Corneal UV Protective Effects Of A Topical Antioxidant Formulation: A Pilot Study On In Vivo Rabbits

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Abstract

Background The purpose of this study has been to evaluate the protective effect of a topical antioxidant and UV shielding action formulation containing riboflavin, d- α -tocopheryl polyethylene glycol (TPGS vitamin E), against corneal UV-induced damage in vivo rabbit eyes. **Methods** For the evaluation, in vivo experiments were performed using twelve male albino rabbits. Animals were divided into 4 groups of 3 animals each: control group (CG) didn't receive any UV irradiation; the first group (IG) was irradiated with an UV-B - UV-A lamp for 30 min; the second (IG30) and the third (IG60) groups received UV irradiation for 30 and 60 minutes, respectively, and were topically treated with 1 drop (approximately 50 μ l) of the antioxidant and shielding formulation every 15 minutes, starting one hour before irradiation, until the end of UV exposure. **Results** The cornea of IG group showed irregular thickening, detachment of residual fragments of the Descemet membrane, stromal fluid swelling with consequent collagen fibers disorganization and disruption, and inflammation. The cornea of G30 group showed edema, a mild thickening of Descemet membrane without fibrillar collagen disruption and focal discoloration, nor inflammation. In the G60 group cornea showed a more severe thickening of the cornea, a more abundant fluid accumulation underneath the Descemet membrane, with focal detachment of the same, and no signs of severe tissue alterations, as those recorded in the IG group. **Conclusion** Our results demonstrate that a topical application of eye drops containing riboflavin, d- α -tocopheryl polyethylene glycol (TPGS vitamin E) counteracts UV corneal injury in exposed rabbits.

Background

Due to the stratospheric ozone layer depletion, human exposure to ultraviolet (UV) is increasing. Ozone is essential for life on Earth because of its ability to absorb ultraviolet radiation (UV). UV can be further divided into four bands, named UV-vacuum (100-200 nm); UV-A (400-311 nm); UV-B (310-280 nm) and UV-C (280-100 nm) [1] and the eye exposure is wavelength-dependent [2, 3]. About 35% of UV-A rays and, approximately, 80% of UV-B rays have the greatest potential damage [4, 5], despite representing less than 1% of the total radiation reaching the Earth's surface. UV-C radiation is fully absorbed in the upper atmosphere.

Many studies demonstrate the risk of eye damage induced by broadband solar radiation [6, 7]: this is becoming an emerging public health problem, and people should be more aware of the potential risk from ocular exposure to solar UV radiation. Unavoidable exposure to photo-stress, i.e. UV light, visible light, and ionizing radiations contribute to damage ocular tissues, mainly triggered by oxidative stress [8].

Ocular surface receives about 59% to 77% of UV light directed to the head. The tear film absorbs wavelengths up to 300 nm, covering much of UV-B spectrum [9]. In general, shorter wavelengths of UV and visible light penetrate tissues less than longer wavelengths [10–13]. Sunlight contains much more UV-A than UV-B, and both of them have no beneficial effects on the eye [14].

Cornea has a high absorption coefficient for UV-B radiation and represents a natural UV-B filter [15], absorbing approximately up to 92% of UV-B at 300 nm, but only 20-40% of the UV-A [9, 16].

The adsorption is age dependent [17]; in fact, at the age of 8-10 years, UV-A and UV-B radiation easily reach the retina, with a maximum at 320 nm [18]. The metabolites of tryptophan within the lens absorb the UV radiation, decreasing the amount of light transmitted from the lens [19]. By the early 20s, only 0,1% of UV ray can penetrate, and by the age of 60 years no UV radiation can reach the retina [20], except in the case of aphakia or pseudophakia [21, 22].

Ocular tissues and fluids contain both enzymatic - catalase, superoxide dismutase, glutathione peroxidase and reductase and non-enzymatic - ascorbic acid, reduced glutathione and alpha-tocopherol antioxidants to protect ocular structures from oxidative damage [23]. However, it has been reported that irradiation of the rabbit eye with UV-A increases corneal hydration and light absorption, while also triggering corneal inflammation [24].

The aim of this study has been to test the protective effect of this antioxidant and shielding action ophthalmic topical formulation against UV damage in corneal tissue of rabbits exposed to UV irradiation.

Methods

2.1 Animals

According to May-Britt Tessem et al. [25] twelve 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 have been performed with albino rabbit eyes [26, 27].

The trial was performed in the experimental rabbit farm at the National Agricultural and Food Centre, Institute of Small Farm Animal (Nitra, Slovak Republic).

Twelve male albino rabbits (New Zealand White, 2.5–3.0 kg) of the same age were used for in vivo experimental studies. They were divided into 4 groups of 3 animals each. Control group (CG) did not receive any irradiation and/or eye drop, while the other three experimental groups were treated as follows: the first group received UV irradiation for 30 minutes, without eye drop formulation (Irradiation group, IG), the second (G30) and the third (G60) groups received UV radiation for 30' and 60', and were topically treated with 1 drop (approximately 50 µl) of the antioxidant and shielding formulation every 15 minutes, starting one hour before irradiation, until the end of UV exposure. The study has been conducted conforming 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), and was approved by the Local Ethic Committee (CTS, Department of Medicine and Health Sciences "V. Tiberio", University of Molise, 15/2019).

At the end of experiment animals were sacrificed 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 [28]. Rabbit eyes were quickly enucleated by veterinary doctor provided by the Research Centre. The dissection of each part of rabbit eye was microscopically performed by expert ophthalmologist, and the single samples were storage -20°C until histochemistry analyses.

2.2 UV irradiation

The animals were euthanized by first, 10 minutes before UV radiation. Rabbits were anaesthetized with an intramuscular injection of Xylazine (20 mg/kg) and Ketamine HCl (5 mg/kg).

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 x 10 cm, one low-pressure lamp at 369 nm (UV-A). The radiant energy was measured with a radiometer (VLX-3W; 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. [28]. The rabbits of IG, G30 and G60 groups were undergone to the radiation (100mW/cm²) for 30 and 60 minutes, respectively. The irradiance on the cornea was 100 mW/cm², with a total fluence of 180 J/cm² and 360 J/cm², respectively. Rabbit eyes of G30 and G60 groups were topically treated with 1 drop (approximately 50 µl) of the antioxidant and shielding formulation every 15 minutes, starting one hour before irradiation, until the end of UV exposure.

2.3 Ophthalmic preparation

The ophthalmic antioxidant and shielding preparation consisted of a riboflavin, d-α-tocopherol polyethylene glycol succinate (TPGS) vitamin E, solution, pH 7.2 (Iromed S.r.l., Italy, patent n° EP 2459186, USP 9192594).

2.4 Histochemistry

Cornea samples were fixed in Davidson solution (alcohol 95%, formaldehyde, glacial acetic acid and distilled water) for 24 hours [29].

Rabbits corneal specimens obtained from rabbits were fixed in buffered 10% formalin, embedded in paraffin and sectioned. Then, 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) for routine staining, as previously described [30–32] . Corneal epithelium thickness was measured with a digital image analysis software.

2.4 Statistical analysis

Significance of means comparison between each pair of studied group was assessed by Student's t-test.

Results

UV irradiation of the cornea caused significantly morphologic changes in exposed rabbits. Histologically, a normal rabbit cornea shows an anterior stratified, squamous, non-keratinizing epithelium sitting on a basement membrane (which resembles the Bowman membrane of humans); under the epithelium there is a large stromal layer (which represents the largest part of the corneal thickness), followed by a membrane that exhibits a homogeneous fine granularity; i.e. Descemet's membrane. Lastly the endothelium is recognized as a flattened layer of cells attached to the Descemet's membrane (**Figure 1a**). Following 30' UV irradiation (study group IG) we observed an irregular corneal thickening, the detachment of residual fragments of the Descemet's membrane, stromal fluid swelling with consequent collagen fibers disorganization and disruption, and inflammation (**Figure 1b**). The G30 irradiated rabbits group showed edema, a mild thickening of Descemet membrane, neither fibrillar collagen disruption and focal discoloration, nor inflammation (**Figure 1c**). The G60 group showed a more severe corneal thickening, a greater fluid accumulation underneath the Descemet membrane with focal detachment of the same, as compared to animals from the G30 group (**Figure 1d**), without the signs of severe tissue alterations found in the irradiated animals not receiving the topical treatment.

Relative epithelium thickness, normalized on control samples, is shown in Table 1 and in Figure 2. As shown in Table 2, differences were significant for all comparisons ($p < 0,01$) except for IG vs IG60 groups ($p = 0,19$). Finally, we measured the relative cell density of corneal epithelium of each studied group. We counted cells in different ROI per each sample and normalized the cell count by the ROI area. Figure 3 and Table 3 show the relative count vs control sample.

Discussion

Exposure to UV-A irradiation is able to induce apoptosis in rabbit corneal epithelial cells and keratocytes [33]. The degree of damage depends on both the wavelength (400-311-nm UV-A) caused more extensive damage to the corneal stroma and endothelium than the wavelength (310-280-nm UV-B) [33] and the fluence [23]. Thus, the UV exposure causes injury to the whole cornea, from the epithelium to the endothelium. Our findings confirm that acute exposure of cornea to UV-A induces cell death in a dose-dependent fashion, as already reported by previous researchers [34].

The constant exposition to UV induces biological damage in absorbing tissues, mainly consisting in: *i*) DNA damage [35]; *ii*) increase of reactive oxygen species (ROS); and, *iii*) decrease of antioxidant protective enzymes [36, 37]. The peculiar position and function of the eye makes the cornea constantly exposed to UV [38] and the UV light at wavelengths of 270-290 nm or less is totally absorbed by the cornea epithelium and Bowman's membrane [39].

Lipid peroxidation of cellular membranes, oxidative changes in proteins, direct oxidative damage to DNA [40–42], decreasing mitochondrial functions and antioxidant protective mechanism, induce apoptosis in corneal tissue [8, 43]. The related histologic finding is represented by micropapillary changes and membrane detachment occurred in our experiments. Although the exposure of irradiated cornea was about 60 times the maximum exposure of the human cornea to UV-A contained in sunlight [44], that is a completely different setting compared to real life scenarios, the protective treatment was able to counteract the known and expected detrimental effects.

Many known ocular surface and corneal diseases are the direct consequence of UV exposure: for acute event, photokeratitis is the most representative one, whereas pterygium, pingueculum and climatic droplet keratopathy are the clinical findings typical of chronic UV exposure [45, 46]. Both corneal epithelium and endothelium (which cannot regenerate) are sensitive to UV radiation. The antioxidant protective mechanism is vulnerable to increased UV-A exposure; in fact, the reduction of protective species results in corneal damage and injury. In this scenario we assess *in vivo* experiments using a topical application of an ophthalmic preparation containing riboflavin, d- α -tocopherol polyethylene glycol succinate (TPGS) vitamin E, to significantly counteract UV corneal injury of exposed rabbits. The choice of this animal model is based on the statement that rabbit eye is relatively large, consequently it has been proved useful for the assessment of both new technologies and ophthalmic surgical procedures in ophthalmic scientific research [47]. Morphologically, the rabbit cornea is very similar to that of humans [48, 49]. However, in our unpublished data (Bartollino et al., 2019), the ophthalmic antioxidant and shielding preparation, consisted of riboflavin, d- α -tocopherol polyethylene glycol succinate (TPGS) vitamin E, solution, (Iromed Group S.r.l., Rome, Italy, patent no. EP 2459186, USP 9192594), showed protective role against UV light damage on the retinal tissue of rabbits exposed to UV irradiation. Ostacolo et al. reported that the ultra-structural analysis of corneal epithelium revealed that the antioxidant and shielding solution was more effective in protecting the corneal epithelium [50].

In fact, microscopic analyses of untreated corneas and of corneas treated with UV irradiation resulted in the destruction of all epithelial layers thereby exposing the Bowman membrane, in comparison with the intact cellular architecture of the control group. In corneas treated with UV irradiation without the ophthalmic antioxidant and shielding solution, there were several cellular gaps in the superficial epithelial layer, mostly due to the rupture of intercellular tight junctions. In addition, in corneas treated with UV irradiation there was a remarkable loss of microvilli and of cytoplasmic nuclei. In corneas treated with UV irradiation with the ophthalmic antioxidant and shielding solution, the epithelial layers, the cell nuclei and the intercellular tight junctions were less damaged. Moreover the reduction in microvilli density was less evident and the surviving microvilli appeared morphologically intact. Giblin demonstrated how a soft

contact lens with class I UV block (senofilcon A) prevented the yellow fluorescence induced by UVA and the loss of NADH in the rabbit lens core in vivo [51].

Vizzari et al. showed that topical application of the antioxidant and shielding solution significantly counteracts the UV-induced oxidative stress in both aqueous humor and lens of exposed rabbits [40]. 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 [52–54].

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 (310-290 nm) and UV-A rays (311-399) 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 and shielding 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 [55].

In cxi procedure the treated cornea is irradiated with ultraviolet A (UV-A; 370 nm) at a variable power between 1- 3mW/cm² for 30 minutes for a total input energy from the UV-A source variable 1.8-5.4 J/cm². 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% [56] of the direct UV intensity on earth is always less than 1 mW / cm². 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 [53, 57, 58].

In addition, as specified above, TPGS vitamin E 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 TPGS vitamin E on the riboflavin corneal permeability and consequently its protective effect against free radicals have been evaluated.

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

Our studies on spectrophotometry results of the ophthalmic antioxidant and shielding action formulation are currently under review.

Our data showed a marked increase in corneal epithelium thickness and cell density following exposure to UV. Both parameters were significantly reduced by topical treatment with ophthalmic antioxidant and shielding preparation, confirming our findings at histological examination. The cumulative UV dose employed in our experiments is hundreds of times higher than those occurring in real life or in exposed outdoor workers [59].

Hwang and Kim [60] have recently demonstrated that the transmittance of cross-linked corneas was 10%–20% lower as compared to control untreated corneas, concluding that riboflavin treatment exerts a protective effect against ultraviolet penetration in rabbit cornea. However, riboflavin has also antioxidant properties; this vitamin neutralizes lipid peroxidation throughout the glutathione redox cycle [61]. These two effects could explain the defensive role exerted by riboflavin in our experiments. TPGS vitamin E acts synergistically: TPGS is a synthetic amphiphilic that undergoes enzymatic cleavage to deliver the lipophilic antioxidant, α -tocopherol (vitamin E TPGS) 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 [62]. Vitamin E TPGS itself, or via glutathione cycle [63–65], acts as a potent scavenger of free radicals, that through its lipid-soluble properties is able to play a pivotal role in membrane preservation against lipid peroxidation, blocking the radical chain and creating a low-reactivity derivative unable to attack lipid substrates [66]. UV exposure significantly increases corneal epithelium thickness and cell density. Both parameters were significantly reduced by topical treatment, justifying the morphological picture.

Conclusions

In conclusion, our findings indicate that an ophthalmic antioxidant and shielding preparation, containing riboflavin, d- α -tocopherol polyethylene glycol succinate (TPGS) vitamin E is able to counteract the detrimental effects of UV on rabbit cornea and its use should be considered as a possible medical preventive treatment.

Declarations

COMPETING INTEREST

The authors declare that they have no conflict of interest.

INFORMED CONSENT

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

STATEMENTS OF HUMAN RIGHTS

No humans were involved in this research.

STATEMENTS 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.

Authors' contribution

S.B., F.V. and M.P performed experiments, analysed the data.

G.G, F.M., D.C, and C. Caruso analysed the data.

S.B. and C. Caruso wrote this manuscript.

C. Costagliola designed the experiment and approved the final manuscript.

CoMPETING interests

The author declare they don't have conflict of interests (political, personal, religious, ideological, academic, intellectual, commercial or otherwise) regarding the publication of the paper.

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Tables

TABLE 1

CTRL	IG	G30	G60	
1	1,768667	1,40292	1,676709	Fold increase vs CTRL
423600	749207	594276	710254	Mean
427500	759386	600771	714339	Median
387000	564455	536799	633306	MIN
456000	841418	623610	751708	MAX
69000	276963	86811	118402	RANGE

TABLE 2

Student's T Test p value	
0,0000000005	CTRL vs IG
0,00000000001	CTRL vs G30
0,000000000000012	CTRL vs G60
0,00003	IG vs G30
0,19	IG vs G60

TABLE 3

Cell count				
CTRL	IG	G30	G60	
33	143	76	141	count
24393	49504	34611	49504	area
0,001352847	0,002889	0,002196	0,002848	density
1	2,14	1,62	2,11	Relative cell density

Figures

Figure 1

a) H & E staining of the corneal tissue obtained from the rabbit eyes of Control Group (CG). Normal corneal thickness and normal morphology of the corneal layers in the control sample. Original magnification x 20. b) H & E staining of the corneal tissue obtained from the rabbit eyes of Irradiated Group (IG). Serious increase in thickness, detachment of residual fragments of Descemet membrane, which appears disappearance for destruction and micropapillary changes. Original magnification x 20. c) H & E staining of the corneal tissue obtained from the rabbit eyes of Irradiated Group (IG30). Moderate increase in thickness, mild edema of the endothelial layer and slight increase in thickness of Descemet membrane. Original magnification x 20. d) H & E staining of the corneal tissue obtained from the rabbit eyes of Irradiated Group (IG60). Severe increase in thickness, detachment of the Descemet membrane and edema of the endothelial layer. Original magnification x 20.

FIGURE 2

Figure 2

The Chart shows the fold increase of Irradiated, untreated group (IG), and irradiated treated groups (G30 and G60) vs CTRL (unexposed group).

Figure 3

Relative cell density. Cell density has been calculated counting nuclei in different ROI and normalizing vs ROI area.

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