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Antioxidant protection in cultured corneal cells and whole corneas submitted to UV-B exposure

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Abstract

Several corneal pathologies are characterized by the presence of reactive oxygen species (ROS); therefore, we evaluated the protection afforded by pirenoxine and melatonin to corneal cell culture and whole rabbit cornea from ultraviolet exposure and other oxidant systems.

Rabbit cornea cell (SIRC) plates and whole corneas were exposed to UV-B (80 or 800 mJ/cm^2) or incubated with fMLP-stimulated autologous macrophages, in the presence or absence of pirenoxine or melatonin (10^{-5} M). The protective activity of compounds was assessed by measuring superoxide anion formation, inhibition of oxidation and mitochondrial viability. Moreover the ex vivo protective effect of pirenoxine and melatonin was verified in the whole cornea submitted to UV-B exposure in vitro.

Our experimental data demonstrate that pirenoxine and melatonin were able to inhibit the superoxide formation and oxidative effect in cell culture and whole rabbit corneas submitted to UV-B exposure or to incubation with fMLP-stimulated autologous macrophages.

Mitochondrial viability was restored in epithelial cells of rabbit cornea but not in SIRCs.

Moreover, both compounds are also able to increase ex vivo epithelial corneal cell defences against the in vitro UV-B induced lipid peroxidation.
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Keywords: Cornea; UV-B; Alveolar macrophages; Reactive oxygen species; Pirenoxine; Melatonin

1. Introduction

Pathological conditions of corneal tissue, characterized by the presence of reactive oxygen species (ROS) with a consistent increase in oxidative insult and a decrease in antioxidant system components, are produced by physiological and non-physiological environmental stress, such as ultraviolet (UV) radiation, aging, bacterial and viral infections, hypoxia-reoxygenation and trauma. Indeed, the effect of UV light has been extensively investigated [1–8]. It has been reported that biotoxic epiphenomena may take place when the threshold level of radiation absorbed in corneal tissues is exceeded, and, consequently, permanent keratocyte damage or loss, epithelium destruction, endothelium edema and necrosis occur, as well as biochemical changes, such as ROS production, protein cross-linking, enzyme inactivation, DNA strand breaks and chemotactic factor release [9–13]. The presence and epiphenomena of ROS, polymorphonuclear cell recruitment, keratocyte loss and development of a diffuse anterior stromal cornea haze are also observed when excimer laser therapy is applied in treating myopia, astigmatism and corneal opacities [14–17].

Moreover, the extension of stromal healing response characterized by an augmentation of subepithelial haze in corneas submitted to excimer laser treatment followed by UV-B exposure could be ascribable to ROS activity prevailing over the epithelial antioxidant system [18,19]. Thus, physiological scavenging agents, such as superoxide dismutase (SOD), catalase, vitamin E, glutathione, ascorbic acid, as well as steroidal or non-steroidal anti-inflammatory molecules, have been tested in order

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to inhibit the harmful action of UV-B radiation or the side effects of excimer laser therapy on the cornea [20–25].

In this context, the aim of our research was to evaluate in vitro the protection afforded by some radical scavengers to an eye derived cell line in culture and tissues of rabbit cornea when free radical attacks were triggered using UV-B radiation or other oxidant systems.

Therefore, we utilized SIRC cultures and whole rabbit corneas. Although in experimental models, SIRCs are described and used as being of epithelial origin [26–28], their morphology, ultrastructure and antigenic properties indicate a fibroblast origin for this cell line [29].

Since in our former work we have already demonstrated that pirenoxine (1-hydroxy-5H-pyrido-(3,2-α)-phenoxazin-5-one-3-carboxylic acid sodium salt), likely via a free radical scavenging mechanism, is able to prevent or inhibit lipid peroxidative degeneration of lenses which can eventually lead to cataract formation [30], in the present study we analyse the protective effect of this molecule in corneal cell culture and whole cornea in both in vitro and ex vivo experiments.

Pirenoxine is a xanthomatin (an ommochrome found in the ocular pigment of several insects) derived compound, already known for its therapeutic effectiveness in suppressing the progress of senile cataract in human eyes (Catalin®: Takeda et al.) [31–33].

Moreover, we evaluate the effect of the pineal neurohormone melatonin (*N*-acetyl-5-methoxy-tryptamine). Melatonin is synthesized also in retinal photoreceptor cells and in the ciliary body of the eye and appears to exert protective actions against light-stimulated production of ROS [34–36]. In some tests, we compare the protective effect of pirenoxine and melatonin to that of α-tocopherol (physiological scavenger of hydroxyl radical) and thioctic acid (a scavenger molecule also able to neutralize superoxide anion) [37,38]. Our experimental data suggest that pirenoxine and melatonin are able to decrease lipid peroxidation and protect the mitochondrial viability of corneal epithelial cells or the whole rabbit corneas when the ROS attack is triggered by UV light or other ROS-producing systems in vitro. Moreover both compounds are able to increase epithelial corneal cell defences in ex vivo.

2. Materials and methods

2.1. Materials

Melatonin, (±) α-tocopherol, DL-6,8-thioctic acid (reduced form), SOD (EC 1.15.1.1), minimum essential medium (MEM), Hanks' balanced salt solution (HBSS) without phenol red, reduced glutathione (GSH) and antibiotic-antimycotic solution were purchased from Sigma Chemical Co. (MO, USA). All reagents were of the highest commercially available analytical grade. Pirenoxine sodium salt was kindly donated by Farmigea (Pisa, Italy).

2.2. Isolated cultured eye cells

SIRCs were initially plated at a density of 4×10^4 cells/cm^2 in 25 cm^2 flasks with MEM–HBSS (2 mM L-glutamine, 1% non-essential amino acid solution, 10% foetal bovine serum, 100 U/ml penicillin G, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B; pH 7) and allowed to proliferate until 100% confluence in 5% CO_2 incubator at 37 °C (medium was changed every 2 days). After treatment with 0.25% trypsin and 0.02% EDTA, the detached cells were harvested by centrifugation at 230*g* for 5 min and split 1:3 in order to provide a sufficient cell stock that can be frozen for repeated studies. Forty-eight hours before the experiment, the SIRCs were passed into 12-well plates at a density of 1.5×10^4 cells/well plate and used for experiment when a 70–80% of confluence was achieved.

2.3. Tissue preparation

The investigation conforms to the European Community rules for the care and use of laboratory animals.

Male pigmented rabbits (Pampaloni, Pisa, Italy) weighing approximately 1.3–1.7 kg upon arrival were kept in comfortable cages under a constant light–dark cycle (light on between 8.00 A.M. and 20.00 P.M.) and left undisturbed for three days in order to acclimate. Unlimited food and water were supplied. All animals were used only once in the experiments.

For the in vitro procedure we used 10 μM pirenoxine that we found to be the most effective concentration for the inhibition of lipid peroxidation of lens homogenate [33]. Melatonin was used in vitro at the same molecular concentration.

For the ex vivo procedure, rabbit right eyes were topically treated every hour for 8 h for 2 days with 10 nmol for administration of both compounds (i.e., 2 drops of 0.005% pirenoxine = 1.5 μg or 0.0038% melatonin = 1.13 μg in 0.145 M NaCl; 1 drop ≈30 μl), whereas the left eyes received only drops of the diluent. The topical treatment ended 1 h before the sacrifice. The 0.005% concentration of the topically administered pirenoxine was that of the commercial ophthalmic solution bearing the trade-name Catalin® [30–32].

All rabbits were killed with an overdose of sodium pentobarbital (100 mg/kg e.v.) and the eyes removed. Full thickness corneas were excised at the limbus with a pair of scissors and processed according to the test.

2.4. Irradiation with UV-B rays of SIRC

SIRCs showing 80% confluence were incubated at 37 °C for 1 h in 5% CO_2 incubator in MEM–HBSS containing 0.1% foetal bovine serum (FBS) in the presence or absence of the pirenoxine, melatonin, α-tocopherol and thioctic acid (each at the concentration of 10 μM). Then, the medium was replaced with HBSS containing 0.1% FBS and cells were submitted for 36 s at room temperature to UV exposure (Vilber Lourmat Lamp, Cedex, France: wavelength range set at 280–350 nm and peak at 312 nm), receiving in these conditions a total equivalent energy of 80 mJ/cm^2 under constant intensity. This energy dosage was chosen since in-between to the energy quoted in vivo to induce keratitis (140 mJ/cm^2) and subkeratitis (30 mJ/cm^2) [6]. After 3 h, the medium was further substituted and SIRCs were incubated for 18 h in a medium containing 10% FBS in the absence (control) or presence of aforesaid substances. Plated SIRCs, without UV-B irradiation (basal value), were likewise treated. To evaluate lipid peroxidation, cells were rinsed with HBSS, scraped, harvested by centrifugation, homogenized in HBSS and lipid soluble fluorescent compounds were measured. SOD-inhibitable superoxide anion formation and mitochondrial viability were determined as described.

2.5. Irradiation with UV-B rays of corneal tissue

Whole corneas were previously incubated for 1 h at 37 °C in 100 μM phosphate buffer (pH 7.4) in the absence (control) or presence of 10 μM melatonin, pirenoxine, thioctic acid and α-tocopherol, and then exposed (epithelial side) for 36 or 360 s to UV-B light (80 or 800 mJ/cm^2) under constant intensity, wetting the surface every 60 s with 1–2 drops of physiological solution when exposure was continued for 360 s. Since in several tests we were unable to detect significant biochemical modifications in control conditions submitting corneas to 80 mJ/cm^2 UV-B radiation, we mainly performed experiments using a 800 mJ/cm^2 UV-B light.

When ex vivo procedure was performed, corneas from eyes submitted in vivo to topical instillations were directly exposed to UV-B light without further treatment.

All specimens were cut into small pieces and incubated for 18 h at 37 °C in 100 μM phosphate buffer (pH 7.4) in the presence of 1000 U/ml collagenase type IA (EC3.4.24.3) and 5 μM $CaCl_2$. The obtained cell suspension was centrifuged (800*g* at 0 °C for 10 min) and the pellet was resuspended in phosphate buffer and washed twice by centrifugation. Cells were then homogenized in phosphate buffer by Ultraturrax (3 × 10 s at 0 °C). Lipid soluble fluorescent compounds, conjugated diene formation and GSH and oxidized glutathione (GSSG) levels were evaluated. Corneas without UV-light exposure were likewise treated (basal value).

The SOD-inhibitable superoxide anion formation in pre-treated and 800 mJ/cm^2 exposed cornea was directly assayed after incubation in 0.1% FBS medium for 3 h.

Corneal epithelial cell mitochondrial viability was evaluated in 80 and 800 mJ/cm^2 UV-B exposed and pre-treated corneas after incubation in 0.1% FBS medium for 3 h. The corneal tissue was then treated with dispase (EC3.4.24.4: 2 U/ml) for 1 h at 4 °C and the obtained epithelial cell sheets digested in 0.25% trypsin at 37 °C for 5 min. After centrifugation at 230*g* for 5 min, the cell pellet was suspended in 10% FBS medium and incubated for 18 h, in the presence or absence of scavenger compounds and, finally, processed for MTT assay.

In order to investigate if the protective effect of pirenoxine and melatonin (10 μM) was exerted during a delayed phase of damage, cells were incubated for 18 h with drugs added only after UV-B-exposure.

2.6. Fenton reaction on SIRC and corneal tissue homogenate

Several plates of SIRC were washed, scraped and harvested with HBSS in order to obtain a suspension (approximately 2.5×10^6 cells/ml) and homogenized. Corneal tissue was weighed and homogenized in HBSS (10% w/v) by Ultraturrax (3 × 10 s at 0 °C). Homogenate aliquots (500 μl) were incubated with 10 μM $FeCl_3$ and 100 μM ascorbic acid in the absence (control) or presence of pirenoxine, melatonin, α-tocopherol or thioctic acid (each molecule at a concentrations of 10 μM) at 37 °C for 30 min in a shaking bath. Lipid soluble fluorescent compounds formation was measured. Basal values were calculated running parallel experiments without the ROS generating system.

2.7. fMLP-stimulated macrophages activity on corneal tissue

Rabbit corneas were incubated (37 °C, 5% CO_2 incubator for 2 h) with autologous alveolar macrophages (10^6 cell/dish; obtained according to Mugnai et al.) [39]. Experiments were performed using resting macrophages (control conditions) or in 10 nM fMLP-stimulated macrophages, with or without (control) 10 μM melatonin and pirenoxine. Afterwards, the specimens were removed from the reaction mixture and rinsed in isotonic saline solution containing 23 μM BHT. After the enzymatic treatment for epithelial component separation, the cellular pellet was homogenized and processed for lipid soluble fluorescent compounds determination.

2.8. Superoxide anion assay

SIRC and corneal tissue, previously rinsed with HBSS, were incubated at 37 °C for 1 h with 1 ml cytochrome *C* (1 mg/ml) solution in the presence and

absence of SOD (300 U/ml). The optical density at 550 nm was spectrophotometrically measured and the superoxide amount calculated by using an extinction coefficient of 2.1×10^4 M^{-1} cm^{-1} and expressed as nmol/h.

2.9. Fluorescence assay

All samples were extracted with chloroform:methanol mixture (2:1 v/v) according to the method of Fletcher et al. [40]. Chloroform extract fluorescence was read by a Perkin–Elmer 650-10 S apparatus (ex. 370 nm, em. 430 nm) and expressed as arbitrary fluorescence units/mg proteins.

2.10. Conjugated diene assay

Homogenates were extracted [30] with chloroform/methanol mixture (2:1 v/v) and centrifuged (1900*g* for 10 min). An aliquot of the chloroform phase was evaporated under an argon stream and the total lipid extract dissolved in cyclohexane. The measurement was performed by scanning the absorbance spectrum between 220 and 350 nm (against cyclohexane blank) and estimating the amount of conjugated dienes at 232 nm after the elimination of the background due to Rayleigh scattering. Data were expressed as differences between the sample and basal absorbance values using a molar absorption coefficient of 2.52×10^4 M^{-1} cm^{-1}.

2.11. Glutathione assay

The determination of GSH and GSSG was conducted essentially according to Hissin and Hilf [41]. The cell pellet was homogenized in buffered solution (3 ml of 0.1 M sodium phosphate, 0.005 M EDTA buffer, pH 8, plus 1 ml of 25% (w/v) HPO_3). For the GSH assay, after centrifugation at 4 °C at 100,000*g* for 30 min, 0.5 ml of the supernatant was diluted with phosphate-EDTA buffer (pH 8) to a 5 ml final volume. For the GSSG evaluation, 200 μl of 0.04 M *N*-ethylmaleimide were added to 0.5 ml of the supernatant, incubated for 30 min and diluted with 0.1 N NaOH to a volume of 5 ml.

Phosphate-EDTA buffer and 0.1 N NaOH (1.8 ml, respectively) and ortho-phthalaldehyde (OTP: 100 μl) solution (1 mg/ml) were added to 100 μl of both mixtures. The mixtures were mixed and incubated at room temperature for 15 min. The fluorescence developed from the GSH–OTP or GSSG–OTP reaction was determined (420 nm emission and 350 nm excitation). Samples were calibrated using a standard curve of GSH at different concentrations (0–10 μg/ml).

2.12. Mitochondrial viability

The MTT assay [42] was used in order to evaluate the mitochondrial viability of UV-treated corneal cells. Corneal cell medium derived either from culture and tissue was replaced with 800 μl DMEM without phenol red and replenished with 400 μg thiazolyl blue. After incubation (3 h at 37 °C in 5% CO_2 incubator), the cells were rinsed with HBSS and dissolved in 1 ml of 0.01 N HCl in isopropyl alcohol. The dark purple formazan generated was diluted 1:5 with isopropyl alcohol and the optical density of the samples was then measured at 570 nm.

2.13. Protein assay

Protein concentration was determined by the method of Sedmak and Grossberg [43] using Coomassie brilliant blue G-250 to detect microgram amounts of protein.

2.14. Statistical analysis

Data were reported as mean values ± SD. One-way analysis of variance (ANOVA) followed by Bonferroni's *t* test was performed in order to estimate significant differences among groups. Indeed as indicated, comparisons were performed among the basal, control and treatment groups. Statistical tests were performed using PRIMER®, version 3.02 software (McGraw-Hill Companies Inc.).

3. Results

3.1. Effects of UV-B on SIRC

UV-B exposure (80 mJ/cm^2) significantly increased superoxide anion formation in SIRC cells to 326% of basal value (Table 1). Pirenoxine and melatonin prevented superoxide production with a similar effectiveness and superoxide production in treated samples was not different from basal value. Moreover, both compounds inhibited the UV-B-induced increase in lipid soluble fluorescent compounds (Table 1). Indeed, fluorescence obtained in treated and exposed cells was not significantly different from basal values (unexposed cells). α-Tocopherol and thioctic acid (10 μM each), used in both experimental protocols as reference compounds, significantly reduced the production of superoxide to 4.2 ± 1.8 and 1.7 ± 0.4 nmol/10^5 cells/h, respectively, and lipid soluble fluorescent compounds to 5.1 ± 0.8 and 7.3 ± 2 U/mg protein, respectively.

However, mitochondrial viability after UV-B exposure was not increased by pirenoxine and melatonin. Indeed, the mitochondrial viability, as evaluated by MTT test, was $63.3 \pm 10\%$, $61.3 \pm 8.2\%$ and $71 \pm 10\%$ of the basal value in control conditions, in the presence of pirenoxine or melatonin, respectively. When drug concentrations were increased up to 100 μM, no different result occurred.

Table 1
In vitro formation of SOD inhibitable superoxide and lipid soluble fluorescent compounds in SIRC cells submitted to 80 mJ/cm^2 UV-B exposure with and without pirenoxine or melatonin (10 μM)

	Superoxide (nmol/10^5 cells/h)	Fluorescence (arbitrary units/mg protein)
Without exposure (basal value)	1.9 ± 0.7	5.8 ± 0.8
With exposure (control)	$6.2 \pm 1.4^*$	$14.3 \pm 1.5^*$
+pirenoxine	3.3 ± 1.0	5.7 ± 1.6
+melatonin	3.3 ± 0.8	5.3 ± 2.0

Mean values ± SD were obtained from at least four tests performed in duplicate. SOD presence decreased UV-B-induced superoxide to the basal values (1.8 ± 1 nmol/10^5 cell/h). One-way ANOVA $F = 12.79$, $P < 0.001$ and $F = 32.12$, $P < 0.001$ superoxide and fluorescence tests, respectively.
$^*P < 0.05$ different from all other groups, whereas other groups were not significantly different each other (Bonferroni's analysis).

3.2. Effect of UV-B on whole corneas

UV-B exposure (800 mJ/cm^2) also significantly increased superoxide anion in tissue culture of rabbit corneas (up to 138% of basal value; Table 2). Pirenoxine and melatonin (each at the concentration of 10 μM) totally prevented UV-B-induced superoxide anion production. Moreover, we observed a significant increase (179% of basal value) in lipid soluble fluorescent substances in light-exposed corneas, whereas, when the aforesaid scavenging compounds were present in the incubation mixture, no valuable change was observed with respect to unexposed tissues (Table 2). α-Tocopherol and thioctic acid (10 μM each) were also able to significantly decrease the light-induced fluorescent compounds showing the value of 2.1 ± 1.2 and 2.8 ± 0.7 arbitrary fluorescence units/mg protein, respectively.

Also the significant increase in conjugated dienes was inhibited by 52.7% and 73.3%, respectively, in the presence of pirenoxine or melatonin in the incubation mixture. Both compounds significantly prevented GSH oxidation, showing a GSH/GSSG ratio slightly higher than that obtained in basal condition (Table 3).

Pirenoxine and melatonin were also able to improve mitochondrial viability of epithelial cells obtained from 80 and 800 mJ/cm^2 UV-B-exposed corneas (Fig. 1), being pirenoxine (10 μM) slightly more effective than melatonin after exposure to 800 mJ/cm^2 UV-B. Increasing melatonin concentration up to 50 μM (4 corneas), the mitochondrial activity rescue resulted in $93 \pm 14\%$ of basal value ($P < 0.05$ vs. control). When epithelial corneal cells were incubated with scavenger compounds only after 800 mJ/cm^2 UV-B light exposure, pirenoxine was still effective in preserving mitochondrial viability ($F = 4.23$, $P < 0.05$ ANOVA, pirenoxine vs. other groups $P < 0.05$, Bonferroni's t test) while melatonin treated group was not different from control. Indeed, mitochondrial viability was $40.4 \pm 17\%$ (10 corneas) in control condition and $80.8 \pm 12\%$ ($P < 0.05$ vs control) and $61.8 \pm 22\%$ (5 corneas) in the presence of pirenoxine and melatonin, respectively.

3.3. Effects of the Fenton reaction on SIRC and cornea

The lipid soluble fluorescent compound formation, induced in SIRC cells and rabbit corneal tissue homogenates by Fe(III)/ascorbate oxidant system, was significantly inhibited by scavenger compounds (Table 4). In the presence of pirenoxine and melatonin, the fluorescence value of SIRC homogenates attained the basal value. Thioctic acid behaved similarly (11.6 ± 1 arbitrary fluorescence units/mg protein), while α-tocopherol (14.2 ± 1.6 arbitrary fluorescence units/mg protein) was less effective. Fluorescence of corneal tissue homogenates was twice as much that of SIRC (Table 4). Similarly, pirenoxine and melatonin significantly dammed the increase in lipid soluble fluorescent substances in corneas as well as α-tocopherol (31.1 ± 5.2 arbitrary

Table 2
Pirenoxine and melatonin affect SOD-inhibitable superoxide anion, conjugated diene increase and lipid soluble fluorescent compound formation in rabbit cornea submitted to 800 mJ/cm^2 UV-B exposure

	Superoxide anion (nmol/hemicornea/h)	Conjugated dienes□ (nmol/mg protein)	Fluorescence (arbitrary units/mg protein)
Corneal tissue (basal value)	92.3 ± 16 (4)	16.6 ± 5.2 (8)	2.4 ± 0.3 (8)
UV exposed corneal tissue (control)	$127.4 \pm 22^*$ (10)	$13.1 \pm 6.4^*$ (14)	$4.3 \pm 1.1^*$ (8)
+pirenoxine	80.4 ± 14 (4)	6.2 ± 2.3 (6)	2.2 ± 0.4 (4)
+melatonin	91.5 ± 12 (4)	3.5 ± 1.2 (6)	2.5 ± 1 (4)

Each number ± SD represents the mean value (in brackets the number of processed corneas). SOD presence decreased UV-B-induced superoxide to the basal values (93.2 ± 17.6 nmol/hemicornea/h) □: expressed by the difference between the sample and basal values. One-way ANOVA $F = 8.59$, $P < 0.001$, $F = 9.38$, $P < 0.001$ and $F = 10.57$, $P < 0.001$ superoxide, conjugated dienes and fluorescent compounds tests, respectively.
$^*P < 0.05$ different from all other groups, whereas other groups were not significantly different each other (Bonferroni's analysis). One-way ANOVA for conjugated dienes was performed among control, pirenoxine and melatonin groups.

Table 3
Pirenoxine and melatonin affect reduced/oxidized glutathione rate in rabbit cornea submitted to 800 mJ/cm^2 UV-B exposure

	Reduced	Oxidized	Reduced/Oxidized
	Glutathione (μg/mg protein)		Glutathione rate
Corneal tissue (basal value; n. 6)	1.5 ± 0.2	0.5 ± 0.1	3.0
UV exposed corneal tissue (control; n. 8)	1.0 ± 0.2*	0.6 ± 0.1	1.7
+pirenoxine (4)	1.5 ± 0.3	0.4 ± 0.1	3.7
+melatonin (4)	1.6 ± 0.4	0.5 ± 0.1	3.2

Each number ± SD represents the mean value (the number of processed corneas is in brackets). One-way ANOVA $F = 7.08$, $P < 0.002$ reduced glutathione test.
* $P < 0.05$ different from all other groups, whereas other groups were not significantly different each other (Bonferroni's analysis).

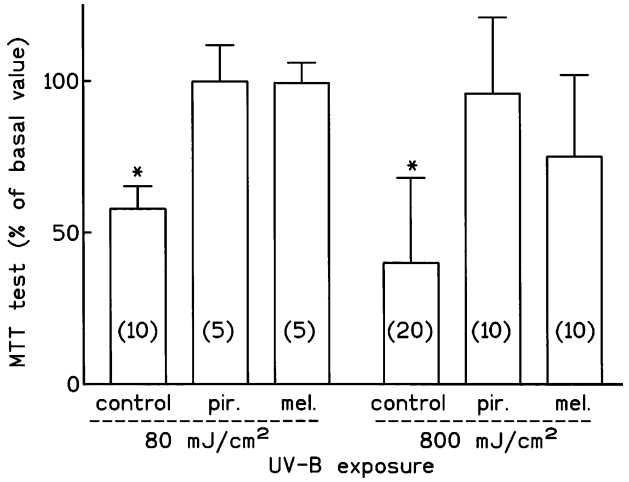

Fig. 1. Effect of pirenoxine (pir.) or melatonin (mel.) on epithelial cell mitochondrial viability of rabbit cornea after exposure to 80 or 800 mJ/cm^2 UV-B. Data are means ± SD (the number of processed corneas is in brackets). Corneas (20) without UV-B exposure (basal value: 100%) showed 0.442 ± 0.083 absorbance/mg protein at 570 nm. One-way ANOVA (performed among control, pirenoxine and melatonin groups) $F = 58.71$, $P < 0.001$ and $F = 15.53$, $P < 0.001$ for 80 and 800 mJ/cm^2 exposed cornea respectively. *$P < 0.05$ different from all other groups, whereas other groups were not significantly different from each other (Bonferroni's analysis).

Table 4
Effect of 10 μM pirenoxine or melatonin on the extent of peroxidation induced by ascorbate/Fe^{3+} in the homogenates of SIRC and tissues of rabbit corneas

	Fluorescence (arbitrary units/mg protein)	
	SIRC	Corneal tissue
Homogenate (basal value)	10.8 ± 1.0	26.6 ± 2.9
Iron-stimulated homogenate (control)	18.2 ± 1.8*	41.9 ± 4.0*
+pirenoxine	11.5 ± 1.4	32.1 ± 3.6
+melatonin	11.8 ± 1.0	34.0 ± 3.4

Each value ± SD represents the mean of at least four determinations (performed in duplicate). One-way ANOVA $F = 26.33$, $P < 0.001$ and $F = 13.11$, $P < 0.001$ SIRC and corneal tissue fluorescence, respectively.
* $P < 0.05$ different from all other groups, whereas other groups were not significantly different each other (Bonferroni's analysis).

fluorescence units/mg protein) and thioctic acid (29.3 ± 6 arbitrary fluorescence units/mg protein).

3.4. Effects of autologous macrophages action on the cornea

Incubation of rabbit corneas with fMLP-stimulated autologous macrophages produced a 142% increase in lipid soluble fluorescent compound formation with respect to the cornea value obtained after incubation with unstimulated macrophages. This increase was inhibited by adding 10 μM pirenoxine or melatonin to the incubation mixture (Fig. 2).

3.5. Ex vivo effects of pirenoxine on the cornea

Pirenoxine and melatonin (topically administered in vivo to rabbit eyes) induced a significant decrease (75.2% and 55.3%, respectively) in conjugated diene

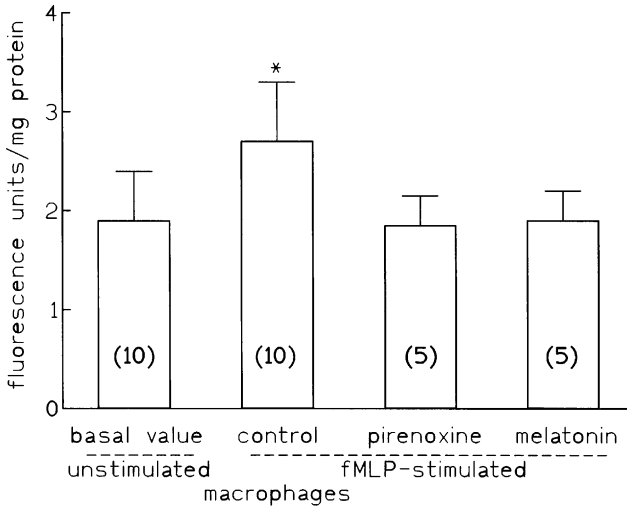

Fig. 2. Effect of 10 μM pirenoxine and melatonin on lipid soluble fluorescent compound formation in the epithelium of rabbit corneas after incubation with fMLP-stimulated autologous macrophages. Data ± SD represent the mean values (the number of processed corneas is in brackets). One-way ANOVA $F = 6.16$, $P < 0.01$. *$P < 0.05$ different from all other groups, whereas other groups were not significantly different each other (Bonferroni's analysis).

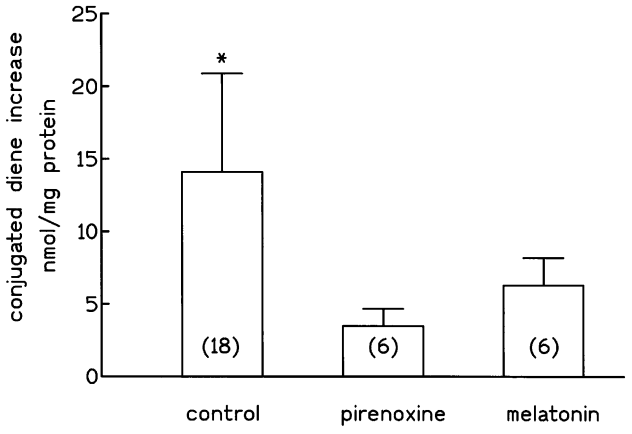

Fig. 3. Ex vivo effects of pirenoxine or melatonin instillations in rabbit eyes on the conjugated dienes increase in corneas submitted to 800 mJ/cm^2 UV-B light in vitro. Each value ± SD represents the mean value, expressed by difference between the sample and basal value (corneas from eyes with saline instillation, but without UV-B exposure: 23.9 ± 5.4 nmol/mg protein). The number of processed specimens is in brackets. One-way ANOVA (performed among control, pirenoxine and melatonin groups) $F = 20.49$, $P < 0.01$. *$P < 0.05$ different from all other groups, whereas other groups were not significantly different each other (Bonferroni's analysis).

formation in in vitro UV-B-treated corneas in comparison with those of saline-instilled eyes (Fig. 3).

4. Discussion

Our data demonstrate that scavenger compounds as pirenoxine and melatonin protect SIRC and the whole rabbit cornea from ROS activity in vitro induced by different systems, such as UV-B radiation, Fe(III)/ascorbate and macrophage oxidative burst.

In our experimental model, UV-B radiation significantly increased superoxide formation in exposed SIRC and rabbit corneas, probably indicating a functional damage to the electron transport chain of mitochondria [44] and elevated xanthine oxidase activity [45,46]. All tested molecules successfully inhibited superoxide anion formation. However, since pirenoxine [30], melatonin [47] and α-tocopherol are reported to be hydroxyl radical scavengers, it seems likely that neutralization of these hyperactive substances, produced after UV-B exposure, can protect the mitochondrial apparatus and prevent an increase in xanthine oxidase activity. On the other hand, thioctic acid, a compound also having superoxide-scavenger activity [38] and, in this case, working with a potency similar to SOD, totally prevented $O_2^{\cdot-}$ formation, suggesting that the UV-B injury of SIRC and corneas can be due to an excessive superoxide production ($O_2^{\cdot-} \rightarrow H_2O_2 \rightarrow HO^{\cdot}$).

A similar mechanism of hydroxyl radical neutralization can also explain the prevention of the lipid-soluble fluorescent compound formation by pirenoxine, melatonin, α-tocopherol and thioctic acid in SIRC and whole rabbit corneas. Lipid soluble fluorescent substances (produced by the reactions of degraded lipids with the amino groups of proteins) are believed to be epiphenomena of ROS activity and a sign of stable oxidation end-products [48].

Other ROS-dependent alterations (i.e., conjugated diene increase and contemporaneous depletion of physiological cellular glutathione) found in rabbit corneal tissue submitted to 800 mJ/cm^2 UV-B exposure are also ascribable to an initial superoxide production followed by hydrogen peroxide and hydroxyl radical formation. Since the conjugated diene increase is an early sign of polyunsaturated lipid structure alteration [49] and glutathione depletion is an indicator of cellular oxidative stress [50], our experimental data demonstrate that pirenoxine and melatonin protect membrane lipid components and cellular antioxidant defences by exerting their ROS scavenging activities.

The protective effect of pirenoxine, melatonin and other scavengers as α-tocopherol and thioctic acid in both SIRC and whole corneas, are also confirmed in vitro using an aggressive metal-catalysed oxidant system. The fluorescence calculated as arbitrary units per milligram of protein is higher in the corneal tissue than in SIRC. Indeed it is likely that the release of other fluorescent substances produced by the ROS-generating system on articulate arrangement of corneas can justify this increase [8,51,52].

By producing extracellular matrix, fibroblasts are known to pilot cell differentiation, migration and adhesion, with pro-healing and anti-inflammatory effects [18,53,54], therefore the protection against UV-B or Fenton reaction-induced ROS, exerted by both compounds on SIRC, may acquire a considerable importance. Indeed SIRC, being basically fibroblasts (keratocytes), possess a low (or absent) expression of some epithelial enzymes suitable to inhibit the ROS attack [48,55,56].

Our data show that the mitochondrial viability is reduced in SIRC culture and cornea by UV-B exposure, but the presence of pirenoxine and melatonin totally prevent the mitochondrial viability decrease in cells of corneas after exposition to 80 or 800 mJ/cm^2 UV-B. Moreover, it is interesting to note that pirenoxine exerts a full protection when it is present in the incubation milieu before and after UV-B exposure, and redoubles mitochondrial viability in comparison to control, when added after irradiation. On the other hand, melatonin protects mitochondrial viability only if the compound is present during the pre-treatment and incubation. In studies on the retina some researchers hypothesize that melatonin can attain efficacious concentrations in the retina micro-environment able to protect biomolecules from free radical damage and counteract apoptosis, since it is synthesized in situ and can therefore attain

high local concentration. Moreover it is reported that, as compared to in vivo concentration, higher melatonin dosages are necessary in vitro in order to fully scavenge free radicals and to rescue antioxidant defences [35,57,58]. We hypothesize that the lack of efficacy of melatonin may be ascribable to an inadequate concentration of melatonin attainable into the corneal cells.

On the other hand, both compounds are ineffective in preserving mitochondrial viability in SIRC after UV-B exposure, although they are able to reduce ROS formation and lipid peroxidation. This different behaviour between SIRC and corneal epithelial cells may be dependent on cell features. Indeed when exposed to UV-B corneal fibroblastic cells demonstrate an inadequate protection in comparison with epithelial cells and it is also reported that whole corneas tolerate higher doses of UV-B radiation than cultured keratocytes [1,13,59,60]. This is further consistent with the different mitochondrial viability obtained when SIRC cell monolayer structure and whole corneas are exposed to UV-B rays, since, in the first case, the high energy of radiation can hit directly all plated cells, whereas only the superficial epithelial cell layer is exposed in corneas (the anterior layers of human corneas are particularly important in preventing damage by UV-B radiation) [61].

With a similar aforesaid mechanism, pirenoxine and melatonin can prevent the formation of lipid soluble fluorescent compounds in rabbit cornea epithelium submitted to the oxidative burst of fMLP-stimulated autologous macrophages. Since corneal damage during inflammation is characterized by monocytes/macrophages and neutrophils invasion that can release ROS, proteolytic enzymes and arachidonic acid [25,60,62]. The indirect anti-inflammatory effect of pirenoxine and melatonin, dependent on their scavenger activities, can counteract several deleterious effects of inflammation.

Moreover, ex vivo experiments show that pirenoxine and melatonin repeatedly instilled in rabbit eye can increase or support the antioxidant defence of the whole cornea. Therefore, it is reasonable to assume that both compounds topically administered in vivo in the eyes can reach a concentration high enough to protect them from oxidative attacks and, consequently, may be considered available tools in the treatment of eye pathologies characterized by the presence of ROS. Studies are being currently carried out in our laboratory in order to better clarify the in vivo mechanism by which pirenoxine and melatonin protect the cornea.

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