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ORIGINAL PAPER

Effects of an antioxidant protective topical formulation on retinal tissue of UV-exposed rabbits

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Abstract
Purpose The aim of this study has been to evaluate the protective effect of a topical antioxidant formulation containing riboflavin, d-α-tocopheryl polyethylene glycol (TPGS vitamin E), proline, glycine, lysine, and leucine against UV-B-induced damage in in vivo rabbit retina.
Methods Twenty male albino rabbits were used. Animals were divided into four groups of five animals each. Control group did not receive any UV irradiation. The first group (IG) was irradiated with a UV-A lamp for 30 min; the second (IG30) and the third (IG60) groups received UV irradiation for 30 and 60 min, respectively, and were topically treated with 1 drop (approximately 50 µl) of the antioxidant formulation, every 15 min, starting 1 h before irradiation, until the end of the UC exposure.
Results The retina of IG group showed extensive destruction of the retinal pigment epithelium (RPE) and of the cones and rods layer. The retina of G30 group showed a lesser destruction of both RPE and cones and rods layer. In the G60 group, retina showed an irregular thickening of the RPE, with massive edema of the inner and outer layer immediately adjacent together with a significant reduction of the photoreceptor number.
Conclusion Our results demonstrate that a topical application of eye drops containing riboflavin, d-α-tocopheryl polyethylene glycol (TPGS vitamin E), proline, glycine, lysine, and leucine counteracts UV retinal injury in exposed retina rabbits.

Keywords UV damage · Retina protection · Topic formulation · Riboflavin · Vitamin E

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Introduction

Human exposure to solar radiation, mainly due to the stratospheric ozone layer depletion, is worryingly increasing [1]. The negative health effects of sunlight exposure include sunburn and increased risk of skin

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cancers (melanoma, lip cancer, and keratinocyte cancers) and ocular disease (cataracts, pterygium, ultraviolet keratitis, and conjunctival neoplasm) [2].

The solar spectrum is commonly divided into three bands: the ultraviolet light (290–380 nm); visible light (380–780 nm) (Vis); and infrared (IR) light (780–2500 nm). The energy distribution, within the solar spectrum, is approximately 2% UV, 47% visible, and 51% infrared [3]. UV is further divided into four bands: UV-vacuum (range 100–200 nm), UV-A (400–315 nm), UV-B (315–280 nm), and UV-C (280–100 nm) [4, 5]. Generally, the amount and spectral composition of UV, impinging on the ocular surface is influenced by the following: (1) time of day (zenith angle); (2) latitudinal location; (3) surface reflectance properties; (4) pupillary or squinting reflexes; and (5) transmission characteristics of the various ocular media [6]. However, it is well known that sunlight contains much more UV-A than UV-B and both radiations do not exert beneficial effects on eyes [7]. Phototoxicity decreases with wavelength increasing: so UV is more hazardous than violet light, which is in turn more hazardous than longer-wavelength blue light [8]. In particular, UV-B displays the greatest potential damage [9], despite its radiation is less than 1% of the total radiation reaching the earth's surface [10–12].

Absorption of UV by epidermal cells in skin and eye leads the production of reactive oxygen (ROS) and nitrogen species, which can damage biomolecules such as membrane lipids and deoxyribonucleic acid (DNA) [13]. UV (both UV-A and UV-B wavelengths) directly damages DNA through the formation of pyrimidine dimers. In particular, UV-A and UV-B radiations are considered potent genotoxic agents and environmental mutagens [14], leading the production of two kinds of helix-distorting photo-lesion to DNA, peculiarly consisting in C to T transition and CC to TT transitions, during the DNA replication [15]. UV-A radiation also damages DNA by the following: (1) inducing ROS formation that causes the oxidation of DNA bases and (2) activating the mitogen-activated protein kinase-dependent pathway [16]. As consequence, significant changes occur in the retinal pigment epithelium (RPE) [17], structural and functional impairments of the inter-photoreceptor matrix components and their cell surface receptors [18], degradation of sensitive photoreceptor cells and apoptosis occur [19]. Thus, cumulative long exposure to UV is detrimental to retina, leading to age-related macular degeneration (ADM) [18], the major cause of blindness among people older than 65 years, which proportionally increase with aging [18, 20].

As unavoidable and ineluctable exposure to photostress contributes to damage ocular tissues, the use of compounds, able to protect retina from the phototoxic effect of UV exposure, becomes challenging.

The aim of this study has been to test the protective effects of an antioxidant ophthalmic topical formulation on the retina of rabbits exposed to UV irradiation.

Materials and methods

Animals

According to May-Britt Tessem et al. [21], twenty male albino rabbits (New Zealand White, 2.5–3.0 kg) were used.

Although the significance of the pharmacological affinity for melanin in intraocular pharmacokinetic studies has been highlighted, most of this type of studies in the field of ophthalmology has been performed with albino rabbit eyes [22, 23].

Animals were divided into four groups of five animals each. Control group (CG) did not receive any irradiation and/or eye drop. The other three experimental groups were treated as follows: the first group received the UV irradiation for 30 min, without eye drop supplement (irradiation group, IG), the second (G30) and third (G60) groups received UV radiation for 30 and 60 min, respectively, and were topically treated with 1 drop (approximately 50 µl) of the antioxidant formulation every 15 min, starting 1 h before irradiation, until the end of UV exposure.

The study has been conformed to the ARVO Statement for use of animals in ophthalmic and vision research, and in accordance with the guidelines of the European Economic Community for animal care and welfare (EEC Law No. 86/609). The study received approval from the local Ethics Committee (C.T.S. Department of Medicine and Health Science "*V. Tiberio,*" University of Molise, Campobasso, Italy, No 15/2019). Rabbits were anesthetized using an intramuscular injection of xylazine (20 mg/kg) and ketamine HCl (5 mg/kg).

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UV irradiation

Animals were killed by injection of an overdose of sodium pentobarbital, preceded by anesthesia with xylazine (20 mg/kg) and ketamine HCl (5 mg/kg), as previously described [24]. During irradiation, rabbits were confined in a special cage so that only the head remained exposed to UV radiation. Eyes of anesthetized rabbits were exposed to UV radiation using a Philips medical UV lamp (Serial LTN4006B; Philips S.p.A. 20126 Milano, Italy) with the following properties: irradiation field 10 × 10 cm, one low-pressure lamp at 370 nm (UV-A). The radiant energy was measured with a radiometer (VLX-3 W; Cole-Parmer, Vernon Hills, IL). A distance of 7 cm from the cornea was chosen, according to the procedure previously described by Giblin et al. [24, 25]. The rabbits of IG, G30, and G60 groups were undergone to the radiation (100mW/cm2) for 30 and 60 min, respectively. The irradiance on the cornea was 100 mW/cmq, with a total fluence of 180 J/cm^2 and 360 J/cm^2, respectively. Rabbit eyes of G30 and G60 groups were topically treated with 1 topical drop (approximately 50 µl) of the antioxidant formulation, every 15 min, starting 1 h before irradiation, until the end of UV exposure.

Ophthalmic preparation

The ophthalmic antioxidant preparation consisted of riboflavin, d-α-tocopheryl polyethylene glycol (TPGS vitamin E), proline, glycine, lysine, and leucine solution, pH 7.2 (Iromed Group S.r.l., Rome, Italy, patent no. EP 2459186, USP 9192594).

Histochemistry

The animals were killed 3 days after the end of the tests, and the eyes were enucleated and fixed in Davidson solution (alcohol 95%, formaldehyde, glacial acetic acid, and distilled water) for 24 h [26]. Rabbit retinal specimens were fixed in buffered 10% formalin, embedded in paraffin, and sectioned using the macular part. 5 µm thick serial sections of corneal specimens were deparaffinized and treated for hematoxylin and eosin (H&E) (haematoxylin: Fluka, AG, Switzerland, Buchs SG; Eosin Y: alcohol and water soluble, Winlap, UK), routine staining, as described elsewhere [27–29].

Image and statistical analysis

Images were analyzed by ImageJ software [30]. Statistical analysis (Student's *t* test) and graph drawing were performed with Microsoft Excel Software.

Results

UV irradiation induces significant morphologic changes on the retina of exposed rabbits. Histologically, while control rabbit samples showed a normal appearance of all retina layers (Fig. 1a), 30′ UV irradiation (study group IG) was able to cause a general reduction of retina layers, with extensive destruction of both retinal pigment epithelium (RPE) and cones and rods layers (Fig. 1b). In the IG30 group (irradiated and supplemented with topical eye drops), the damage recorded was at a lower extent when compared to that occurring in the IG group (Fig. 1c). The changes in the IG60 group were more pronounced than those observed in the IG30 group (Fig. 1d). In fact, the signs of tissue alterations were not severe as those found in the irradiated animals not receiving the topical treatment (IG group). The topical protective treatment was more effective in the IG 30 than in the IG60 group, even if a lowest degree of protection was observed also in this group.

Relative thickness of the retinal layers after UV exposure, normalized on control samples and expressed in arbitrary units, is reported in Table 1 and displayed in Fig. 2. As shown in Table 1, differences were significant for all comparisons, except for RPE and the external nuclear layers of IG30 groups. However, the variations in the IG30 group were less significant than those observed in the IG and IG 60 groups, where a marked reduction of retinal thickness and cell density occurred. The reduction was more evident at the level of the external (outer) plexiform layer. The topical treatment with ophthalmic antioxidant formulation significantly attenuated the UV-induced retinal damage.

Discussion

Our findings demonstrated that UV exposure is able to damage retinal integrity in exposed rabbits and that a topical treatment with antioxidant eye drops exerts a

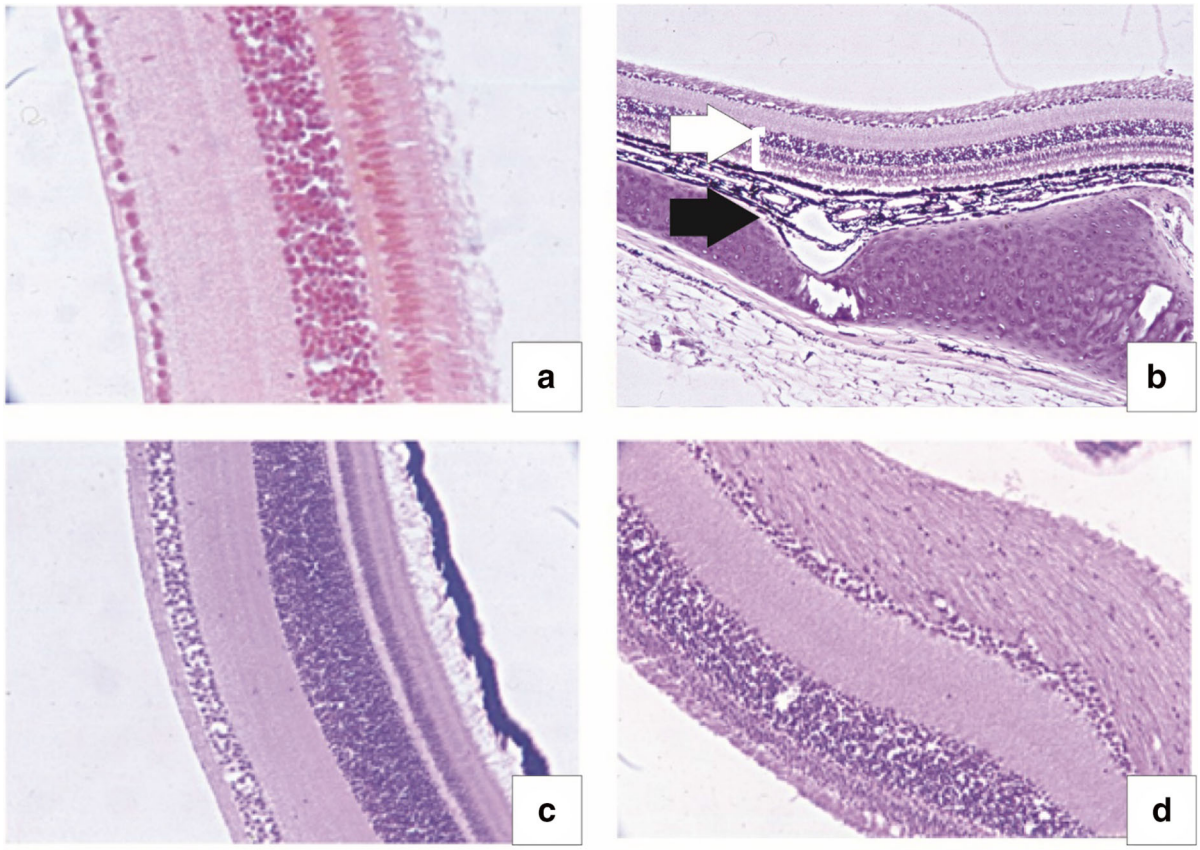

Fig. 1 Retinal morphological changes induced by UV exposure. **a** Control group (CG): normal retina for structure and morphology in the macular region where the multi-stratified layer of ganglion cells is visible; **b** irradiated group (IG): extensive destruction of RPE (black arrow) and of the cones and rods layer (white arrow); **c** irradiated group (IG30) (30′ UV exposure): lesser destruction of the retinal pigment epithelium and of the cones and rods layer; **d** irradiated group (IG60) (60′ UV exposure): irregular thickening of the retinal pigment epithelium (RPE) with massive edema of the inner and outer layer immediately adjacent to the retinal pigment epithelium corresponding to the layer of the cones and rods

Table 1 Thickness of the retinal layers after UV exposure

Retinal layer	A	B	C	D
RPE	95.061	17.223**	93.401	80.582*
PHOTORECEPTOR LAYER	446.797	69.040***	361.653*	278.471**
OUT NUCL LAYER	261.609	54.483**	260.508	220.729*
OUT PLEX LAYER	51.356	7.952**	38.579*	8.713**
inn NUCL + PLEX LAYER	366.880	64.278***	249.755*	134.445**

(A) Control group (CG); (B) irradiated group (IG); (C) irradiated group for 30′, treated with eye drop every 15 min, starting one hour before irradiation, until the end of UV exposure starting one hour before irradiation, until the end of UV exposure starting one hour before irradiation, until the end of UV exposure (IG30); (D) irradiated group for 60′, treated with eye drop every 15 min starting one hour before irradiation, until the end of UV exposure

OUT NUCL OUTER NUCLEAR, *OUT PLEX* OUTER PLEXIFORM *INN* INNER

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, Student's t test, control group (A) versus treated groups

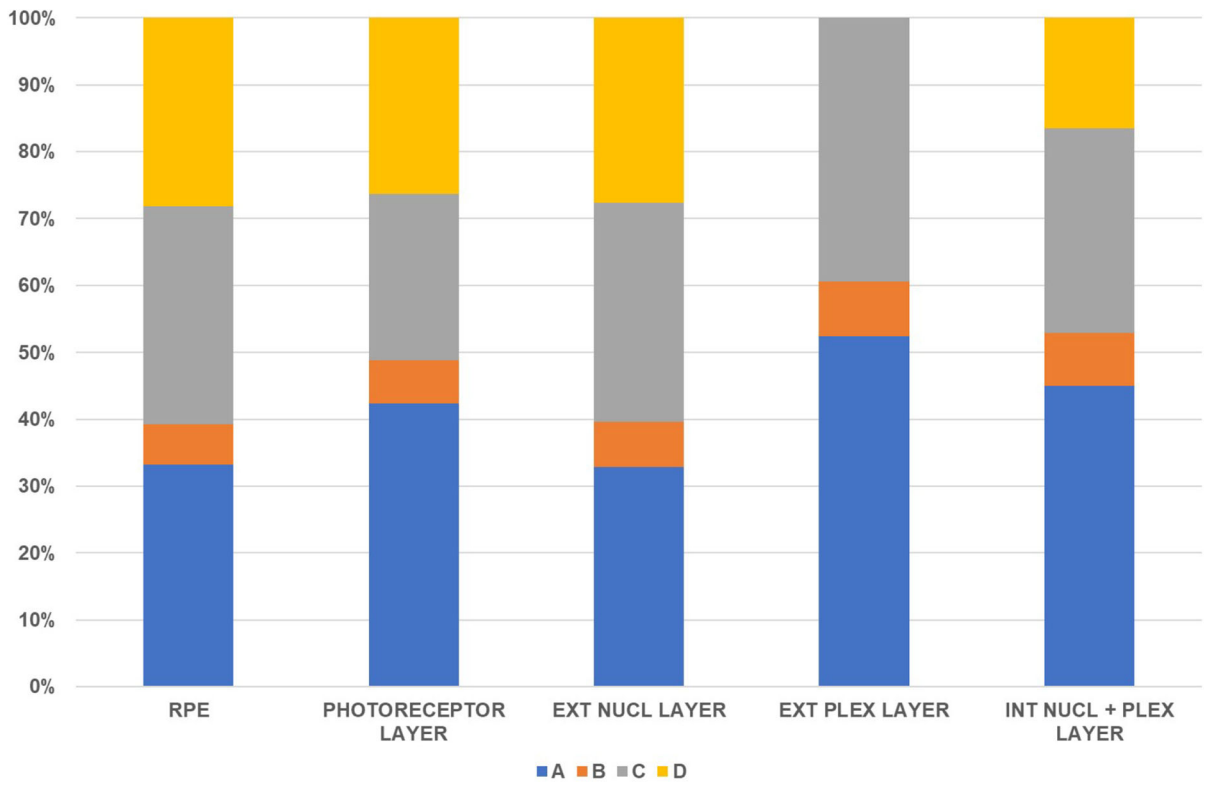

Fig. 2 Relative thickness of layers in the examined samples (EXT NUCL = OUTER NUCLEAR; EXT PLEX = OUTER PLEXIFORM; INT = INNER; RPE = RETINAL PIGMENT EPITHELIUM). (A) Control group (CG); (B) irradiated group (IG); (C) irradiated group for 30′, treated with eye drop every 15 min, starting one hour before irradiation, until the end of UV exposure (IG30); (D) irradiated group for 60′, treated with eye drop every 15 min, starting one hour before irradiation, until the end of UV exposure (IG60)

significant protective effect on the retina of exposed rabbits.

Eyes represent the most specialized structure able to counteract light injury, being designed to focus the incoming light rays to form images on the neutral retina. The constant exposition to UV wavelengths induces biological damage in absorbing tissues. As a consequence, light delivering a radiant exposure insufficient to produce skin damage may indeed cause injury when focused on the retina, by the optics of the eye [31]. The peculiar position and function of the eye make the retina constantly exposed to UV [32], although cornea and lens are known to absorb most of these wavelengths [33]. The retina appears particularly sensitive to photo-oxidative damage, because of its high oxygen consumption and metabolic rate. High amounts of ROS, especially singlet oxygen, are generated in retinal cells during photo-oxidative damage, and this event contributes to the development of macular degeneration [34]. For a collimated beam of a visible light incident on the cornea, and focused to a minimal spot on the retina, the increase in energy density (expressed as J/cm^2) may be as high as 10^5 [31]. Thus, retinal light damage threshold is much lower than that recorded for skin or other external tissues, with an easier occurrence of related retinal pathological changes, i.e., degenerative retinopathy and solar retinitis [25, 35–37]. Ocular tissue and fluids contain both enzymatic (catalase, superoxide dismutase, glutathione peroxidase, and reductase) and non-enzymatic antioxidants (ascorbic acid, reduced glutathione, and alpha-tocopherol) which provide protection from oxidative light-induced damage [38]. Over a lifetime, chronic exposure induces accumulative photo-oxidative damage via singlet oxygen and free radical production, that leads to damage of DNA, proteins, and lipids. Over time, the amount of antioxidants gradually diminishes, making retina more

sensitive to free radical damage [39]. This occurs not only for aging processes, but also after acute exposure to UV radiation [40, 41]. Considering the general trend of prolonged life expectancy [20, 42], strategies to limit oxidation in retina may be important in preventing the development of retinal degenerative diseases. For this purpose, we assess in vivo experiments using a topical application containing riboflavin, d-α-tocopheryl polyethylene glycol (TPGS vitamin E), proline, glycine, lysine, and leucine, to counteract UV retinal damage in exposed eyes.

In our published study, the ophthalmic antioxidant preparation, consisted of riboflavin, d-α-tocopheryl polyethylene glycol (TPGS vitamin E), proline, glycine, lysine, and leucine solution, (Iromed Group S.r.l., Rome, Italy, patent no. EP 2459186, USP 9192594), showed protective role against UV light damage in rabbit eyes. In fact, the results showed that its topical application significantly counteracts the UV-induced oxidative stress in both aqueous humor and lens of exposed rabbits [25]. Furthermore, riboflavin shows an indirect antioxidant capacity because it has a shielding action (limits the damage caused by the UV-A irradiation) as demonstrated in our studies on cross-linking [43–46]. In fact, the existence of ocular biochemical damages due to acute and chronic exposure to UV-B and UV-A has been widely demonstrated. The UV-B rays (320–290 nm) and UV-A rays (321–399) are cytotoxic to ocular tissues; this is the main environmental source of photo-oxidation, due to the oxidative stress.

Thanks to the cited shielding action, the ophthalmic antioxidant formulation, used in this study, is the first and only product certified both as a medical device (Directive 93/42/EEC and subsequent amendments) and as Personal Protective Equipment (Directive 89/686/EEC) against UV and blue light. The safety of the corneal cross-linking procedure depends on the addition of more riboflavin during the UV-A irradiation process. The instilled riboflavin forms the tear film on the cornea and absorbs most of the UV-A energy input, thus protecting the ocular tissues. But the addition of more riboflavin to the corneal surface produces a variable thickness over time of riboflavin on the corneal surface that blocks UV-A absorption in an erratic way and highly variable over time, acting as a "sun protection" for UV-A transmission during treatment. The thickness of the riboflavin film varies constantly, inducing large variations in UV-A intensity and transmission to the corneal stroma where corneal reinforcement is needed [47]. In corneal cross-linking procedure, the treated cornea is irradiated with ultraviolet A (UV-A; 370 nm) at a variable power between 1 and 3mW/cm^2 for 30 min for a total input energy from the UV-A source variable 1.8–5.4 J/cm^2. These are the values necessary to trigger the oxidation of riboflavin and the formation of free radicals necessary for the creation of interfibrillary bridges. The albedo of a beautiful sunny day that represents 20% [48] of the direct UV intensity on earth is always less than 1 mW/cm^2. This value does not induce the oxidation of riboflavin, which instead exerts its shield action so much that the most of the UV-A absorption comes from unoxidized riboflavin inside the corneal tissue [44, 45, 49].

The UV-A block, produced by the riboflavin film on the corneal surface, can vary; a more curved cornea produces a tear film of lesser riboflavin than a flatter cornea. Furthermore, the UV-A block also depends on the cross-linking procedure used. This variation could cause a differential stiffening effect at different depths within the treated corneas and between treated eyes. In addition, as specified above, vitamin E TPGS is widely used as a drug penetration enhancer through different biological barriers, while exerting a protective effect on biological membranes against free radical damage. This is why the effects of VE-TPGS on the riboflavin corneal permeability and consequently its protective effect against free radicals have been evaluated.

Since the protective role of the topical antioxidant formulation in the anterior segment has been demonstrated, an antioxidant capacity in the macula was consequently hypothesized.

Our studies on spectrophotometry and on corneal anatomical results of the ophthalmic antioxidant formulation are currently in peer review.

Acute UV exposure significantly reduces the thickness of retinal layers (Table 1 and Fig. 2). The reduction was more evident at the level of the external (outer) plexiform layer, the layer of synapses between dendrites of bipolar and horizontal cells from the inner nuclear layer and photoreceptor terminal axons from the outer nuclear layer. Topical application of antioxidant eye drops makes the ultrastructural modifications less dramatic, even at the highest exposure. The cumulative UV dose employed in our experiments is hundreds of time higher than those occurring in real life or in exposed outdoor workers (U.S.

Environmental Protection Agency. UV Index. http://www.epa.gov/sunwise/uvindex.html).

Among the eye drop components, riboflavin and TPGS vitamin E represent the crucial factors. In fact, riboflavin blocks the UV transmittance acting as a filter on the cornea. Recently, Hwang and Kim [50] demonstrated that the transmittance of cross-linked corneas was 10–20% lower compared to controls, concluding that riboflavin treatment exerts a protective effect against ultraviolet penetration in rabbit cornea. However, riboflavin has also antioxidant properties, neutralizing lipid peroxidation throughout the glutathione redox cycle [51]. TPGS vitamin E acts synergistically. TPGS is a synthetic amphiphile that undergoes enzymatic cleavage to deliver the lipophilic antioxidant, α-tocopherol (vitamin E) to cell membranes. The antioxidant properties of TPGS are based on cellular enzymatic hydrolysis by cytoplasmic esterases that liberate free α-tocopherol, which then penetrates in cell membrane and through free radical quenching protects the membrane from lipid peroxidation and oxidative damage [52]. Vitamin E itself, or via glutathione cycle [53], is a potent scavenger of free radicals, playing a fundamental role against lipid peroxidation [54]. According to Ostacolo and coworkers [55], TPGS vitamin E increases riboflavin corneal penetration, further enhancing the protective role of the topical formulation.

Furthermore, several studies have shown that UV irradiation caused statistically significant metabolic changes in rabbit ocular tissues; a decrease in metabolites as amino acids (proline, glycine, lysine, and leucine) was observed [21]. Then, some small molecule substances, such as lysine, have been shown to block the glycation reaction of tissue proteins [56].

In conclusion, UV exposure significantly decreases retinal thickness and photoreceptors density in rabbit retina. Topical treatment is able to counteract the detrimental effects of UV on the retina of exposed animals, minimizing the ultrastructural changes. Thus, its use should be proposed as a preventive treatment in outdoor workers and in elderly aphakic and pseudophakic subjects.

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Authors' contribution SB and MP performed experiments, analyzed the data, and wrote this manuscript. BP, FM, BP, and CC analyzed the data. FS and GG designed the experiment and wrote the manuscript. CC supervised and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Statement of human rights No human was involved in this research.

Statement on the welfare of animals All applicable international, national, and/or institutional guidelines for the care and the use of animals were followed. All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Informed consent This article does not contain any studies with human participants performed by any of the authors.

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