

# Saffron Supplement Maintains Morphology and Function after Exposure to Damaging Light in Mammalian Retina

Rita Maccarone,<sup>1</sup> Stefano Di Marco,<sup>1</sup> and Silvia Bisti<sup>1,2,3</sup>

**PURPOSE.** To test whether the saffron extract (*Crocus sativus* L.) given as a dietary supplement counteracts the effects of continuous light exposure in the albino rat retina.

**METHODS.** Three experimental groups of Sprague-Dawley rats were used. Experimental animals were prefed either saffron or  $\beta$ -carotene (1 mg extract/kg/d) before they were exposed to bright continuous light (BCL) for 24 hours. Flash electroretinograms (fERGs) were recorded in control and treated rats the day before and 1 week after light exposure. At the end of the second recording session, the animals were killed and the retinas were quickly removed, fixed, cryosectioned, and labeled so that the thickness of the outer nuclear layer (ONL) could be analyzed. Changes in protein level and cellular localization of fibroblast growth factor (FGF)2 were determined by Western blot analysis and retinal immunohistochemistry, respectively. In a second series of experiments, rats were killed at the end of light exposure, and the amount of apoptotic figures in the ONL was assessed by terminal transferase-mediated deoxyuridine triphosphate (d-UTP)-biotin nick-end labeling (TUNEL). BCL induced DNA fragmentation, characteristic of dying cells, almost exclusively in the photoreceptor layer. The rate of photoreceptor death induced by BCL is expressed as the frequency of TUNEL-positive profiles per millimeter.

**RESULTS.** The photoreceptor layer was largely preserved in saffron-treated animals because it was the fERG response. In addition, the rate of photoreceptor death induced by BCL appeared drastically reduced in treated animals. In  $\beta$ -carotene prefeeding experiments, morphologic analysis showed preservation of the ONL similar to that obtained with saffron prefeeding, whereas the fERG response was unrecordable. Western blot analysis showed that exposure to light induced a strong upregulation of FGF2 in control and  $\beta$ -carotene-treated rats, but no change was noted in saffron-treated rats.

**CONCLUSIONS.** These results show that saffron may protect photoreceptors from retinal stress, maintaining both morphology and function and probably acting as a regulator of programmed cell death. (*Invest Ophthalmol Vis Sci.* 2008;49:1254-1261) DOI:10.1167/iovs.07-0438

Apoptosis is the main mechanism of neuronal cell death in induced and inherited retinal degenerations; photoreceptors die by a process resembling programmed cell death rather than necrosis.<sup>1</sup> Photoreceptors are retinal cells highly specialized to transform quanta of light into electric signals and eventually into visual perception. The energetic cost to perform this task makes these cells vulnerable to a range of oxidative, mechanical, and dysmetabolic stresses, including exposure to high-intensity light. On the other hand, it is well known that the mature nervous system, including the retina, responds to sublethal environmental stress and injury by mounting a self-protective mechanism that enhances neuronal survival in the face of subsequent insult. This phenomenon of conditioning in photoreceptors and other neurons is thought to involve the stress-mediated release of growth factors and cytokines and the subsequent activation of intracellular signaling pathways.<sup>2-4</sup> Ciliary neurotrophic factor (CNTF) and fibroblast growth factor (FGF)2 are two major components of the survival factor response to injury, and they have been proved to protect photoreceptors either exogenously applied or endogenously upregulated.<sup>4</sup> Their membrane receptors are strategically located and are upregulated after retinal stress.<sup>3,5,6</sup> It must be noted that the activation of self-protective mechanisms is one of the strategies used to rescue photoreceptors from death after light damage.<sup>7</sup> The neuroprotective mechanisms provided by cytokines are not completely understood. Numerous treatment strategies and compounds could, at least partially, prevent retinal degeneration in animal models of retinitis pigmentosa. The reduction of oxidative stress by the application of antioxidants is one of those.<sup>8-12</sup> With few exceptions, however, the efficacy is relative and the mechanisms of action remain to be identified. There does not seem to be a single treatment that can prevent retinal degeneration in humans; rather, the best outcome to date is a slowing of degenerative changes. Consequently, any treatment capable of interrupting the initiation or execution, or both, of apoptosis may be of value in these neurodegenerative diseases. Recent apoptosis research clearly revealed a multitude of apoptotic stimuli, which is reflected in a great diversity of proapoptotic cellular signaling and initiation of the death cascade.<sup>1</sup> It thus may emerge that different apoptotic stimuli and pathways might require different rescue strategies, conceivably even a combination of several approaches.<sup>13</sup> In recent years, the impact of nutrition on the manifestation and progression of retinal diseases has become an important and controversial topic.<sup>14</sup> Nevertheless, many potentially beneficial nutritional effects on retinal diseases have not been proven in prospective clinical trials. For most patients, the impact of nutritional factors is still insufficiently understood. Among others,  $\beta$ -carotene, the precursor of vitamin A, has been tested in the treatment of retinal disease.<sup>12,15</sup> Recently, our attention was focused on saffron (an extract from *Crocus sativus*), which has been largely used in traditional medicine<sup>16</sup> for its anticarcinogenic and antitoxic properties but which has never been studied in neurodegenerative disease. The antiapoptotic characteristics of the major components of saffron<sup>17</sup> and its role in oxygen diffusibility<sup>18</sup>

From the <sup>1</sup>Department of Science and Biomedical Technology, University of L'Aquila, L'Aquila, Italy; <sup>2</sup>ARC Centre of Excellence in Visual Science, Canberra, Australia; and the <sup>3</sup>Istituto Nazionale Biostrutture e Biosistemi (INBB), Rome, Italy.

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Corresponding author: Silvia Bisti, Department of Science and Biomedical Technology, University of L'Aquila, L'Aquila, Italy; bisti@univaq.it.

made it of interest for conditions that affect retinal photoreceptors. Here we report data showing that prefeeding with saffron provides striking protection against the death of photoreceptors induced by exposure to continuous bright light. Preliminary results have been reported in abstract form (MacCarone R. *IOVS* 2005;46:ARVO E-Abstract B145).

## METHODS

All experiments were performed in compliance with the Animal Care and Use Committee guidelines and in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All efforts were made to minimize the number of animals used and their suffering. The data presented in this article were obtained from experiments carried out on 69 Sprague-Dawley rats (2 months old; 23 controls, 26 saffron supplementation diet, 20  $\beta$ -carotene supplementation diet). The animals were raised in cyclic (12 hours at 5 lux, 12 hours dark) light.

## Diet Supplementation

Stigmas of saffron (*L'Aquila Saffron*; *Antica Azienda Agricola Peltuinum, Prata D'Ansiondia, Italy*) were dissolved in water to obtain a suspension, and 1 mg/kg was directly offered every day through a syringe. Animals learned easily to suck directly from the tip. A similar procedure was adopted for  $\beta$ -carotene (extracted from *Dunalietta salina* by Norbiotech Chile S.A. la Tirana, Iquique, Chile) dissolved in olive oil; the daily dose was 1 mg/kg.

## Electroretinographic Recording

Albino rats were dark adapted for a 12-hour period overnight, and electroretinograms (ERGs) were recorded in a completely darkened room in control and treated animals 1 day before and 1 week after exposure to bright, damaging light using a procedure previously described.<sup>19</sup> Briefly, animals were anesthetized with intramuscular injection of 100 mg/kg ketamine/12 mg/kg xylazine, corneas were anesthetized with a drop of novocaine, and pupils were dilated with 1% tropicamide. The rat was placed on a pad heated to 37°C. Recordings were made from the left eyes, with a gold electrode placed on the cornea while the right eye was fully covered. The reference electrode was placed subcutaneously in the anterior scalp between the eyes, and the ground electrode was inserted into the tail. Responses were amplified differentially, bandpass filtered at 0.3 to 500 Hz, digitized at 0.25- to 0.5-ms intervals by a personal computer interface (LabVIEW 5.1; National Instruments, Milan, Italy), and stored on a disc for processing. Responses were averaged ( $n = 5$ ), with an interstimulus interval ranging from 60 seconds for dim lights to 5 minutes for the three brightest flashes. An electronic flash unit (SUNPAK B3600 DX; Tocad Company, Tokyo, Japan) generated a stimulus whose energy decayed in time with a  $\tau$  of 1.7 ms. Calibrated neutral-density filters were used to attenuate the intensity of the flashes. The flash luminance at the corneal plane was measured in photometric units, as previously described.<sup>19</sup> The maximum flash intensity obtained by this procedure corresponded to 1488 cd/m<sup>2</sup>. The estimated maximum retinal illuminance of 18,689 scotopic td/s, therefore, corresponded to  $5.7 \times 10^5 \phi$ . At the end of the second recording session, animals were humanely killed by anesthetic (urethane) overdose.

## Light Exposure

Sprague-Dawley rats were placed in individual acrylic glass (Plexiglas) cages with food available on the floor and water in plastic bottles. Animals were dark adapted overnight, and at 9 AM they were exposed to continuous bright light (1000 lux) for the next 24 hours. One experimental group was humanely killed at the end of the exposure, and the eyes were enucleated and fixed in 4% paraformaldehyde. Frozen sections were prepared as described<sup>7</sup> to label apoptotic cells using the TUNEL (terminal deoxynucleotidyl transferase-mediated

dUTP-nick end labeling) procedure. The second group was allowed to recover for 1 week before ERG recordings were performed.

## Immunohistochemistry and Morphologic Evaluation by Quantitative Histology

One week after bright light exposure (at the end of the recording session), the eyes were enucleated and fixed in 4% paraformaldehyde for 1 hour, washed in 0.1 M phosphate-buffered saline (PBS, pH 7.4), and cryoprotected by immersion in 15% sucrose overnight. Eyes were embedded in optimum cutting temperature (OCT) compound (Tissue-Tek; Qiagen, Valencia, CA), snap frozen in liquid nitrogen/isopentane, and cryosectioned at 20  $\mu$ m. Sections were collected on gelatin- and poly-L-lysine-coated slides and immunolabeled for FGF2 protein (Upstate Biotechnology, Lake Placid, NY).

To block nonspecific binding, 1% bovine serum (BSA) was used. Sections were incubated overnight with mouse monoclonal FGF2 antibody diluted 1:200 in 1% BSA at 4°C. Secondary antibody was anti-mouse IgG conjugated to green fluorescent dye (Alexa Fluor 488; Molecular Probes, Invitrogen, Carlsbad, CA) diluted 1:200 in 1% BSA and incubated at 37°C for 2 hours. This was followed by three 5-minute washes in PBS and counterstaining with DNA-specific label, bisbenzamide (Hoechst) 1:10,000, for 1 minute at room temperature (RT) to measure the thickness of the photoreceptor layer.<sup>7</sup> Outer nuclear layer (ONL) thickness was measured starting at the dorsal edge along the vertical meridian crossing the optic nerve head according to a procedure already described.<sup>20</sup> Measurements were reported at 1-mm intervals (each point was the mean of four measurements at 250- $\mu$ m intervals). In each retina, we measured two sections. Images were taken by confocal microscope (Nikon, Tokyo, Japan) and fluorescence microscope (Nikon).

## Western Blot Analysis

One week after bright light exposure, retinas were rapidly extracted and independently homogenized with a syringe in ice-cold lysis buffer (0.1% sodium dodecyl sulfate, sodium desossicolate 0.5%, sodium phosphate dibasic, sodium phosphate monobasic 1.7 mM, 150 mM sodium chloride, 1% NP-40), supplemented with protease inhibitors (1 tablet Complete Mini aprotinin 30  $\mu$ L/mL and sodium orthovanadate 10  $\mu$ L/mL; both Roche Diagnostics, Basel, Switzerland). Retinal lysates were incubated on ice for 30 minutes and then centrifuged at 14,000 rpm for 20 minutes, after which the supernatant was collected. Protein concentration was determined by a BCA protein assay kit (Pierce, Rockford, IL). For each sample, 50  $\mu$ g protein was resolved by electrophoresis on 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and was transferred to nitrocellulose membranes (Protran; Whatman Schleicher and Schuell, Dassel, Germany). Blots were incubated in 10 mM Tris (pH 8), 150 mM NaCl, 0.1% Tween 20 (TBST), and 10% nonfat dried milk (Euroclone, Pero, Italy) for 1 hour at room temperature to block nonspecific signals. Membranes were incubated overnight at 4°C with monoclonal anti-human FGF2 (type II, clone bFM-2; 2  $\mu$ g/mL; Upstate Biotechnology). Membranes were washed with TBST and then incubated for 1 hour at room temperature with anti-mouse peroxidase-linked secondary antibodies (0.4  $\mu$ g/mL; SC-2031; Santa Cruz Biotechnology, Santa Cruz, CA). Protein signals were detected with a chemiluminescent reagent (ECL; Super Signal West Pico Chemiluminescent substrate; Pierce) followed by exposure of membranes to autoradiograph (Biomax Xar; Eastman Kodak, Rochester, NY) imaging film. Membranes were reprobbed with an anti- $\alpha$ -actinin polyclonal antibody (1:1000, 0.2  $\mu$ g/mL; SC-7454; Santa Cruz Biotechnology) as housekeeping gene, followed by incubation with an anti-goat peroxidase-linked secondary antibody (0.4  $\mu$ g/mL; SC-2033; Santa Cruz Biotechnology). Densitometric analysis was performed (Image J software) on scanned autoradiographic films obtained from a series of three independent Western blot analyses, each performed with retinal samples from distinct experimental groups. Densitometric values obtained for FGF2 were normalized with respect to  $\alpha$ -actinin level in the same blot.

## Statistical Analysis

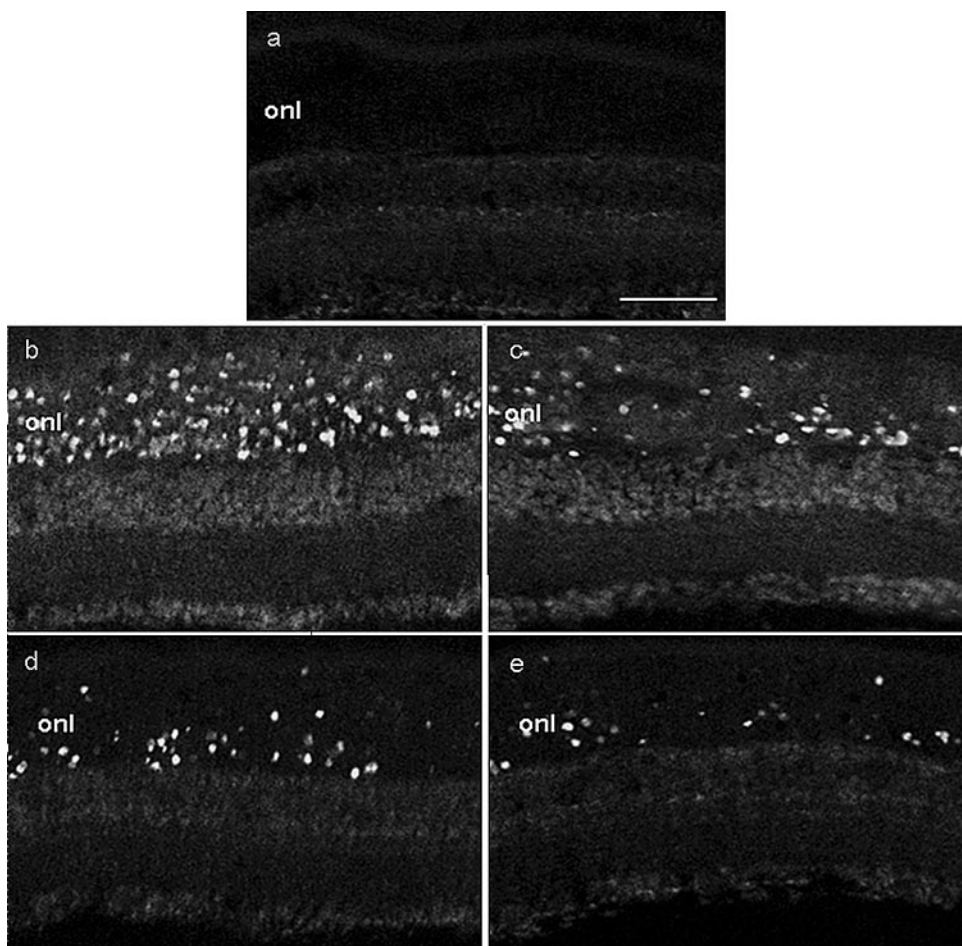
The statistical significance of differences between the average values of control and treated animals was investigated by individual Student's *t*-test and one-way ANOVA test followed by Dunnett post hoc test. Results are expressed as the mean  $\pm$  SE (but as mean  $\pm$  SD in Fig. 8).  $P < 0.05$  was considered significant.

## RESULTS

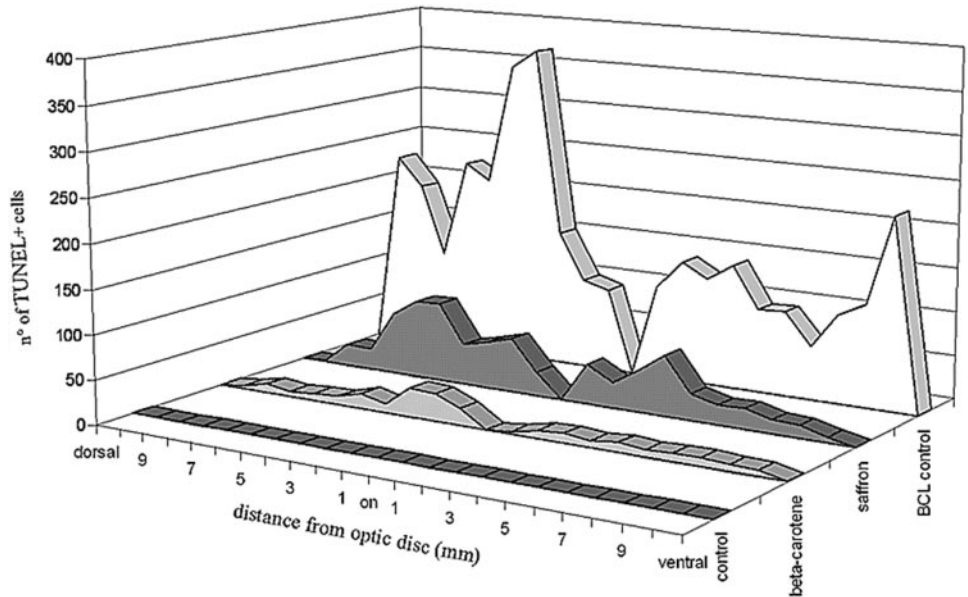
### Histology, Cell Death, and Structure

An artificial induction of apoptosis can be obtained in photoreceptors of albino rats by exposure to high-intensity light. Cell death was thought to have resulted from oxidative stress induced by prolonged increase in oxygen tension and photo-oxidation. We used this model to test whether a saffron supplement with the diet may increase photoreceptor resistance to light damage. Sprague-Dawley rats were fed with saffron 6 weeks before exposure to bright continuous light (BCL; 1000 lux for 24 hours), and we assessed apoptosis using the TUNEL technique. Parallel experiments following the same protocol with  $\beta$ -carotene supplementation were used as a control.  $\beta$ -Carotene is a natural carotenoid widely used and recommended in the diet.<sup>12</sup> As previously reported,<sup>7</sup> BCL induced the DNA fragmentation characteristic of dying cells almost exclusively in the photoreceptor layer. As shown in Figure 1a, no dying neurons were detected in control retina, whereas the stressed retina presented TUNEL-positive cells with a clear dorsal (Fig. 1b) to ventral (Fig. 1c) gradient. After 6 weeks of saffron treatment, the number of dying cells was significantly

reduced in the dorsal (Fig. 1d) and in the ventral (Fig. 1e) retina (regions corresponding to 1 mm from the optic disc are shown in control and treated retinas). Representative results in four experimental conditions are reported in Figure 2; dying neurons were counted along the entire extension of the retina, from dorsal to ventral, in control, in  $\beta$ -carotene, and in saffron prefed rats after BCL exposure and in normal control rats. Data obtained in BCL saffron,  $\beta$ -carotene, and control animals were pooled and reported in Figure 3. After 6 weeks of treatment (saffron and  $\beta$ -carotene), the number of dying cells was reduced, and the difference was statistically significant. Two-day treatment reduced the rate of cell death (though not to a significant level), a result that suggested progressive activation of protective mechanisms. In Figure 4, nuclear staining demonstrates that saffron (Fig. 4c) and  $\beta$ -carotene (Fig. 4d) treatment maintained the morphology of the outer nuclear layer (ONL) in comparison with control (Fig. 4a) and light-damaged retina (Fig. 4b). Representative photomicrographs are taken in corresponding retinal regions (dorsal retina 1 mm from the optic disc). The thickness of the ONL was measured in 1-mm steps from the dorsal to the ventral edge, in sections that passed through the optic disc. Data have been averaged and reported in Figure 5a for saffron and in Figure 5b for  $\beta$ -carotene. As previously reported,<sup>20</sup> the ONL thickness was reduced by light exposure (controls), and the reduction was systematically counteracted by prefeeding either with saffron or with  $\beta$ -carotene. The light-induced damage and the saffron/ $\beta$ -carotene induced rescue was greater in dorsal retina, even with 2-day treatment, and this difference was statistically significant. In the ventral retina, only  $\beta$ -carotene induced statistically a



**FIGURE 1.** Representative sections of retina from control (a) and rats exposed to bright light for an extended period (BCL, 1000 lux for 24 hours) without (b, c) and with (d, e) saffron supplementation. (b, d) Dorsal retina 1 mm from optic disc. (c, e) Ventral retina 1 mm from optic disc. Fragmentation of DNA induced by BCL is labeled with the TUNEL technique. DNA fragmentation was specific to the ONL and therefore to photoreceptors. Scale bar, 50  $\mu$ m.



**FIGURE 2.** BCL-induced TUNEL labeling of the ONL expressed as TUNEL-positive cells as function of the dorso-ventral position along the retina in four representative experimental conditions.

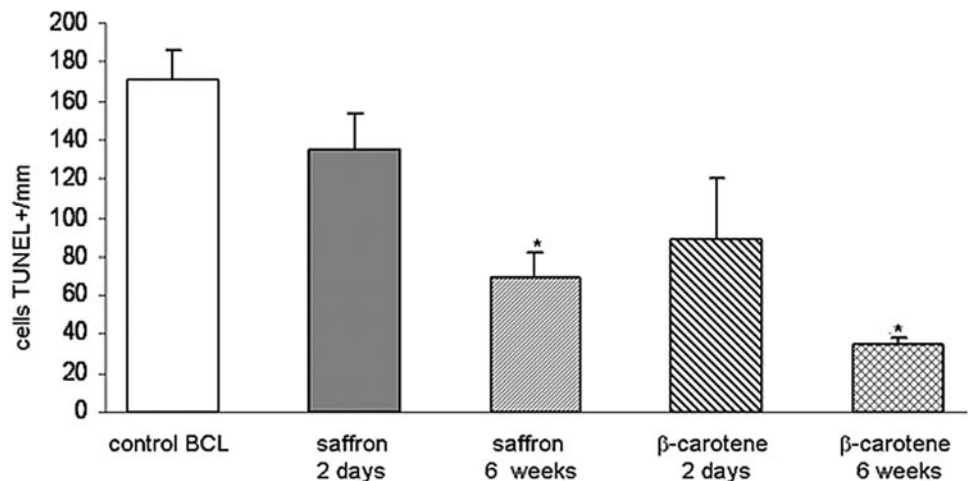
significant difference in ONL thickness. Six-week treatment led to ONL thickness in the range of normal controls.<sup>20</sup>

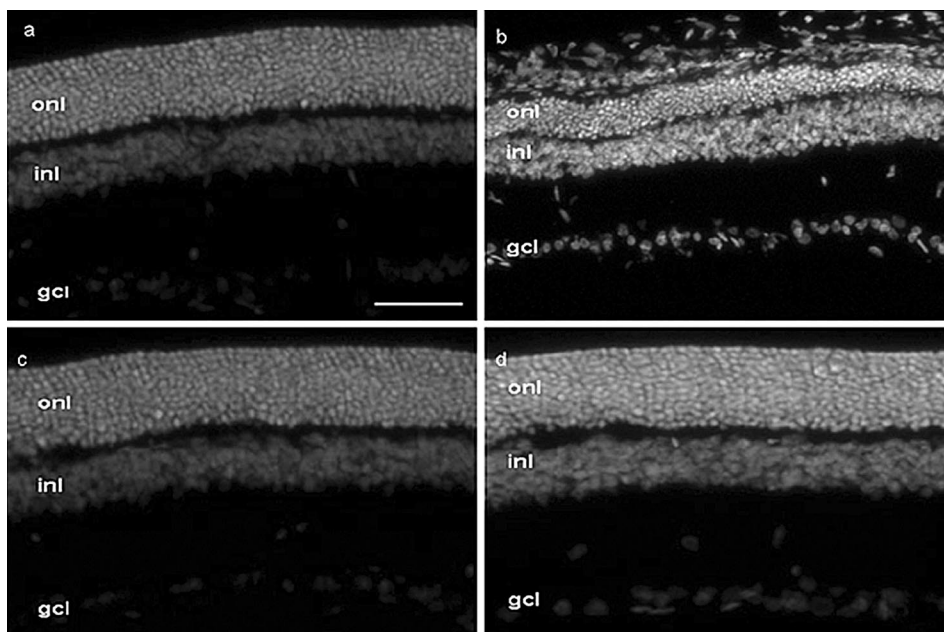
**Functional Evaluation of Photoreceptor Cell Protection**

The functional consequences of prefeeding animals with saffron or  $\beta$ -carotene were evaluated by recording, in each experimental animal, the electroretinographic response to a flash of light (fERG) in dark-adapted conditions before and 1 week after exposure to damaging light. Although we recorded responses as a function of increasing luminance from threshold to saturation, here we report data obtained at a fixed value of luminance ( $5.7 \times 10^2 \phi$ ) in the linear range before saturation. Results for saffron prefeeding are summarized in Figure 6 (a, b, e, *middle inset*). Data were pooled and reported as mean amplitude (in mV; Fig. 6a) or as a percentage of pre-damage amplitude (Fig. 6b). The amplitude of the b-wave in control was strongly reduced after exposure to damaging light (*upper inset*), in agreement with morphologic results, whereas in treated animals, the b-wave showed preservation, and the amplitude improved with increasing periods of treatment. The reduction in the b-wave amplitude in treated animals was not statistically significant after exposure to bright light, suggesting

that saffron treatment largely preserved morphology and function.  $\beta$ -Carotene prefeeding led to an interesting result, summarized in Figures 6c-e (*lower inset*). Increasing the time of treatment led to the disappearance of the visual response, though histologic examination showed even better preservation than was seen in saffron-treated animals. Based on previous results of the impact of trophic factor upregulation on visual function, we tested whether prefeeding with saffron or  $\beta$ -carotene has a differential effect on the expression of FGF2. FGF2 has been shown to be protective for stressed photoreceptors but deleterious for visual response to light.<sup>7,19,21,22</sup> Immunostaining of stressed and unstressed retinas showed that though FGF2 protein in the control retina was localized in Müller cells, in retinas exposed to damaging light (Figs. 7a, 7a1), it filled the photoreceptor cell bodies in the ONL (Figs. 7b, 7b1). No ONL label could be detected in rat retinas prefed saffron (Figs. 7c, 7c1), whereas after  $\beta$ -carotene feeding, the amount of FGF2 in photoreceptors was greatly increased (Figs. 7d, 7d1). To test whether the presence of FGF2 in ONL was attributed to a translocation of the protein from Müller cells or to an increase in protein level, we performed Western blot analysis; results are shown in Figure 8. The antibody labeled two distinct fragments (22–24 kDa and 18 kDa); we performed

**FIGURE 3.** BCL-induced TUNEL labeling of ONL expressed as TUNEL-positive cells per millimeter are averaged over the full length of the section, and results from all animals used in the five experimental conditions are pooled together. BCL control, 11 retinas; saffron 2 days, 6 retinas; saffron 6 weeks, 9 retinas;  $\beta$ -carotene 2 days, 7 retinas;  $\beta$ -carotene 6 weeks, 10 retinas. For each experimental group, the mean  $\pm$  SE is reported. Only retinas after 6 weeks of treatment with saffron and  $\beta$ -carotene presented a significant reduction in TUNEL-positive figures versus BCL control. \* $P < 0.05$ , one-way ANOVA followed by Dunnet post hoc test.





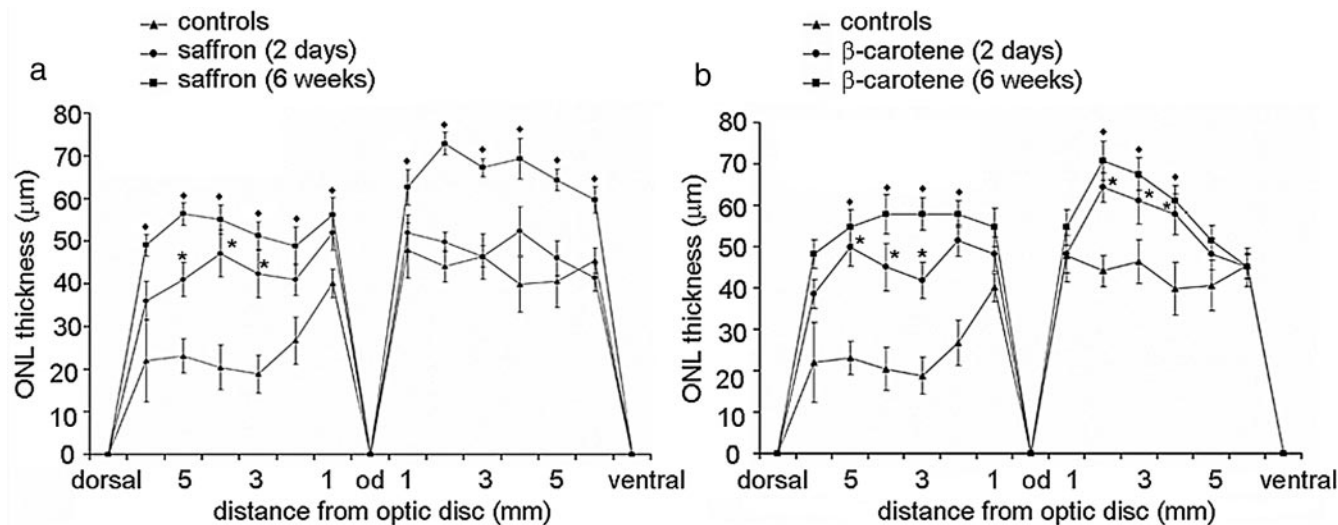
**FIGURE 4.** Representative bisbenzimidide labeling of control (a), BCL (b), 6-week saffron-treated (c), and 6-week  $\beta$ -carotene-treated retinas (d). One millimeter dorsal from optic disc. Scale bar, 50  $\mu$ m.

densitometric analysis on both, and we reported in the figure the result obtained on an 18-kDa fragment as a ratio FGF2 and  $\alpha$ -actinin. Saffron-treated retinas showed a statistically significant difference from BCL controls, but there was no difference from normal controls.  $\beta$ -Carotene-treated and BCL control retinas showed a comparable increase in FGF2 expression.

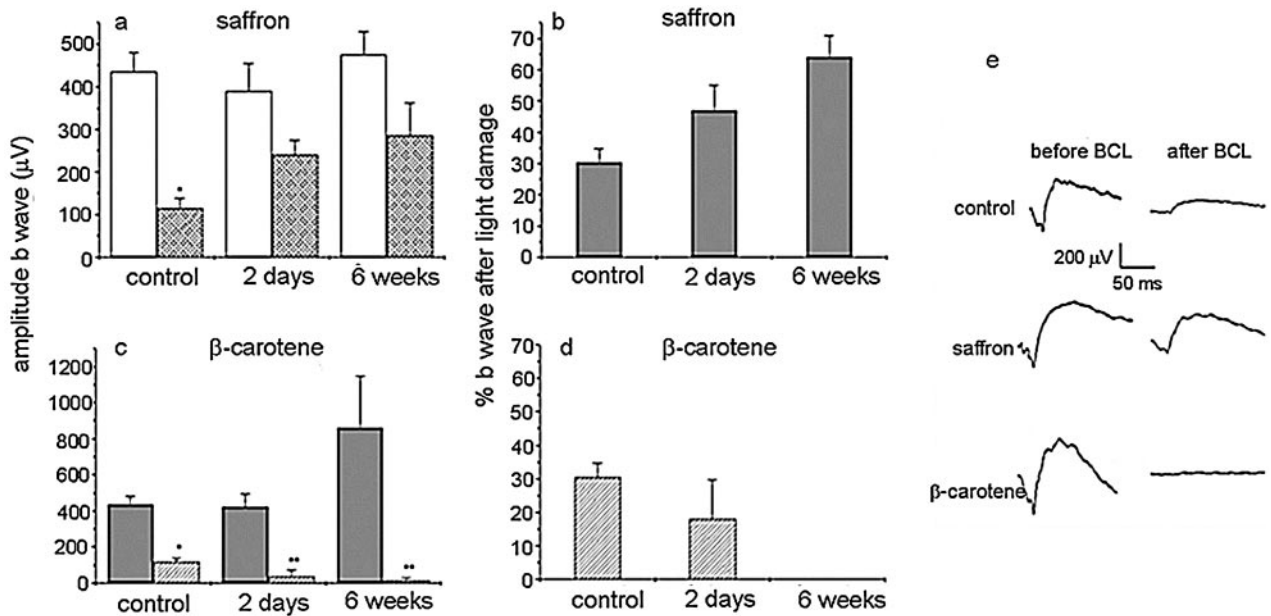
**DISCUSSION**

The main result reported in the present paper is that saffron diet supplementation mitigates retinal damage induced by exposure to continuous bright light. The morphology and the function of the retina are highly preserved in animals prefed saffron. Saffron, the dried stigma of *C. sativus*, was widely used for many centuries in traditional medicine, following the common pathways of many drugs. Recently, its crude extract and purified chemicals have been demonstrated to prevent tumor

formation, atherosclerosis, hepatic damage, and renal damage.<sup>18</sup> The mechanism of action of the crocin, a carotenoid pigment of saffron, has been extensively investigated in PC12 cells under serum-free and hypoxic conditions.<sup>23</sup> Crocin significantly suppressed cell death, membrane lipid peroxidation, and caspase-3 activation in serum-deprived and hypoxic PC12 cells. Crocin increases GSH levels and prevents the activation of the JNK pathway, which has a role in the signaling cascade downstream ceramide. Because reduced GSH levels make cells sensitive to various apoptosis-inducing agents, the restoration or maintenance of intracellular GSH levels by increased synthesis may be expected to protect cells from damage or death.<sup>24</sup> Interestingly, crocin seems to act only in living cells after the induction of gene expression.<sup>23</sup> The antiapoptotic characteristic of saffron components makes them interesting candidates in the treatment of retinal neurodegenerative disease. Recently, crocin was proved to reduce apoptosis in photoreceptors iso-



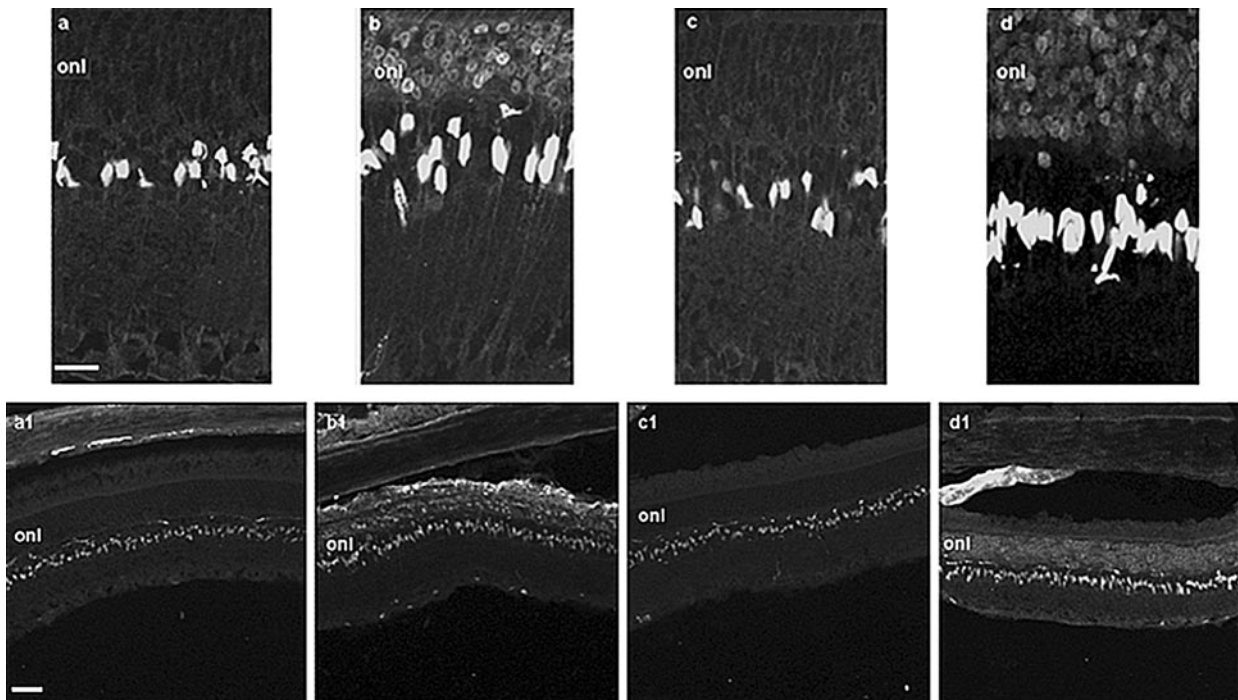
**FIGURE 5.** Thickness of the ONL was measured in BCL control (6 retinas); saffron 2 days (5 retinas); saffron 6 weeks (6 retinas);  $\beta$ -carotene 2 days (3 retinas);  $\beta$ -carotene 6 weeks (3 retinas). At each point, experimental data have been pooled and the SE of the mean is reported. The thickness was reduced by light exposure (controls), and the reduction was systematically countered by prefeeding with saffron (a) and  $\beta$ -carotene (b). \* $P < 0.05$ : 2-day treatment versus control.  $\blacklozenge P < 0.05$ : 6-week treatment versus control (one-way ANOVA followed by Dunnet post hoc test).



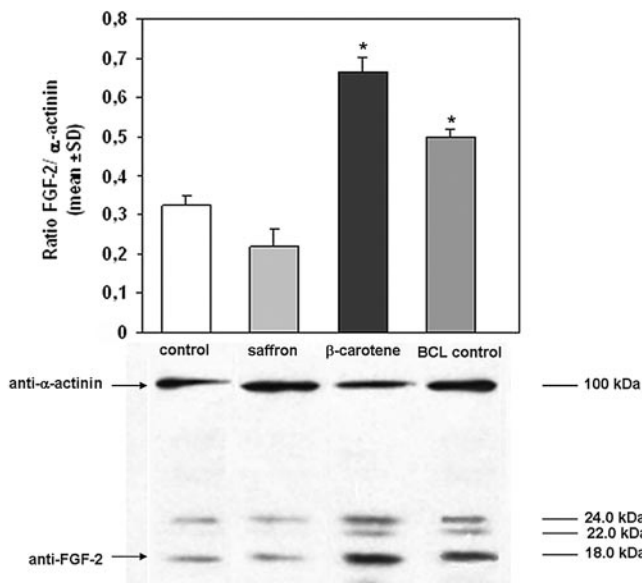
**FIGURE 6.** (a) The b-wave amplitude of ERG is reported before and after light exposure in control, saffron (2 days and 6 weeks) and (c)  $\beta$ -carotene treated (2 days and 6 weeks). (b, d) Data as in (a) and (c); here the b-wave is reported as a percentage of the amplitude recorded before BCL. (e) Representative ERG recordings in three experimental conditions before and after light exposure in BCL control, saffron, and  $\beta$ -carotene 6-week treatment. \* $P < 0.05$ ; \*\* $P < 0.001$ . BCL control, 10 animals; saffron 2 days, 6 animals; saffron 6 weeks, 7 animals;  $\beta$ -carotene 2 days, 4 animals;  $\beta$ -carotene 6 weeks, 4 animals (Student's *t*-test).

lated in primary retinal cell cultures and exposed to damaging blue light,<sup>25</sup> supporting this hypothesis. The possibility of protecting photoreceptors using a diet supplement has never been tested before. Here we provide evidence that saffron extract strongly reduces photoreceptor death induced by environmental stresses, suggesting that this effect could be attributed to the crocin introduced with the diet. For this to occur,

crocin has to be absorbed at the intestinal level and reach the retina in a sufficient concentration. In addition, it has been shown that intravenous injection of crocin reduced the infarct volume in an ischemia-reperfusion brain model; therefore, crocin may pass the blood-brain barrier.<sup>23</sup> With respect to the mechanisms of intestinal absorption, some evidence indicates that orally administered crocin is hydrolyzed to crocetin before



**FIGURE 7.** Representative FGF2 immunolabeling in control (a, a1), BCL retina (b, b1), saffron-treated retina (c, c1), and  $\beta$ -carotene-treated retina (d, d1). One millimeter dorsal from OD. (a-d, high magnification) Scale bars: (a-d) 10  $\mu$ m; (a1-d1) 50  $\mu$ m.



**FIGURE 8.** Western blot analysis of FGF2 protein level. Retinas were collected from normal control, saffron,  $\beta$ -carotene (6-week treatment), and BCL control 1 week after exposure to bright light. Quantification was performed by normalizing the FGF2 level with that of  $\alpha$ -actinin (1:1 ratio, FGF2/ $\alpha$ -actinin). We reported a molecular mass in kilodaltons for each band. For FGF2, the signal was constituted by a doublet (22 and 24 kDa) and by a unique band at 18 kDa that was used for quantification. Six retinas at each data point. Statistical analysis was performed with one-way ANOVA followed by Dunnett post hoc comparing the three experimental conditions with normal control. \* $P < 0.05$ ; comparing  $\beta$ -carotene with BCL control was not significant.

or during intestinal absorption. Absorbed crocetin is partly metabolized to monoglucuronide and diglucuronide conjugates,<sup>26</sup> suggesting either a more complicated mechanism of action or a different intestinal absorption when crocins are supplied together with the other saffron components. Further experiments are necessary to elucidate these questions. The relevance of the present data comes from the growing evidence of the pivotal role of the diet in maintaining health. Improving our knowledge on the chemical characteristics of food intake means thinking not only in terms of calories but in terms of activation of metabolic cellular and molecular mechanisms. As already reported for "in vitro" experiments on cell lines,<sup>23</sup> crocins are able to activate metabolic pathways to protect cells from apoptosis. An alternative mechanism of action of saffron components is suggested by experimental evidence that crocetin increases oxygen diffusivity through liquids, such as plasma. Considering the high metabolic rate of photoreceptors, the availability of oxygen may be a critical factor in protecting them from death.<sup>18</sup> Interestingly, according to our results on FGF2 immunolocalization and Western blot analysis, it seems that saffron does not follow the protective pathway of upregulating trophic factors. It has recently been shown that the amplitude of the ERG main components (a and b waves) can be regulated by an upregulation of trophic factors; specifically, CNTF acts at the photoreceptor level reducing the dark current<sup>19</sup> while FGF2 controls the amplitude of the b wave by modulating synaptic contacts.<sup>5,19,21,22</sup> As already indicated, rescue of photoreceptor morphology is not necessarily correlated with rescue of function.<sup>19,21,22</sup> Thus, it appears essential that any rescue strategy that yields satisfactory preservation of morphology is verified by testing retinal function. This statement is confirmed by the present results, which demonstrate that although treatment with  $\beta$ -carotene was effective in preserving retinal morphology, it was ineffec-

tive in maintaining function. This may explain conflicting results reported in the literature on the efficacy of vitamin A supplementation.<sup>12</sup> In this study we have shown that saffron treatment preserves morphology and function, and this result may offer new possibilities in the treatment of retinal neurodegeneration. Many open questions, however, remain to be answered, among them whether different pathologies activate different death pathways, making it more difficult to develop rescue strategies.

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