to be tightly regulated to maintain corneal transparency. The corneal endothelium (CE) functions in this regulation. The CE is a monolayer of squamous epithelial cells[2] that constitute the posterior margin of the cornea.[1] Though the CE represents a small portion of the cornea's total volume, its so-called "pump-leak" functionality makes it critical in the prevention of stromal clouding and the maintenance of proper vision. Corneal endothelial cells (CECs) are connected to adjacent CEC's by focal tight junctions[2] that restrict the forward movement of aqueous humor from the anterior chamber into the relatively dehydrated corneal stroma. However, osmotic pressure tends to draw fluid from the anterior chamber into the stroma via interendothelial spaces. Sodium bicarbonate pumps within the CE counter this attraction by actively removing ions from the cornea. This balance prevents stromal edema under normal conditions.[2]

Corneal Response to Injury. The CE shows very little mitotic activity after birth; thus, any endothelial cell loss results in the growth and migration of neighboring cells, rather than cell division.[3,4,5,6] Under normal physiological conditions, the amitotic CE sustains a gradual reduction of cell density over the organism's lifespan.[7] When the CE receives a traumatic injury, this basal rate of cell loss is accelerated.[3,4] Cells may behave in two manners to compensate for the resultant defect. Those at the wound edge separate from adjacent cells and migrate into the area of cell loss, while those farther removed from the wound edge enlarge and flatten as a unit to reduce the overall area of the deficit.[8] This phenomenon is observed *in vivo* and can be mimicked *in vitro* (See Materials and Methods for further discussion). These modes of wound repair may be adequate to restore endothelial functioning and morphology when a significant population of corneal endothelial cells (CEC's) survives and is capable of maintaining the tissue's barrier and pump functions.[9] This is true of injuries that are primarily limited to the epithelium and stroma, with little permeation into the endothelial layer. Under more invasive conditions, monolayer integrity may be permanently impaired. In such cases, stromal edema and corneal opacity can only be remedied via penetrating keratoplasty, a corneal transplantation procedure which is limited by the supply of donor corneas.[8]

When endothelial function is compromised, the avascular CE actively recruits lymphocytes, macrophages, and polymorphonuclear cells (PMN's) to the site of the defect.[10] These inflammatory cells accumulate on the surface of the CE as keratic precipitates (KPs), which are clinical hallmarks of inflammation.[11,12]

Though inflammation is a normal systemic reaction to injury, excessive infiltration of inflammatory cells into the CE threatens irreversible oxidative damage. Oxygen free radicals produced by PMN's and monocytes are cytotoxic.[11,13] In response to injury, CE cell membranes also release prostaglandins, which generate reactive oxygen species (ROS) that compound

oxidative stress on the tissue.[14,15] Authors credit the accelerated cell loss accompanying inflammation to the effects of ROS.[11,16,17] It has also been observed that—like the ion pumps of other ocular tissues—the CEC's sodium and potassium pumps are disrupted by oxygenated metabolites.[18,19] Endothelial cell morphological changes[6,17] and enlarged intercellular gaps are also characteristics of CE injury. Coupled with reduced cell density, these events challenge the barrier and pump functions of the CE and contribute to stromal edema that may temporarily or permanently impair vision.[9,12,19] Oxidative damage has been implicated in ocular diseases affecting other tissues of the eye—including glaucoma and cataracts[11,18,19]—as well as diseases affecting other body organs, notably cardiovascular disease and certain forms of cancer.[20,21]

The Antioxidant Connection. The corneal endothelium has a natural antioxidant defense system that effectively neutralizes the basal levels of ROS in the normal CE.[22] The combination of intracellular antioxidant enzymes and nonenzymatic extracellular antioxidants—such as α-tocopherol (Vitamin E) and ascorbate (Vitamin C)—protect the tissue from accelerated cell density decline due to ROS.[11,17,19] Damage to the CE seems to occur when ROS overwhelm this system. This concept is evidenced by studies showing dose-dependent CE transport inhibition in the presence of high concentrations of oxidized ascorbate[18] and diminishing concentration of ascorbic acid in the aqueous humor after the experimental induction of uveitis in bovine corneas.[19]

Enhanced survival time of extracted corneas stored in antioxidants—including α-tocopherol and ascorbate—has been observed in humans and rabbits.[23] It has now been shown that this effect is a manifestation of prolonged survival of CE pump function that prohibits stromal edema, combating the detrimental effects of inflammation.[20]

Though the antioxidant vitamins have been investigated in conjunction with inflammatory ocular conditions, there is little research available investigating other dietary antioxidants with respect to this system. In the past two decades especially, bioavailable phytochemicals previously identified in abundance in fruits and vegetables have been explored for their antioxidant capacity in other systems. The most copious plant sources of antioxidants in the Western diet are polyphenols, with daily intakes approximating one gram.[24] Polyphenols are classified into four groups based on the structure of their carbon skeleton: flavonoids and phenolic acids comprise most of the group while stilbenes and lignans are rarer.[24]

Quercetin. Flavonoids function as superoxide and peroxyl radical scavengers, lipid peroxidation inhibitors, and anti-inflammatory agents.[25, 26] The flavonols are some of the most abundant polyphenols—found in almost every plant[25]—and are considered the most effective antioxidant flavonoids. Quercetin is a dietary flavonoid belonging to the subclass flavonol. It is present in small amounts in a broad range of fruits, vegetables, and beverages; however it is most abundant in onions and tea.[24] Although quercetin is among the least abundant of the flavonols—representing less than two percent of the total polyphenol intake[24]—its potent activity and longevity make it one of the most well studied.

The intact absorption and elimination half-life of quercetin are unparalleled among the flavonoids. Up to eighty-one percent of quercetin can be absorbed in the small intestine from the metabolism of quercetin glucosides, and up to fifty-three percent absorption has been reported for intact quercetin.[27,28] The half-life of quercetin—a standard which is positively correlated with drug efficacy—has been documented to be up to 72 hours,[28] compared to 1-2 hours for most flavonoids.[24]

Quercetin's attributes include significantly greater anticarcinogenic and anti-inflammatory[26] properties than many other flavonoids.[29] It was selected for this study as a possible stimulator of corneal endothelial wound-healing because of its demonstrated inhibition of ROS proliferation and neutrophil-mediated inflammatory action.

Neutrophils, chief phagocytic leukocytes that comprise a significant portion of the KP's present at the site of CE injury, can cause tissue damage by several methods. During phagocytosis, neutrophils generate superoxide-anion radicals, which then produce a broad array of ROS, including peroxide, the hydroxyl radical and peroxinitrite.[30] Potential free-radical damage is compounded by the activation of myeloperoxidase (MPO), an enzyme in phagocytic cells that catalyzes formation of chloride radicals via peroxide oxidation.[31]. Neutrophil-generated lysozymal enzymes can also contribute to tissue injury.[32] Quercetin hinders neutrophil activation and degranulation[30] and effectively combats all three of these pathways. It inhibits MPO activity more dramatically than available pharmacological inhibitors[33], suppresses superoxide production and lysozymal enzyme release, and neutralizes chlorinated radicals.[32]

Beyond blocking the generation of free radicals, quercetin combats some of their harmful effects. Lipid peroxidation is initiated by an ROS attack on cell membranes that form lipid hydroperoxides. The subsequent radical chain reaction on adjacent lipids in the membrane is implicated in inflammation. Several studies have identified quercetin as an inhibitor of the initiation sequence, as well as a chain-breaking antioxidant against its propagation.[30]
The other distinct role of quercetin as a modulator of inflammation is inhibition of the intercellular signaling pathway that triggers PMN invasion of an injured tissue. Lipogenase, an enzyme responsible for upregulating the synthesis of intercellular leukotrienes, is uniquely quercetin-sensitive. The effect of other flavonoids on the system is an order of magnitude less than that of quercetin.[34] This gives quercetin a distinct anti-inflammatory advantage over

compounds which are solely antioxidants and therefore can not impact this system until the ROS species have been produced.

Resveratrol. Like quercetin, resveratrol is a polyphenol with antioxidant properties. More formally known as 3,4',5-trihydroxystilbene,[35] it is a member of the rarer stilbene subclass[24]. Resveratrol is present in low concentrations in red wine and few other foods[36], but was first discovered in medicinal plants of the *Polygonum* species[35]. It has gained much attention, despite relatively low preponderance in foods, because of its potent actions. Investigations of human populations that maintain low incidences of cardiovascular disease despite relatively high lipid consumption, such as France and Crete, identified resveratrol in red wine as one of the potential sources of cardiovascular protection.[35,37] Since that time, its list of known biological actions has expanded dramatically. In his analysis of the traditional Mediterranean diet, Simopoulous summarizes many of resveratrol's most noted actions. For example, resveratrol may confer cardiovascular protection via inhibition of lipogenesis in hepatocytes and adipocytes. It also attenuates lipoxygenase products and platelet formation. Its anticarcinogenic properties include tumor growth reduction and the induction of apoptosis among cancerous cells via multiple mechanisms.[38,39]

Resveratrol's relevance to this project is based on its antioxidant and anti-inflammatory actions. In studies of post-ischemic blood vessels of rats, resveratrol reportedly limits leukocyte recruitment.[40] Furthermore, it attenuates the degranulation of PMN leukocytes[35]. Both of these findings suggest that resveratrol could be beneficial in preventing inflammatory damage. Investigations on animal organs including the brain cortex, aortic smooth muscle, and the ovaries suggest that resveratrol inhibits the production of lipid peroxidation products and the aqueous free radicals peroxide and hydroxyl.[41,42,43]

Rationale and Hypothesis

The known protective roles of alpha-tocopherol and ascorbic acid against inflammatory ROS damage in the CE led to the hypothesis that quercetin and resveratrol would improve the rate of wound-healing for injured cells of this tissue. It is known that ROS are generated by recruitment of PMN leukocytes into the avascular cornea after injury, and that the resultant oxidative stress damages the tissue. Both polyphenols attenuate leukocyte recruitment and activation in other systems as outlined in the introduction and exhibit low reducing potentials. In the amitotic CE, these characteristics are critical for minimizing tissue damage and recovering the ion-pump activity required for corneal clarity.

Using primary cultures of bovine corneal endothelial cells, this project compared rates of wound closure between control cells, cells treated with hydrogen peroxide, and cells treated with hydrogen peroxide and quercetin or resveratrol. An *in vitro* model of cultured cells was preferable to other methods for this experiment for several reasons. First, it eliminated the cost and ethical concerns that must be addressed when experimenting with live animals. More importantly, isolating CECs allowed observation of those cells independent of potential influences from surrounding tissues. Researchers who use this technique suggest that the ability to generate many independent wounded areas from the same corneas minimizes other variables that may influence the experimental results.[44]

Materials and Methods

Hydrogen peroxide (3%) was obtained from a local pharmacy. All other chemicals were obtained from Sigma Aldrich (St. Louis, MO).

Corneas were isolated from bovine eyes obtained from a local abattoir and incubated for ninety minutes with 0.25% mixed crude protease (dispase) in Earle's Balanced Salt Solution at 37°C. Bovine corneal endothelial cells (BCEC's) were mechanically dislodged from the underlying Descemet's membrane using a spatula with a tapered silicon surgical tip. The isolated cells were aspirated and centrifuged, and the pellet resuspended in a solution of 5mL Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum, 100μM fungizone, and 100μM gentamicin (S^+ media). The BCEC's were placed in 25cm^2 flasks and received 5 ml of S^+ media and were incubated at 37°C. The culture media was replaced three times a week with S^+ media. After reaching confluence (approximately one week), they were sub-cultivated. Cells were harvested after a ten-minute incubation with a trypsin-EDTA solution, pipetted into a test tube, and centrifuged. The pellet was resuspended in S^+ media and transferred into twelve-well plates, with each well having a surface area of 3.8cm^2 and receiving 2 mL of the solution. Each well received 2 ml of S+ media three times a week until the cultures reached confluence.

All experiments were performed on confluent BCEC's, twenty-four hours post feeding with S^+ media. In experiments to determine a peroxide-induced delay in wound-healing, cultures were rinsed in serum-free (S^-) media immediately before wounding. To test the effects of

antioxidants in the inflammatory model, cultures were bathed in S^- media two hours before wounding to eliminate traces of growth factors or other nutrients that could potentially contribute to cell proliferation. Although the CE is mitotically inactive in humans, some low levels of mitosis have been documented in other species, including bovine.[45] This feature allows them to be grown in culture. Studies conducted on *in vitro* BCEC's have shown that, under serum free conditions, no mitotic cells are detected post-wounding.[45] Using this model, wound-healing occurs in a non-proliferative fashion, similar to a human cornea *in vivo.*

Because there is no standard procedure for wounding cultured CEC's, the specific protocol used for wounding these cultures was developed by this research group. The wounding device constructed for this experiment was a silica-gel tip fitted over a dissecting probe. The silica tip measured approximately 1.5 cm x 0.5 cm and was tapered to a thickness of approximately 1 mm at the wounding edge. This construction provided the rigidity necessary to generate a straight wound of a uniform width. The silica's flexibility was preferable to other materials that were tried, such as wood and stainless steel, which created scratches in the tissue-culture plastic during wounding. Mechanical wounds were produced in each culture by dragging the sterile tool across the entire diameter of each well. This resulted in a rectangular area approximately 1mm wide that was denuded of cells (Figure 1).

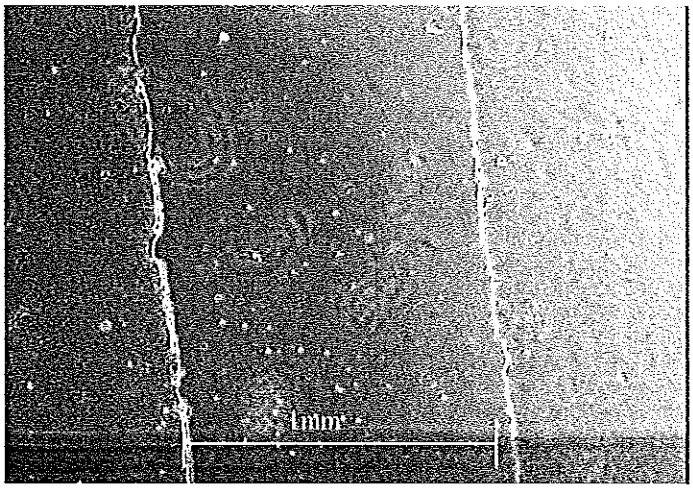

Figure 1: Confluent cultures of bovine corneal endothelial cells were wounded with a silica-gel-tipped device. Immediately after wounding, an area denuded of cells was apparent in each culture. The wound site was approximately 1mm wide in all trials and was bordered on both sides by areas of confluent cells. Photograph was taken at 40x magnification.

Immediately after wounding, the S- media was removed and each of four wells received 2 mL of different treatment media: S^- media, S^- supplemented with 100 μM hydrogen peroxide, or the latter treatment with the addition of 40μM resveratrol or 50μM quercetin. The antioxidants had previously been solubilized in dimethyl sulfoxide (DMSO) to generate stock solutions of 40mM and 50mM, respectively, and the final concentration of DMSO in the treatment media was 0.1%. The effective concentration for resveratrol was determined to be 38.5μM in studies on isolated ischemic and reperfused rat hearts.[46] Quercetin at 50μM has been shown to decrease intracellular ROS levels by 75% in osteoclasts.[47] The treatment media was replaced every forty-eight hours for the duration of the experiment. Later experiments utilized resveratrol and quercetin at 2x and 5x their reported effective concentrations. 100μM and 200μM resveratrol and 100μM and 250μM quercetin were prepared from the same stock solutions as above.

Cell cultures were visualized with a phase-contrast microscope at 100x or 40x magnification. A mark on the underside of the tissue culture plastic served as a landmark for photographing a reproducible area at the center of each wound (Figure 1).[44] Photographs were taken immediately after wounding, and at twenty-four hour intervals thereafter. For purposes of accuracy, images of a micrometer scale were printed at the same magnification as each photograph. Wound-healing was quantified from the printed images by using a ruler to measure the distance between confluent wound edges at ten locations along the length of the wound. Measurements were made at locations assigned with a random numbers generator (randomnumbers.org), and these locations remained constant for all trials within each experimental set.

Results

Despite their noted role as free-radical scavengers, neither quercetin nor resveratrol were observed to increase the rate of wound-healing in bovine corneal endothelial cells subjected to mechanical injury in the presence of peroxide. Figure 2 illustrates an apparent inhibition of wound-healing in the presence of peroxide in comparison with both the cultures receiving media supplemented with fetal calf serum and the serum-free media. However, both quercetin and resveratrol appear to further inhibit wound-healing, rather than reversing the inhibition. Resveratrol appears to elicit an early wound-healing inhibition that continues through the experiment. Quercetin does not seem to affect wound-healing initially, but generates a more dramatic inhibition than resveratrol at forty-eight hours post-wounding and beyond.

One-way analysis of variance (ANOVA) yielded a variance ratio (F) of 0.056, which does not exceed the tabulated value of F (2.5). This suggests that the differences in the wound-healing patterns of the five treatments were not significant. Unpaired student's t-tests of average wound width in the serum free cultures and those to which 100μM H_2O_2 had been added generated p values of 0.005 and 0.001 at twenty-four and forty-eight hours, respectively. T-tests did not reveal a significant difference between the peroxide-treated cultures and those supplemented with either resveratrol or quercetin. Taken together, these results suggest that the presence of peroxide in wounded bovine CEC's in culture suppresses wound-healing. The addition of resveratrol and quercetin does not impact this phenomenon.

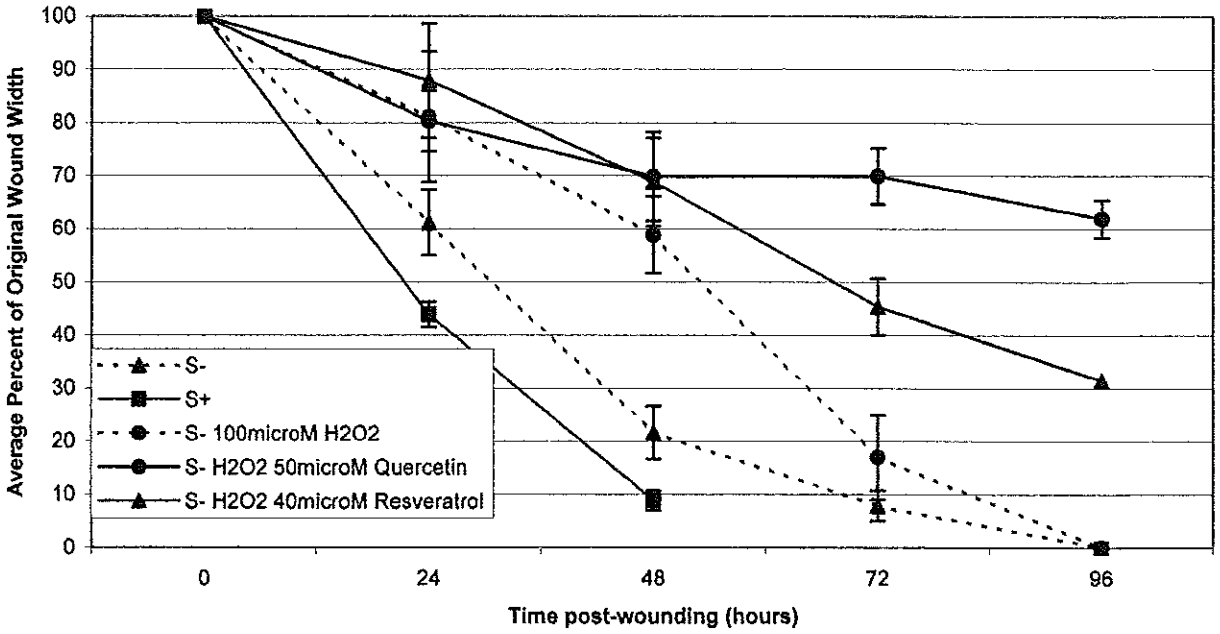

Figure 2: Wounded bovine corneal endothelial cells bathed in media supplemented with 10% fetal calf serum (S+) produced the fastest rate of wound closure (solid line with squares). During the first 24 hours, wound-healing in the quercetin-treated cultures (solid line with circles) approximated the speed of those treated with peroxide alone. However, after day one, wound closure in the quercetin-treated cultures slows, while the peroxide-treated culture continues to close. Resveratrol treatment (solid line with triangles) also appears to inhibit wound closure compared to peroxide treated cultures. ANOVA analysis suggested that these trends are not significant. Unpaired Student's t-tests reveal a significant difference between the serum-free group (dashed line with triangles) and those treated with peroxide (dashed line with circles); however, no further difference was detected between peroxide-treated and either the resveratrol or quercetin-supplemented groups. Error bars represent SEM. The sample size varied between treatment groups and between time intervals within each treatment group as indicated in Table 1.

Table 1	**Average percentage of original wound width at time intervals post-wounding**			
Post-wounding treatment	**24 hours**	**48 hours**	**72 hours**	**96 hours**
Serum Free	61.19 +/- 6.23 n = 7	21.68 +/- 4.97 n = 7	7.91 +/- 2.90 n = 4	0 n = 2
With Serum	43.88 +/- 2.35 n = 9	8.92 +/- 1.88 n = 9	n/a	n/a
Serum Free 100μM H2O2	81.09 +/- 12.23 n = 8	58.88 +/- 7.18 n = 8	17.05 +/- 7.93 n = 4	0 n = 3
Serum Free 100μM H2O2 50μM Quercetin	66.09 +/- 12.23 n = 5	50.87 +/- 7.18 n = 5	n/a	n/a
Serum Free 100μM H2O2 40μM Resveratrol	87.91 +/- 10.76 n = 5	68.86 +/- 8.29 n = 5	45.37 +/- 8.29 n = 3	31.41 +/- 5.31 n = 3

Because investigations of resveratrol and quercetin at their reported effective concentrations did not yield positive results, subsequent experiments were designed to study higher concentrations of these antioxidants. Treatment media containing 100μM and 250μM quercetin were used on cultures with the same protocol as before. These dosages resulted in a significant wound-healing inhibition compared to peroxide-treated cultures ($p<0.001$ at forty-eight hours post wounding). This suggests dose-dependent inhibition of wound healing (See figure 3).

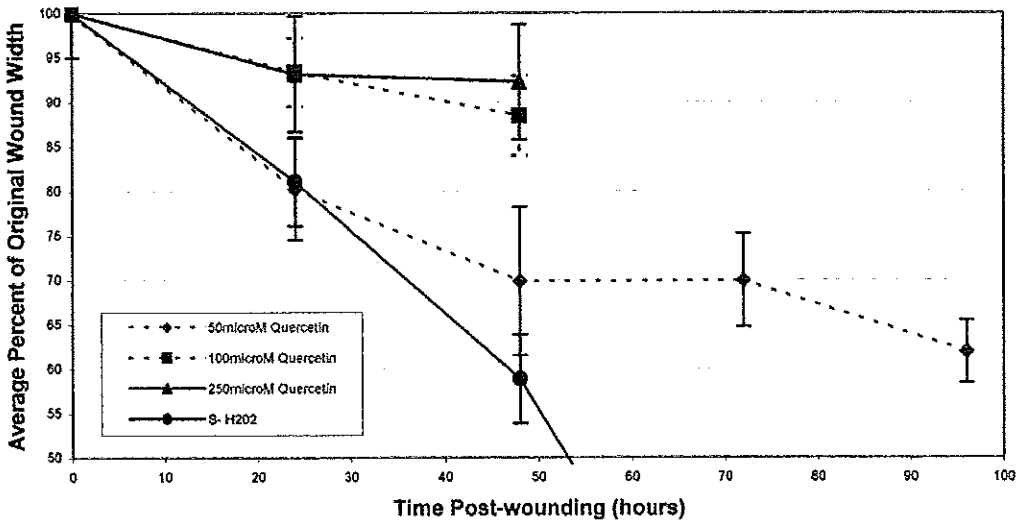

Figure 3: Wounded cultured bovine corneal endothelial cells in the presence of peroxide with and without quercetin. Increasing the concentration of quercetin applied to peroxide-treated cultures post wounding leads to a significant wound-healing inhibition. While there was no significant difference in the rate of wound closing for peroxide alone and peroxide in the presence of 50 μM quercetin, both 100 and 250μM quercetin significantly retarded wound-healing ($p <0.001$). Error bars represent SEM.

Increasing the resveratrol dosage in the media supplied to cultures post-wounding likewise compounded the inhibitory effect observed with peroxide alone. Resveratrol was added to treatment media to a final concentration of 100μM and 200μM. Figure 3 illustrates the increased inhibition at the 100μM compared to peroxide-treated cultures and those receiving

resveratrol at 40μM. The difference between the 100μM resveratrol group and the peroxide group was significant ($p < 0.001$ at forty-eight hours post wounding). Treating cultures with 200μM resveratrol resulted in widespread necrosis, generating non-confluent patches of cells throughout each well with no clear wound margin. These results suggest that wound-healing inhibition in the presence of resveratrol is concentration dependent and that high concentrations are cytotoxic.

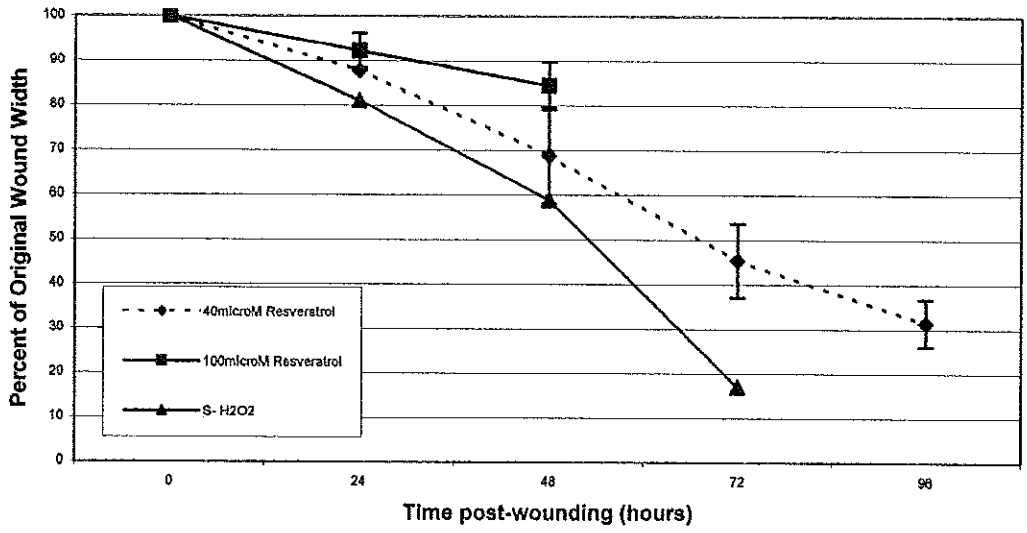

Figure 4: Wound-healing in cultured bovine corneal endothelial cells was delayed by increasing the concentration of resveratrol in peroxide-containing media applied after wounding. With the addition of 100μM resveratrol, 92.3% and 84.5% of the wound width remained denuded of cells after 24 and 48 hours, respectively (squares, solid line), compared to 87.9% and 68.9% when treated with 40μM resveratrol (dashed line, diamonds). Error bars represent SEM.

Discussion

Some injuries sustained by the cornea do not penetrate into the corneal endothelium and neither do certain corrective surgeries—including laser-assisted *in situ* keratomileusis (LASIK) and photorefractive keratectomy (PRK). In these cases, epithelial and stromal tissue loss is responsible for accompanying inflammation, and regeneration of these tissue layers is sufficient to reduce stromal swelling and correct vision impairment[9]. Damage to the corneal endothelium, however, can cause permanent vision loss because of the nonregenerative nature of the tissue and its importance in the pump-leak regulation of stromal volume.

Endothelial damage can occur via several mechanisms. Bourne summarizes the three primary causes of CE damage: trauma, primary corneal endotheliopathy, and corrective surgery. The most commonly occurring traumas to the CE are cell loss from ruptures in Descemet's membrane caused by forceps during delivery of a newborn, and corneal contusions resulting from explosive accidents. There are four types of primary corneal endotheliopathies, as well as intermediate forms of each, which cause endothelial damage. Posterior polymorphous dystrophy (PPD), congenital hereditary endothelial dystrophy (CHED), and Fuch's dystrophy are all autosomal dominant diseases which result in endothelial damage, with Fuch's typically presenting in older adults. Iridocorneal endothelial syndrome (ICE) is also common but is not genetically linked. Of these diseases, only Fuch's has been found to alter endothelial pump

function.[48] For this reason, it is one of the most frequent indicators for penetrating keratoplasty.[49]

Cataract surgery is the second most common cause of corneal transplantation.[49] After the 1970's discovery that cataract extraction caused considerable damage to the CE, safer procedures were developed. However, even these led to CEC density declines up to four times the normal rate for ten years post surgery. The long-term CE damage produced by modern small incision surgery has not yet been determined.[48]

Corneal transplantation has its own associated damage to the CE. Time-dependent cell loss can occur during storage of donor corneas. Surgical manipulation can also contribute to the damage[48]. Bourne reports that endothelial cell loss is twelve times the normal rate for the first five years following transplantation, and remains eight times the normal range for up to a decade following the surgery.[48] This is attributed to low-grade rejection by the recipient's immune system.[50]

The aim of this research was to investigate the effects of two antioxidant phytochemicals on an *in vitro* model of corneal endothelial injury, with the goal of improving recovery time after endothelial damage. Hydrogen peroxide was used to simulate the effects of leukocyte recruitment into the avascular monolayer. Wound-healing inhibition in the presence of peroxide was demonstrated compared to control groups maintained in serum-free media ($p = 0.001$ forty-eight hours post wounding).

Based on their potent antioxidant and anti-inflammatory properties, it was hypothesized that quercetin and resveratrol would reverse peroxide-induced wound-healing inhibition in cultured bovine corneal endothelial cells. The opposite effect was actually observed. At their reported effective concentrations both compounds produced an insignificant reduction in wound

closure, while higher concentrations yielded significant inhibition ($p < 0.001$). This suggests that the polyphenols contribute to oxidative stress, rather than reducing it in this tissue, and that the impact on wound-healing is dose-dependent. Although these results were unexpected, the data for quercetin are in line with some findings by other authors.[25,51,52]

In their review of flavonoid properties, Rice-Evans and colleagues point out that "quercetin's ability to penetrate and interact with lipid bilayers in different systems results in conflicting antioxidant potentials." They further caution that researchers have identified the 3,4 hydroxyl as a potential site of auto-oxidation in some systems.[25] The research of Canada and coworkers identified this phenomenon in aqueous media at pH of 7.5, suggesting that it may occur in physiologically relevant situations.[51] The structural property of many flavonoid compounds may explain occasional unpredicted relationships between antioxidant potential and structure.[25] If auto-oxidation were indeed occurring in the aqueous media in which the wounded CEC's were bathed, this could explain the additive inhibitory effect observed in the presence of peroxide and quercetin.

Some authors suggest that preincubation of quercetin before exposure to an oxidant source is more effective at reducing inflammation than coincubation[52]. It is possible that the methodology utilized in this experiment is inadequate to promote antioxidant action and that a preincubation procedure could have generated more desirable results.

No research is currently available identifying resveratrol as a pro-oxidant. However, the extensive list of resveratrol's actions provided by Simopoulous[35] suggests one of resveratrol's other actions, as discussed in the introduction, may account for the results which cannot be explained by its antioxidant capacity alone.

Though S- and S+ media served as negative and positive controls, respectively, for this experiment, there was an inherent flaw in these controls. Stock solutions of resveratrol and quercetin were initially solubilized in DMSO. The final concentration of DMSO in the cultures was 0.1%, but higher for those experiments exploring the antioxidants at 2x and 5x their reported effective concentrations. It is possible that the solvent rather than the antioxidants contributed to the wound-healing inhibition. The S- and S+ cultures should have received the same concentration of DMSO to rule out this possibility. However, DMSO is a common reagent utilized in this lab, and no previous experiments had noted deleterious effects in the presence of DMSO comparable to those reported here.[53]

Even though the expected wound-healing enhancement was not observed in the presence of resveratrol and quercetin, this study resulted in the development of an *in vitro* model of wounding BCECs. The methods used mimicked the oxidative stress that could result from the inflammatory response that accompanies CE wounds *in vivo*. In the future, this model can be used to investigate other reagents that may affect wound-healing.

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