Protective Effect of Crocin against Blue Light– and White Light–Mediated Photoreceptor Cell Death in Bovine and Primate Retinal Primary Cell Culture

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Purpose. The present study was performed to investigate the effect of crocin on blue light– and white light–induced rod and cone death in primary retinal cell cultures.

Methods. Primary retinal cell cultures were prepared from primate and bovine retinas. Fifteen-day-old cultures were exposed to blue actinic light or to white fluorescent light for 24 hours. Cultures were treated by the addition of different concentrations of crocin for 24 hours before light exposure or for 8 hours after light exposure. Cultures kept in the dark were used as controls. Green nucleic acid stain assay was used to evaluate cell death. Rods and cones were immunolabeled with specific antibodies and counted. TUNEL labeling was used to detect fragmented DNA in fixed cells after light exposure.

Results. Primary retinal cell cultures contained a mixture of retinal cells enriched in photoreceptors, bipolar cells, and Müller cells. Twenty-four-hour exposure to blue and white light induced death in 70% to 80% of the photoreceptors in bovine and primate retinal cell cultures. Crocin protected the photoreceptors against blue light– or white light–mediated damage in a concentration-dependent manner with an EC_{50} of approximately 30 μM. TUNEL assays confirmed that crocin protected photoreceptors from light damage.

Conclusions. These results show that blue and white light selectively induce rod and cone cell death in an in vitro model. Crocin protects retinal photoreceptors against light-induced cell death. (*Invest Ophthalmol Vis Sci.* 2006;47:3156–3163) DOI:10.1167/iovs.05-1621

Oxidative stress is a common contributor to many different neurodegenerative diseases, including retinal disorders such as age-related macular degeneration (AMD).[1,2] The retina is particularly susceptible to oxidative stress because of its high levels of photosensitizers and pigments, its high consumption of oxygen, and its exposure to visible light.[1–3] Multiple factors have been linked to the pathogenesis of AMD. These include inflammation,[4] choroidal neovascularization,[5] genetic factors including complement factor H polymorphisms,[6] and the accumulation of lipofuscin,[7] particularly a *bis*-retinoid, *N*-retinylidene-*N*-retinylethanolamine (A2E), and its photoisomers, the major blue light–absorbing fluorophores of lipofuscin in the RPE believed to be associated with AMD pathogenesis.[7] Several epidemiologic studies suggest that long-term history of exposure to light may have some impact on the incidence of AMD.[8] Exposure to intense light causes photoreceptor death. Light-induced photoreceptor death is mediated by rhodopsin,[9] and the extent of its bleaching and its regeneration and visual transduction proteins determine the degree of damage.[10,11] Blue light– and white light–induced damage of retinal cells have been widely used as in vivo models.[12,13] Although the abnormality of the retinal pigment epithelium is thought to constitute the primary lesion in AMD,[14] it is the dysfunction and death of photoreceptors that accounts for vision loss. Studies performed on nonexudative AMD tissue show that the RPE becomes dysfunctional in persons susceptible to AMD. Secondarily, rod loss continues and cones begin to degenerate. Eventually, only a few cones remain in late-stage AMD.[15]

Several antagonists and antioxidants were used as neuroprotective compounds against light-induced photoreceptor death in vivo.[16–19] The pistils of *Crocus sativus* L (CSE) are generally used in Chinese medicine for anodyne, sedative, and emmenagogue, and its crude extract and purified chemicals have been demonstrated to prevent tumor formation,[20] atherosclerosis,[21] and hepatic damage.[22] They have also been shown to have antinociceptive and anti-inflammatory effects.[23] Crocin is an ethanol-extractable component of CSE shown to prevent ethanol-induced impairment of hippocampal synaptic plasticity[24] and acetaldehyde-induced inhibition of long-term potentiation in dentate gyrus[25] and to selectively antagonize the inhibitory effect of ethanol on *N*-methyl-D-aspartate (NMDA) receptor–mediated responses in hippocampal neurons.[26] However, the precise effect of crocin on the central nervous system, including the retina, has not yet been assessed in vitro. In the present study, we used crocin (component of saffron stigmas) to investigate its effect against light-induced photoreceptor death in an in vitro model.

Materials and Methods

Primary Cell Cultures

Retinal cell cultures were prepared using bovine and primate eyes. Fresh (2-week-old) bovine eyes (Lampert Co., Duvall, WA) and adult cynomolgus monkey (*Macaca fascicularis*) eyes (SNBL Ltd., Everett, WA) were used. After rapid immersion in ethanol, the cornea, lens, and vitreous were removed. The optic nerve disc area was removed, and the rest of the retina was used. The retina was detached and chopped into small fragments in HBSS++ solution (Cambrex Bio-Science, Walkersville, MD). Retinal fragments were incubated for 15 minutes at 37°C in activated papain solution (20 U/mL; Worthington Bioscience, NY). The enzyme reaction was stopped by the addition of 2 mL fetal bovine serum (FBS; Invitrogen, Carlsbad, CA), and tissue aggregates were eliminated by the addition of 200 μL DNaseI (Sigma, St. Louis, MO). Tissues were dissociated, and the cell suspension was then centrifuged at 214*g* for 5 minutes. Cells were resuspended in defined media

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DMEM/F12 plus insulin (25 μg/mL), transferrin (0.1 mg/mL), sodium selenite (5.18 ng/mL), and putrescine (9.66 μg/mL) and were supplemented with 10% FBS. The bovine retinal suspension obtained was plated at a density of 5×10^5 cells/well in 96-well tissue culture plates. The primate retinal suspension was plated in 96-well plates at a density of 2.5×10^5 cells/well. Retinal cell cultures were incubated at 37°C in a humidified atmosphere of 5% carbon dioxide and 95% air. Cells were not fed, and they were used for the experiments at 15 days in culture.

Light Damage

Retinal cell cultures were treated with different concentrations (10–160 μM) of crocin (Indofine Chemical Inc., Hillsborough, NJ) for 24 hours before light exposure, followed by 24 hours of light exposure. In another experiment, cells were treated with 80 μM crocin after 8 hours of light exposure and then exposed continuously to light for 16 hours. A stock solution of 10 mM crocin was prepared in dimethyl sulfoxide (DMSO). Serial dilutions of crocin were prepared in cell culture medium and were added to the cell culture in 96-well plates. Bovine retinal cell cultures were placed in the incubator and exposed for 24 hours to a continuous blue actinic light (Super Actinic/03 fluorescent lamps; Philips Lighting Company, Somerset, NJ) set at 900 lux (420 nm, 1.1 mW/cm^2) or to white fluorescent light (F15T8CW; Ushio, Tokyo, Japan) set at 6000 lux (2.3 mW/cm^2). Primate retinal cell cultures were subjected for 24 hours to a continuous blue actinic light set at 300 lux (420 nm, 0.4 mW/cm^2) or to white fluorescent light set at 4400 lux (1.7 mW/cm^2). Blue and white fluorescent light tubes were mounted on the topside in the incubator kept at 37°C and 5% CO_2. Cell culture controls were taken from the same batch as the ones used for the light damage and were kept in the incubator in the dark. For consistency of the light exposure, the energy and illuminance for different light sources were monitored weekly with the use of a radiometer and a light meter.

Green Nucleic Acid Stain Assays

Green nucleic acid stain assay (Sytox; Molecular Probes, Eugene, OR) was used to monitor cell death. Sytox is a DNA-binding dye that penetrates only dying cells in which the plasma membrane is compromised. The green nucleic acid stain assay was added at 1 μM to 96-well plates and was incubated for 30 minutes at 37°C. Fluorescence was determined using a plate reader with excitation fluorescence at 485 nm and emission fluorescence at 528 nm. This assay was used as the primary assay to assess cell death.

Immunocytochemistry

Immnunocytochemistry was performed directly on cells grown in 96-well plates. Experimental and control cell cultures were fixed with 4% paraformaldehyde for 10 minutes at room temperature followed by cold methanol for 10 minutes at 4°C. After three washes with PBS, cell cultures were incubated with primary antibodies in 0.2% Triton X-100 and 5% goat serum for 1 hour at 37°C. Different antibodies were used to identify cell populations. A monoclonal antibody against rhodopsin (1:2000 dilution; Chemicon International, Temecula, CA), a polyclonal antibody against recoverin (1:3000 dilution; Chemicon International), a polyclonal antibody against protein kinase C (PKCα; 1:1000 dilution; Upstate, Charlottesville, VA), and a monoclonal antibody against vimentin (1:1000 dilution; Chemicon International) were used. Binding of the primary antibody was detected using the appropriate secondary antibodies such as goat antimouse (1:250 dilution) or goat antirabbit (1:250 dilution) conjugated for red emission (Alexa-594; Molecular Probes) or for green emission (Alexa-488; Molecular Probes). Nuclei staining with DAPI was performed by mixing DAPI with the secondary antibodies; this was followed by 1-hour incubation at room temperature. DAPI staining of nuclei allows the analysis of nuclear conformation in cell cultures; condensed and fragmented nuclei are characteristic of apoptotic cells. For double immunofluorescence labeling, the cells were incubated with a mixture of mouse anti–rhodopsin antibody and rabbit anti–recoverin antibody. The cells were washed thoroughly and mounted with fluoromount-G (SouthernBiotech, Birmingham, AL).

TUNEL Assays

An in situ cell death detection kit (Boehringer Mannheim, Mannheim, Germany) was used to identify cell death by the terminal deoxyribonucleotidyltransferase (TdT)–mediated biotin-16-dUTP nick-end labeling (TUNEL) technique.[27] After fixation with 4% paraformaldehyde and methanol, cells were incubated with 0.15 Triton X-100 in 0.15 M sodium citrate solution for 5 minutes on ice. Cells were washed with PBS and incubated with TUNEL reaction mixture for 1 hour at 37°C. Cells were then washed thoroughly and mounted with fluoromount-G.

Cell Counting and Statistical Analyses

Fluorescence labeling was observed with a microscope (Olympus, Tokyo, Japan) under epifluorescence illumination. All images were recorded with the aid of a digital charge-coupled device (CCD) camera (Hamamatsu Photonics, Hamamatsu, Japan), and the images were processed using software (MetaMorph; Universal Imaging Corp., Downingtown, PA).

For evaluation of cell counting, cell imaging was performed with the use of an automated imaging microscope (Cell/Cell Imaging Station for Life Science Microscopy; Olympus), and cell counting was performed using a proprietary image–based cell counting software (Acucela, Inc., Seattle, WA). The cell counting algorithm was based on scoring cells positive for nuclei staining with DAPI and for immunolabeling with recoverin or rhodopsin. Statistical analyses were performed (Origin 7.5 software; OriginLab Corp., Northampton, WA). To determine the EC_{50}, data were plotted (GraphPad Prism; GraphPad Software Inc., San Diego, CA). At least three independent experiments from different cultures were conducted for all studies.

Results

Description of Bovine and Primate Retinal Cells in Cultures

Cells used in this study were primary cultures of mixture retinal cells prepared from bovine or primate retinas. High cell density plating (5×10^5 cells/well) was performed to obtain a mixture of retinal cells that would survive for more than 2 weeks with stable photoreceptors and would be uniformly distributed. During preparation, most photoreceptors lost their outer segments. However, these cells survived, grew neurites, and were viable in culture for at least 2 weeks. Immunocytochemistry was used to characterize different cell types in these cell cultures. Photoreceptors—immunolabeled with rhodopsin (a marker for rods) or recoverin (a marker for photoreceptors and cone bipolar cells in rodents) antibodies—represented the major population of neuronal cells and consisted of approximately 65% rods and 35% cones (of total photoreceptors). Rod bipolar cells, immunolabeled with the PKCα antibody, were the second most abundant population in this culture (data not shown). Müller cells, immunolabeled with the vimentin antibody, represented a monolayer with an undetermined percentage of the cells (data not shown). Few amacrine and ganglion cells were characterized in this cell culture (data not shown). The cell culture preparation contained a low percentage of retinal pigment epithelium (as indicated by their dome shape and melanin pigment, characteristic of RPE cells). This description for different cell types was performed in bovine retinal cell culture. In primate retinal cell cultures, the photoreceptors were mainly rods and represented the most abundant population in addition to Müller cells.

The average photoreceptor count in bovine retinal cell cultures was approximately 1300 per view (0.58 mm^2) at ×100 magnification from day 7 to day 15 in culture (data not shown). Most assays were performed using cell cultures approximately 2 weeks in vitro. Experiments were performed to determine whether different sources of light mediated photoreceptor cell

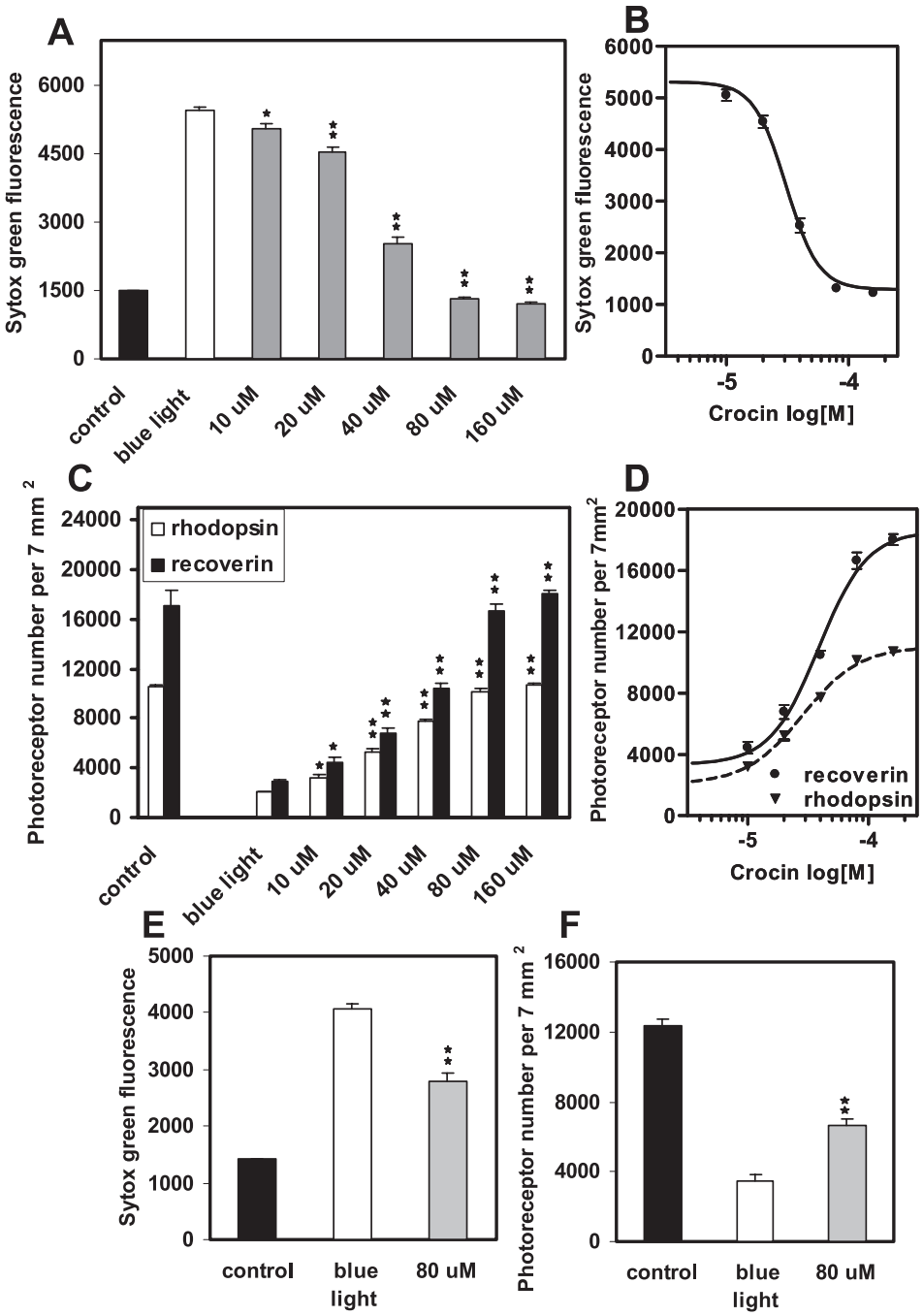

FIGURE 1. Protective effect of crocin against blue light damage in bovine retinal cell cultures. (**A**–**D**) Fifteen-day-old cell cultures were pretreated with different concentrations of crocin (10–160 μM) for 24 hours. Cells were then exposed to blue actinic light for 24 hours. (**A**, **B**) Green fluorescence represents arbitrary units on the *y*-axis. The reduction in green nucleic acid stain fluorescence units was used to assess the extent of neuroprotection. (**B**) Data were plotted. Crocin protected against blue light—mediated cell damage in a concentration-dependent manner with an EC_{50} of approximately 30 μM. (**C**, **D**) Recoverin and DAPI labeling were used to quantify the number of rods and cones using cell-based counting software. (**C**) The number of photoreceptors was significantly decreased in cell culture exposed to blue light compared with control. The number of rods and cones in cultures treated with different concentrations of crocin were significantly increased compared with light-damaged culture (12 views = 7 mm^2; values are mean ± SEM; $n = 6$; $^*P < 0.05$; $^{**}P < 0.001$ versus light damage condition; one-way ANOVA with Bonferroni correction). (**D**) Crocin protected against light-induced photoreceptor damage in a concentration-dependent manner with an EC_{50} of 35 μM for rods and an EC_{50} of 28 μM for total photoreceptors. (**E**, **F**) Cell cultures were treated with 80 μM crocin 8 hours after light exposure and then exposed continuously to blue light for 16 hours. The number of photoreceptors in cell cultures posttreated with crocin was significantly greater than that in light-damaged culture.

death in mixed retinal cell culture and whether this cell death was inhibited with crocin.

Neuroprotective Effect of Crocin against Blue Actinic Light–Induced Photoreceptor Damage in Bovine Retinal Cell Cultures

Exposure of bovine retinal cell cultures to blue actinic light for 24 hours induced approximately 80% photoreceptor cell death. No difference was observed between vehicle and control (data not shown). Cell death was significantly attenuated in cell cultures pretreated with various concentrations of crocin. With the use of green nucleic acid stain assay, complete protection against blue light–induced damage was observed in cell cultures pretreated with 80 μM and 160 μM crocin (Figs. 1A, 1B). This protection was confirmed by cell counts after immunocytochemistry using the recoverin and the rhodopsin antibodies (Figs. 1C, 1D). The number of rods and the total number of photoreceptors increased significantly in cell cultures incubated with various concentrations of crocin relative to samples exposed to blue light alone (Fig. 1C). Crocin inhibited blue light–induced photoreceptor death in a concentration-dependent manner with an EC_{50} of 30 μM based on green nucleic acid stain assay (Fig. 1B) and an EC_{50} of 28 to 35 μM based on cell counts (Fig. 1D). Crocin (80 μM) protected against blue light–mediated photoreceptor cell death in cell culture treated 8 hours after light exposure (Figs. 1E, 1F). However, the percentage of protection (36%) was lower than in cell culture treated with 80 μM crocin for 24 hours before light exposure (100%) (Figs. 1A, 1C). A monolayer of photoreceptors labeled with rhodopsin and recoverin was evident in control cultures (Fig. 2A). Cell cultures exposed for 24 hours to blue light

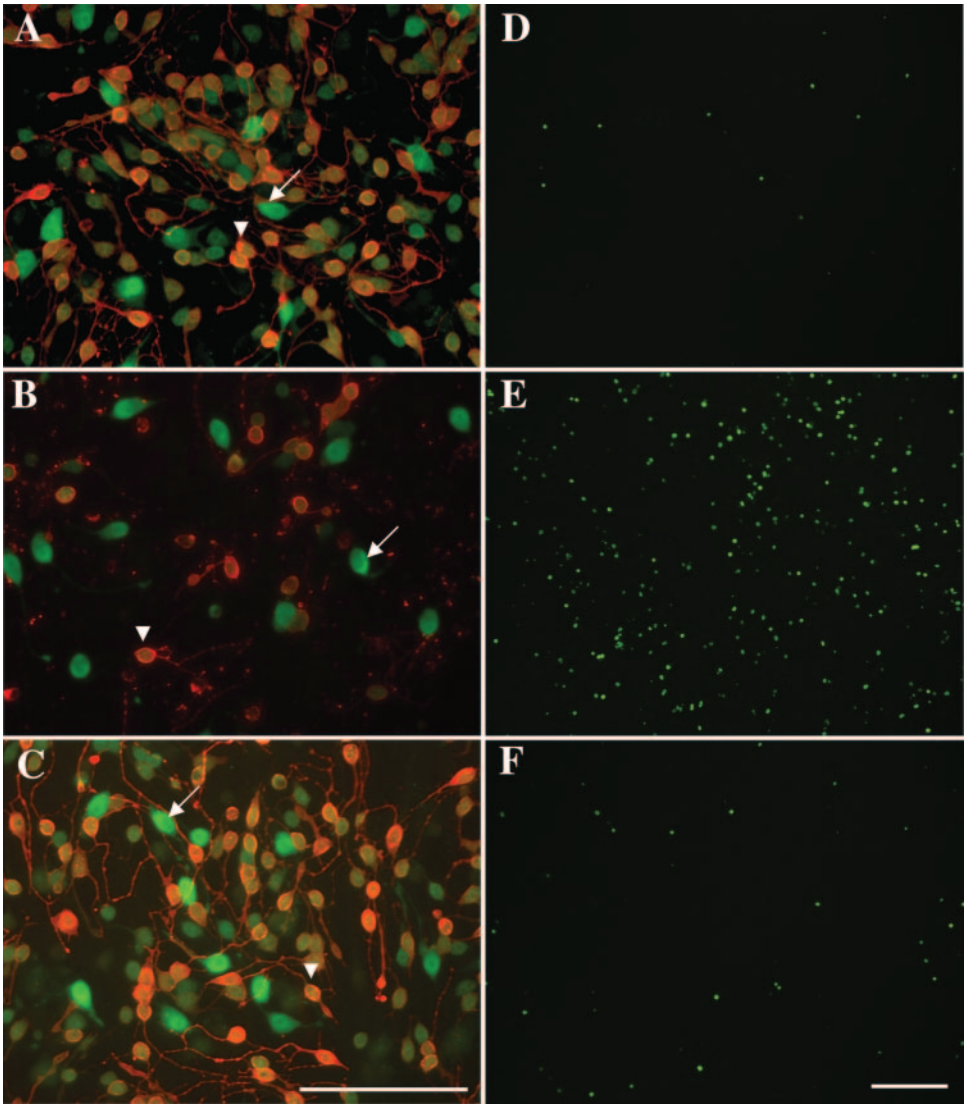

FIGURE 2. Crocin protects bovine photoreceptors against blue light damage. (**A–C**) Bovine retinal cell cultures were labeled with antibodies against recoverin (*green*) and rhodopsin (*red*). (**A**) Control cell culture was kept in the dark. Small round rod photoreceptors are double labeled to rhodopsin and recoverin (*arrowhead*). Larger cells are cone photoreceptors labeled uniquely with recoverin (*arrows*). (B) Cell cultures exposed to blue actinic light for 24 hours. Recoverin immunolabeling was evident in some remaining cones (*arrow*). Rhodopsin immunolabeling was mostly confined in rod cell bodies (*arrowhead*). (**C**) Cells were pretreated with 80 μM crocin. No difference was observed in (**C**) compared with control (**A**). (**D–F**) Localization of TUNEL-positive cells in bovine retinal cell cultures. (**D**) Control cell cultures were kept in the dark. (**E**) Cell cultures were exposed to blue light for 24 hours. (**F**) Cell cultures were pretreated for 24 hours with 80 μM crocin, followed by 24-hour light exposure. Few TUNEL positive cells were detected at high concentrations of crocin (**F**). No difference was seen between 80 μM and 160 μM (data not shown). Scale bars (**A–F**): 10 μm.

displayed extensive photoreceptor damage (Fig. 2B). Few rod and cone photoreceptors remained after exposure to blue light. Crocin prevented morphologic changes and death in a concentration-dependent manner (see, for example, Fig. 2C). At 80 μM crocin, 100% of photoreceptors survived the light damage (Fig. 2C). TUNEL assay confirmed blue light–induced cell death and neuroprotection with crocin compared with control (Figs. 2D–2F). Few cells were TUNEL positive in control cultures, suggesting these cells were undergoing normal cell death. Photoreceptor cell counts permitted the confirmation of TUNEL and green nucleic acid stain assays for the detection of cell death and neuroprotection.

Immunocytochemistry with different protein markers was used to determine the differential damage of photoreceptors compared with other cell types, such as bipolar cells and Müller cells. Blue light did not induce bipolar cell death or Müller cell death compared with control cultures kept in the dark (data not shown).

Neuroprotective Effect of Crocin against White Fluorescent Light–Mediated Photoreceptor Damage in Bovine Retinal Cell Culture

Exposure of bovine retinal cell cultures to white fluorescent light caused approximately 78% photoreceptor cell death (Fig. 3A). Cell death was significantly attenuated in cell cultures treated with various concentrations of crocin. Crocin at 10 μM did not inhibit cell death significantly (Figs. 3A, 3C). However, complete protection against light-induced damage was obtained in cell cultures pretreated with 80 μM and 160 μM crocin (Fig. 3A). Crocin inhibited white light–induced photoreceptor cell death in a concentration-dependent manner with an EC_{50} of 30 μM in green nucleic acid stain assay (Fig. 3B). Protection with crocin was confirmed by cell counts after immunocytochemistry with the use of recoverin and rhodopsin antibodies. Rod and total photoreceptor numbers increased significantly in cell cultures treated with various concentrations of crocin (Fig. 3C). Based on cell counting, crocin inhibited white light–induced photoreceptor cell death in a concentration-dependent manner with an EC_{50} of 35 μM (Fig. 3D). Cell cultures exposed for 24 hours to white light displayed extensive photoreceptor damage (Fig. 4B). Few rod and cone photoreceptors remained in culture after exposure to white light. Crocin prevented morphologic changes and death in a concentration-dependent manner (see, for example, Fig. 4C) compared with light-damaged cells (Fig. 4B). White light did not induce cell death of other cell types, such as bipolar cells and Müller cells (data not shown). TUNEL assay showed an increase in TUNEL-positive cells in cultures exposed to white light (Fig. 4E) compared with control cultures (Fig.

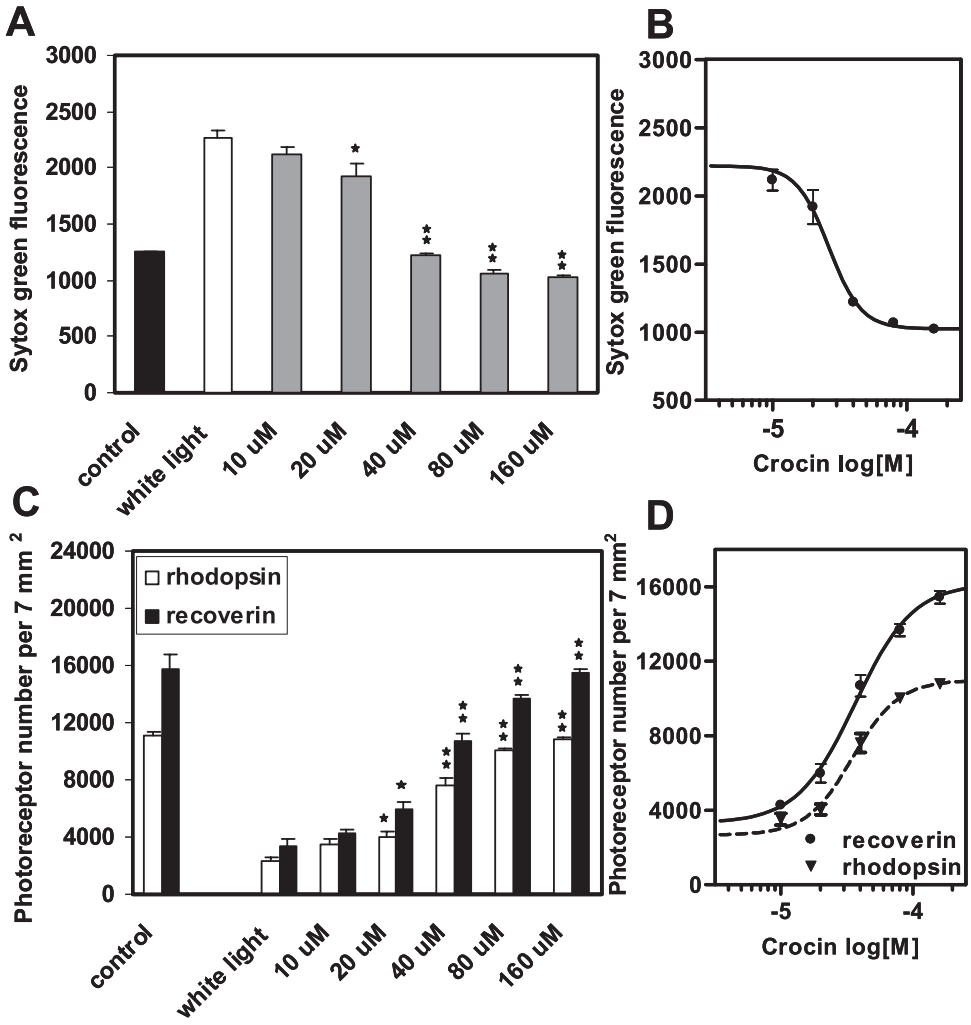

FIGURE 3. Protective effect of crocin against white light damage in bovine retinal cell cultures. (**A**–**D**) Fifteen-day-old bovine retinal cell cultures exposed to white fluorescent light. Different concentrations of crocin (20–160 μM) protected against white fluorescent light exposure using green nucleic acid stain detection (**A**, **B**) and photoreceptor cell counting (**C**, **D**). (**B**) Crocin protected against white light–induced cell damage in a concentration-dependent manner with an EC_{50} of approximately 26 μM; data are the same as in (**A**). Numbers of rods and cones in cultures treated with different concentrations of crocin were significantly increased relative to white light–damaged culture (12 views = 7 mm^2; values are mean ± SEM; $n = 6$; $^*P < 0.01$; $^{**}P < 0.001$ vs light; one-way ANOVA with Bonferroni correction). (**D**) Crocin protected against white light–mediated cell damage in a concentration-dependent manner with an EC_{50} of 35 μM for rods and total photoreceptors.

4D). However, the intensity of the signal and the number of TUNEL-positive cells was higher in cell culture exposed to blue light (Fig. 2E) than in cells exposed to white light (Fig. 4E). The number of TUNEL-positive cells exposed to white light decreased in cell cultures treated with various concentrations of crocin (see, for example, Fig. 4F).

Neuroprotective Effect of Crocin against Blue Actinic Light– and White Fluorescent Light–induced Photoreceptor Damage in Primate Retinal Cell Culture

Exposure of primate retinal cell cultures to blue actinic light or white fluorescent light induced approximately 89% and 70% photoreceptor cell death (Figs. 5A, 5C). Crocin inhibited blue light– and white light–induced photoreceptor cell death in a concentration-dependent manner with EC_{50} of 27 μM and 23 μM (Figs. 5B, 5D), respectively.

DISCUSSION

It is well established that different sources of light can mediate apoptosis of photoreceptors in in vivo animal models.[28,29] Primary retinal cell culture prepared from adult porcine and adult primate retinas were used in several studies.[30,31] In this study, we used primary retinal cell cultures from bovine and primate as a model to test light damage. Bovine retinal cell cultures contained rod (~65%) and cone (~35%) photoreceptors. Most photoreceptors in primate retinal cell culture were rods; few cones were identified in this cell culture condition. Primate retina, which is generally considered to be dominated by cones, actually has a relatively low overall cone/rod ratio. The cone/rod ratio in humans is 1:20,and it is 1:15 in *Macaca* primates.[32] Although no reports have been published of the cone/rod ratio in bovine retina, our data agree with previous findings in porcine retinal cell culture in which the cone density was higher than in human or primate retinal cell cultures.[33] The high density of cones in our bovine retinal cell culture indicates that bovine rather than primate retinal cell culture could be a good in vitro model system for studies aimed at understanding human cone-related diseases such as AMD.

Two sources of light were used in this study. There is ongoing discussion on whether lifelong blue light exposure, as an important component of sunlight, or bright artificial light sources may contribute to pathogenic steps in AMD.[34] It is well known that the condition for light-induced damage of photoreceptors varies among species.[35] We have been able to consistently induce photoreceptor light damage uniformly in primary bovine and primate retinal cell cultures. The total number of photoreceptors damaged was approximately the same in bovine and primate retinal cell cultures exposed to blue light (83% and 89%) or white light (78% and 70%). Cones were more resistant and less vulnerable than rods to blue and white light damage, suggesting that cones have a different sensitivity to blue and white light than rods. Given that there is experimental evidence for the existence of rod-derived cone survival factors,[36,37] the delay in cone cell death might have been

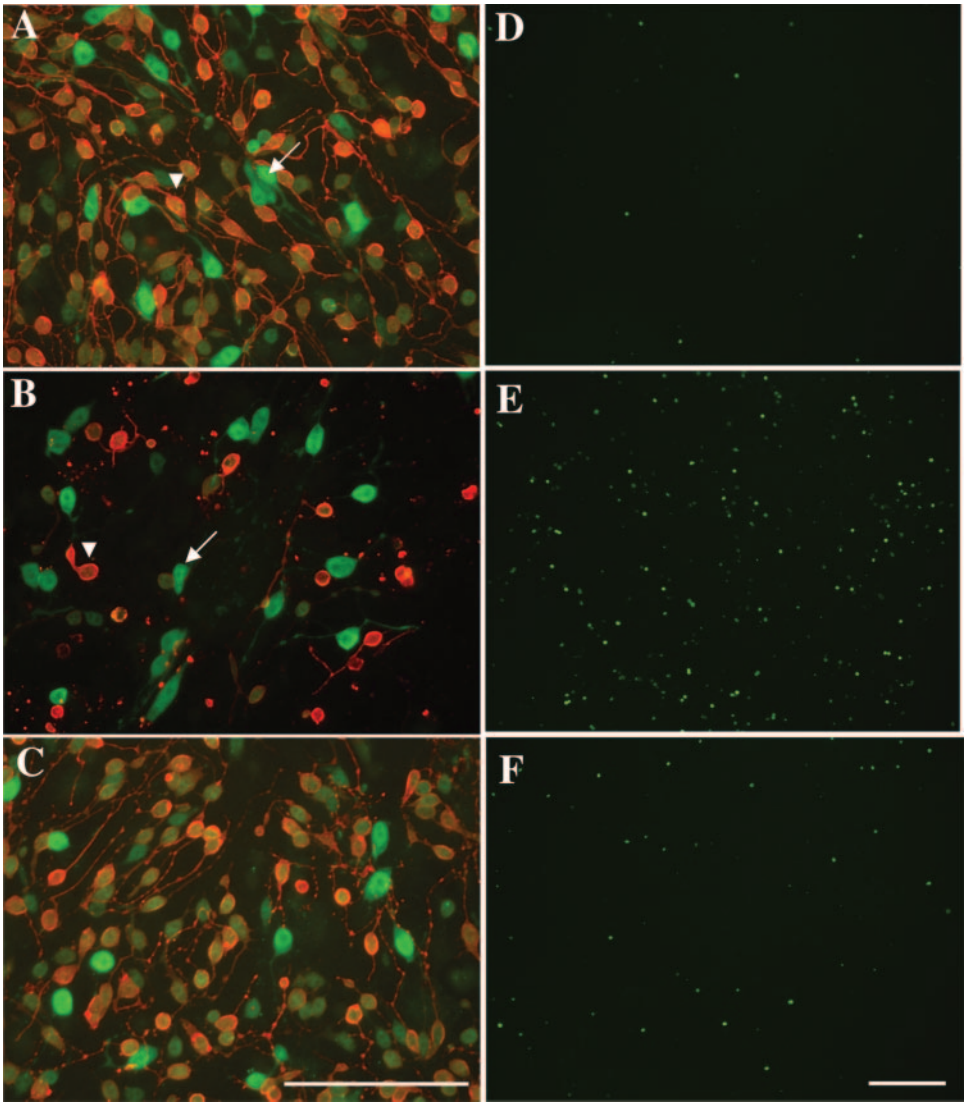

FIGURE 4. Crocin protects bovine photoreceptors against white light damage. (**A–C**) Bovine retinal cell cultures were labeled with recoverin (*green*) and rhodopsin (*red*). (**A**) Control cell culture was kept in the dark. Rod cells were double labeled with rhodopsin and recoverin (*arrowhead*). Cone cells were labeled with recoverin (*arrow*). (**B**) Cell culture was exposed to white fluorescent light for 24 hours. Recoverin immunolabeling was evident in some remaining cones (*arrow*). Rhodopsin immunolabeling was mostly confined in few rod cell bodies (*arrowhead*), and these cells are not labeled with recoverin. (**C**) Cell cultures were pretreated with 80 μM crocin for 24 hours then exposed to light for 24 hours. The number of photoreceptors positive to rhodopsin and recoverin increased with the increase of crocin concentrations added to the cell cultures. (**D–F**) Localization of TUNEL-positive cells in bovine retinal cell cultures. (**D**) Control cell cultures were kept in the dark. (**E**) Cell cultures were exposed to white fluorescent light for 24 hours. (**F**) Cell cultures were pretreated with 80 μM crocin. Number of TUNEL-positive cells was higher in culture exposed to light (**E**) than in control (**D**). A few TUNEL-positive cells were detected at a high concentration of crocin (**F**). No difference was seen between 80 μM and 160 μM (data not shown). Scale bars (**A-F**): 10 μm.

expected. It was reported that visible light exposure to photoreceptor cells (661 W) creates conditions of photo-oxidative stress leading to oxidative damage.[38]

Immunocytochemistry and cell counts confirmed that the exposure of retinal cell culture to blue and white light leads to preferential death of photoreceptor compared with other cell types, such as bipolar cells and Müller cells. In fact, Müller cells spread out to form a type of feeder layer in our cell culture system, consistent with previous in vitro reports.[39,40] It has been shown that glial cells are essential for long-term photoreceptor survival and neurite outgrowth in vitro.[39] This is supported by another study in which porcine retinal photoreceptors in primary culture survived poorly without feeder layers.[40] Almost all the bipolar cells were double labeled with PKCα and PKCα, β, γ (data not shown), suggesting that the bipolar cell population in bovine and primate retinal cell culture consists mostly of rod bipolar cells.[41]

Reduced green fluorescence and increased recoverin- and rhodopsin-positive photoreceptors were used to assess the neuroprotective effects of crocin. The increase in green fluorescence units correlated with the increase in TUNEL-positive cells and the decrease in the photoreceptor number in bovine retinal cell cultures, suggesting that blue and white light preferentially affect photoreceptors. Even though the percentage of photoreceptor death was approximately the same in bovine cell cultures exposed to blue or white light, the green nucleic acid stain assay reading units seem to be higher in cell cultures exposed to blue light than to white light. In addition, more TUNEL-positive cells were seen in cell cultures exposed to blue light. This discrepancy in detecting cell death in different assays between blue and white light may suggest a more rapid degradation of DNA in cell cultures exposed to white light than in those exposed to blue light. Green nucleic acid stain works by detecting the loss of plasma membrane integrity, and TUNEL labels DNA fragmentation. However, the specificity of DNA fragmentation to apoptosis and necrosis is uncertain.[42] Clearly, if lysis occurs and cells are lost, green nucleic acid stain assay and TUNEL have nothing to label. Immunocytochemistry and photoreceptor counts were performed in primate retinal cells as a second model for the study. This confirmed light-induced photoreceptor cell death and the neuroprotective effects of crocin against blue and white light in primate retinal cells. The EC_{50} for crocin protection was lower in primate retinal cell culture; this could have been related to the lower density of the cells in primate cultures compared with bovine retinal cell cultures.

The carotenoid crocin[43] is one of the constituents of saffron stigmas. Crocin protected against white light– and blue light–induced photoreceptor death in bovine and primate retinal cell culture. Approximately 70% to 89% of photoreceptors died after 24-hour exposure to light. More than 50% of photoreceptors survived at approximately 30 μM crocin, and 90% to 100%

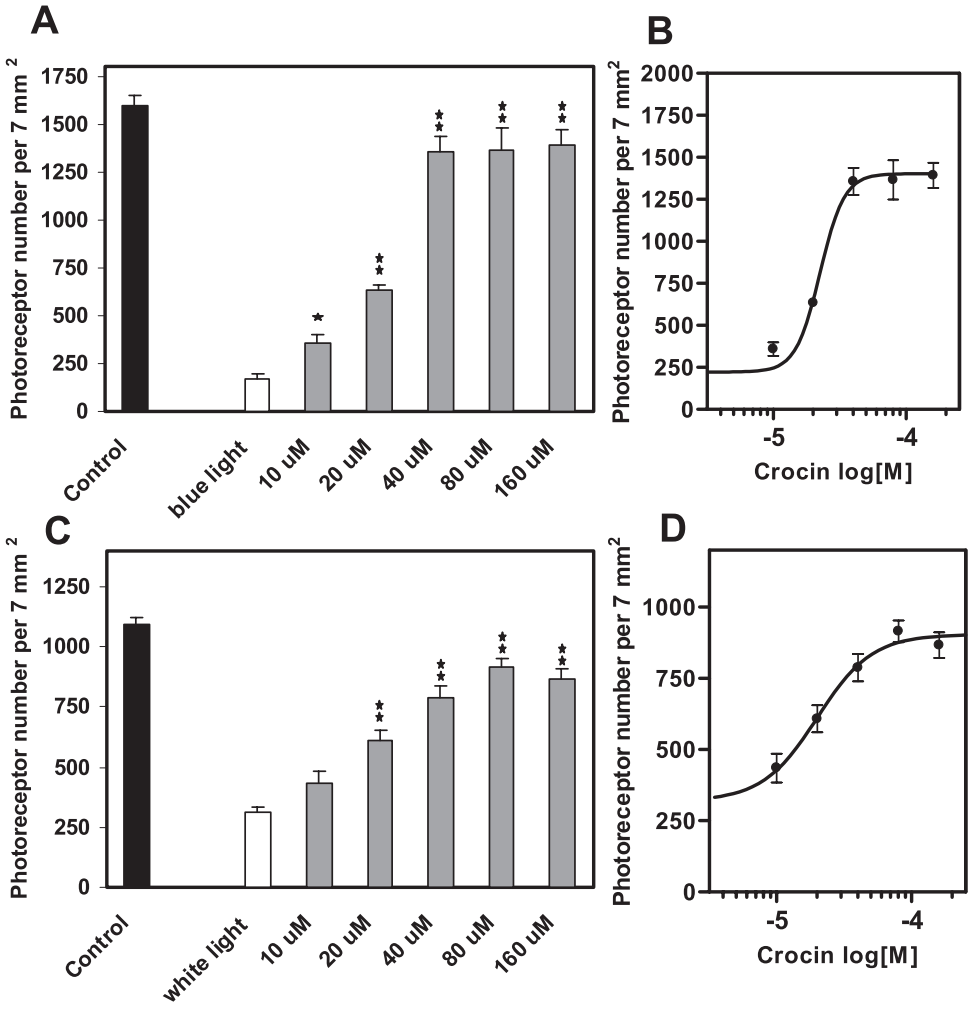

FIGURE 5. Neuroprotective effect of crocin against blue actinic light– or white light–induced photoreceptor damage in primate retinal cell cultures. (**A**, **B**) Fifteen-day-old cell cultures were pretreated with different concentrations of crocin (10–160 μM) for 24 hours, followed by blue light exposure. (**A**) The number of photoreceptors was significantly decreased (89%) in cell cultures exposed to blue light compared with control. (**B**) Crocin protected against light-induced cell damage in a concentration-dependent manner with an EC_{50} of 27 μM. (**C**, **D**) Cell cultures were pretreated with different concentrations of crocin (10–160 μM) for 24 hours, followed by exposure to white fluorescent light for 24 hours. (**C**) Number of photoreceptors was significantly decreased (70%) in cell culture exposed to light compared with control. Number of photoreceptors in cultures treated with different concentrations of crocin was significantly increased relative to light-damaged cell cultures (12 views = 7 mm^2; values are mean ± SEM; $n = 6$; $^*P < 0.05$; $^{**}P < 0.001$ versus light; one-way ANOVA with Bonferroni correction). (**D**) Crocin protected against light-induced cell death in a concentration-dependent manner with an EC_{50} of 23 μM.

photoreceptors survived at 160 μM crocin in bovine and primate retinal cell cultures. Posttreatment with crocin after light exposure was protective but less effective than when used as pretreatment for 24 hours before light exposure. These results suggest that crocin has the ability to suppress light stress and to block photoreceptor cell death in this in vitro model. Crocin has been shown to have antioxidant properties[44,45] and to prevent lipid peroxidation.[46] Further studies have shown that crocin prevents the death of PC12 cells through glutathione pathways and suppresses the activation of caspase-8 caused by serum/glucose deprivation.[47,48] A study[49] has shown that crocin prevents PC-12 cell death when it is induced by tumor necrosis factor-alpha (TNF-α). Crocin suppressed TNF-α–induced expression of Bcl-X_S and LICE (caspase-3) mRNAs and simultaneously restored the cytokine-induced reduction of Bcl-X_L mRNA expression. Crocin also blocked cytochrome *c*–induced activation of caspase-3.[49] In addition to these crocin properties, crocin analogs have a specific action to increase the blood flow in the retina and choroid.[50] Further experiments are required to precisely define the action of crocin at the molecular level for protection against light-induced photoreceptor damage.

In conclusion, these data provide the first experimental evidence that crocin prevents light-induced photoreceptor death in primary retinal cell cultures. These results provide the rationale for further investigation of crocin and its derivatives as a possible therapeutic agent for degenerative diseases of the retina such as AMD.

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References

1. Beatty S, Koh H, Phil M, Henson D, Boulton M. The role of oxidative stress in the pathogenesis of age-related macular degeneration. *Surv Ophthalmol*. 2000;45:115–134.
2. Winkler BS, Boulton ME, Gottsch JD, Sternberg P. Oxidative damage and age-related macular degeneration. *Mol Vis*. 1999;5:32–42.
3. Anderson RE, Kretzer FL, Rapp LM. Free radicals and ocular disease. *Adv Exp Med Biol*. 1994;366:73–86.
4. Donoso LA, Kim D, Frost A, Callahan A, Hageman G. The role of inflammation in the pathogenesis of age-related macular degeneration. *Surv Ophthalmol*. 2006;51:137–152.
5. Frank RN, Amin RH, Puklin JE. Antioxidant enzymes in the macular retinal pigment epithelium of eyes with neovascular age-related macular degeneration. *Am J Ophthalmol*. 1999;127:694–709.
6. Klein RJ, Zeiss C, Chew EY, et al. Complement factor H polymorphism in age-related macular degeneration. *Science*. 2005;308:385–389.
7. Eldred GE, Lasky MR. Retinal age pigments generated by self-assembling lysosomotropic detergents. *Nature*. 361:724–726.
8. Taylor HR, West S, Munoz B, Rosenthal FS, Bressler NM. The long-term effects of visible light on the eye. *Arch Ophthalmol*. 1992;110:99–104.
9. Grimm C, Wenzel A, Hafezi F, Yu S, Redmond TM, Reme CE. Protection of RPE65-deficient mice identifies rhodopsin as a mediator of light-induced retinal degeneration. *Nat Genet*. 2000;25:63–66.

10. Organisciak DT, Xie A, Wang HM, Jiang YI, Darrow RM, Donoso LA. Adaptive changes in visual cell transduction protein levels: effect of light. *Exp Eye Res*. 1991;53:773–779.
11. Keller C, Grimm C, Wenzel A, Hafezi F, Reme CE. Protective effect of halothane anesthesia on retinal light damage: inhibition of metabolic rhodopsin regeneration. *Invest Ophthalmol Vis Sci*. 2001;42:476–480.
12. Grimm C, Reme CE, Rol PO, Williams TP. Blue light's effects on rhodopsin: photoreversal of bleaching in living rat eyes. *Invest Ophthalmol Vis Sci*. 2000;41:3984–3990.
13. Cortina MS, Gordon WC, Lukiw WJ, Bazan NG. Light-induced photoreceptor damage triggers DNA repair: differential fate of rods and cones. *Adv Exp Med Biol*. 2003;533:229–240.
14. Young RW. Pathophysiology of age-related macular degeneration. *Surv Ophthalmol*. 1987;31:291–306.
15. Curcio CA, Medieros NE, Millican CL. Photoreceptors loss in age-related macular degeneration. *Invest Ophthalmol Vis Sci*. 1996; 37:1236–1249.
16. Sugawara T, Sieving PA, Luvone PM, Bush RA. The melatonin antagonist luzindole protects retinal photoreceptors from light damage in the retina. *Invest Ophthalmol Vis Sci*. 1998;39:2458–2465.
17. Organisciak DT, Darrow RA, Barsalou L, Barrow RM, Lininger LA. Light-induced damage in the retina: differential effects of dimethylthiourea on photoreceptor survival, apoptosis and DNA oxidation. *Photochem Photobiol*. 1999;70:261–268.
18. Krinsky NI, Landrum JT, Bone RA. Biologic mechanisms of the protective role of lutein and Zeaxanthin in the eye. *Annu Rev Nutr*. 2003;23:171–201.
19. Tanito M, Masutani H, Kim YC, Nishikawa M, Ohira A, Yodoi J. Sulforaphane induces thioredoxin through the anti-oxidant-responsive element and attenuates retinal light damage in mice. *Invest Ophthalmol Vis Sci*. 2005;46:979–987.
20. Salomi MJ, Nair SC, Pannikar KR. Inhibitory effects of nigella sativa and saffron on chemical carcinogenesis in mice. *Nutr Cancer*. 1991;16:67–72.
21. Gainer JL, Jones JR. The use of crocetin in experimental atherosclerosis. *Experientia*. 1975;31:548–549.
22. Wang CJ, Shiow SJ, Lin JK. Effects of crocetin on the hepatotoxicity and hepatic DNA binding of aflatoxin B1 in rats. *Carcinogenesis*. 1991;12:459–462.
23. Hosseinzadeh H, Younesi H. Antinociceptive and anti-inflammatory effects of *Crocus sativus* L. stigma and petal extracts in mice. *BMC Pharmacol*. 2002;2:7.
24. Sugiura M, Shoyama Y, Saito H, Abe K. Ethanol extract of *Crocus sativus* L. antagonizes the inhibitory action of ethanol on hippocampal long-term potentiation in vivo. *J Pharmacol Exp Ther*. 1994;271:703–707.
25. Abe K, Sugiura M, Yamaguchi S, Shoyama Y, Saito H. Saffron extract prevents acetaldehyde-induced inhibition of long-term potentiation in the rat dentate gyrus in vivo. *Brain Res*. 1999;851: 287–289.
26. Abe K, Sugiura M, Shoyama Y, Saito H. Crocin antagonizes ethanol inhibition of NMDA receptor-mediated responses in rat hippocampal neurons. *Brain Res*. 1998;787:132–138.
27. Gavrieli Y, Sherman Y, Ben-Sasson SA. Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J Cell Biol*. 1992;119:493–501.
28. Yang JH, Basinger SF, Gross RL, Wu SM. Blue light-induced generation of reactive oxygen species in photoreceptor ellipsoids requires mitochondrial electron transport. *Invest Ophthalmol Vis Sci*. 2003;44:1312–1319.
29. Wenzel A, Grimm C, Samardzija M, Reme CE. Molecular mechanisms of light-induced photoreceptor apoptosis and neuroprotection of retinal degeneration. *Prog Retin Eye Res*. 2005;275–306.
30. Traverso V, Kinkl N, Grimm L, Sahel J, Hicks D. Basic fibroblast and epidermal growth factors stimulate survival in adult porcine photoreceptor cell cultures. *Invest Ophthalmol Vis Sci*. 2003;44: 4550–4558.
31. O'Malley MB, MacLeish PR. Monoclonal antibody substrates facilitate long-term regenerative growth of adult primate retinal neurons. *J Neurosci Methods*. 1993;47:61–71.
32. Curcio CA, Hendrickson AE. Organization and development of the primate photoreceptor mosaic. In: Osbourne N, Chader J, eds. *Progress in Retinal Research*. Oxford, UK: Pergamon Press; 1991: 89–120.
33. Hendrickson A, Hicks D. Distribution and density of medium-and short-wavelength selective cones in the domestic pig retina. *Exp Eye Res*. 2002;74:435–444.
34. Margrain TH, Boulton M, Marshall J, Sliney DH. Do blue light filters confer protection against age-related macular degeneration? *Prog Retin Eye Res*. 2004;23:523–531.
35. Wenzel A, Grimm C, Samardzija M, Reme CE. Molecular mechanisms of light-induced photoreceptor apoptosis and neuroprotection for retinal degeneration. *Prog Retin Eye Res*. 2005;24:275–306.
36. Mohand-Said S, Deudon-Combe A, Hicks D, et al. Normal retina releases a diffusible factor stimulating cone survival in the retinal degeneration mouse. *Proc Natl Acad Sci USA*. 1998;95:8357–8362.
37. Fintz AC, Audo I, Hicks D, et al. Partial characterization of retina-derived cone neuroprotection in two culture models of photoreceptor degeneration. *Invest Ophthalmol Vis Sci*. 2003;44:818–825.
38. Krishnamoorthy RR, Crawford MJ, Chaturvedi MM, et al. Photooxidative stress down-modulates the activity of nuclear factor-kB via involvement of caspase-1, leading to apoptosis of photoreceptor cells. *J Biol Chem*. 1999;274:3734–3743.
39. Gaudin C, Forster V, Sahel J, Dreyfus H, Hicks D. Survival and regeneration of adult human and other mammalian photoreceptors in culture. *Invest Ophthalmol Vis Sci*. 1996;37:2258–2268.
40. Picaud S, Pattnaik B, Hicks D, et al. GABAA and GABAC receptors in adult porcine cones: evidence from a photoreceptor-glia coculture model. *J Physiol*. 1998;513:33–42.
41. Haverkamp S, Haeseleer F, Hendrickson A. A comparison of immunocytochemical markers to identify bipolar cell types in human and primate retina. *Vis Neurosci*. 2003;20:589–600.
42. Graslkraupp B, Ruttkaynedecky B, Koudelka H, Bukowska K, Bursch W, Schultehermann R. In situ detection of fragmented DNA (TUNEL assay) fails to discriminate among apoptosis, necrosis, and autolytic cell death: a cautionary note. *Hepatology*. 1995;21:1465–1468.
43. Abdullaev FI. Biological effects of saffron. *Biofactors*. 1993;4:83–86.
44. Pham TO, Cormier F, Farnworth E, Tong VH, Van Calstern MR. Antioxidant properties of crocin from *Gardenia jasminoides* Ellis and study of the reactions of crocin with linoleic acid and crocin with oxygen. *J Agricul Food Chem*. 2000;48:1455–1461.
45. Ochiai T, Ohno S, Soeda S, Tanaka H, Shoyama Y, Shimeno H. Crocin prevents the death of rat pheochromyctoma (PC-12) cells by its antioxidant effects stronger than those of α-tocopherol. *Neurosci Lett*. 2004;362:61–64.
46. Premkumar K, Suresh K, Santhiya ST, Ramesh A. Protective effects of saffron (*Crocus sativus* L) on genotoxins-induced oxidative stress in swiss albino mice. *Phytother Res*. 2003;17:614–617.
47. Ochiai T, Soeda S, Ohno S, Tanaka H, Shoyama Y, Shimeno H. Crocin prevents the death of PC-12 cells through sphingomyelinase-ceramide signaling by increase glutathione synthesis. *Neurochem Int*. 2004;44:321–330.
48. Ashkenazi A, Dixit VM. Death receptors: signaling and modulation. *Science*. 1998;281:1305–1308.
49. Soeda S, Ochiai T, Paopong L, Tanaka H, Shoyama Y, Shimeno H. Crocin suppresses tumor necrosis factor-α -induced cell death of neuronally differentiated PC-12 cells. *Life Sci*. 2001;69:2887–2898.
50. Xuan B, Zhou YH, Li N, Min ZD, Chiou GCY. Effects of crocin analogs on ocular blood flow and retinal function. *J Ocular Pharmacol*. 1999;15:143–152.