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Journal of Photochemistry and Photobiology B: Biology 78 (2005) 35–42
Journal of Photochemistry and Photobiology B:Biology
www.elsevier.com/locate/jphotobiol

Pirenoxine prevents oxidative effects of argon fluoride excimer laser irradiation in rabbit corneas: biochemical, histological and cytofluorimetric evaluations

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Received 30 April 2004; received in revised form 23 July 2004; accepted 13 September 2004
Available online 11 November 2004

Abstract

The production of reactive oxygen species (ROS) associated with excimer laser irradiation is recognized as a possible cause of corneal haze following photorefractive keratectomy (PRK). Our work was aimed at investigating in vitro the oxidative effects induced by subablative laser fluences and at demonstrating the protective effectiveness of pirenoxine. Comparative trials of subablative fluence on rabbit eyes with or without 10^{-5} M pirenoxine were carried out. Superoxide anion ($O_2^{-\bullet}$), conjugated diene (CD), and thiobarbituric acid reagent substance (TBARS) formation were analyzed. Cellular death was evaluated by flow cytometry. Histological examinations were also performed. No appraisable differences in $O_2^{-\bullet}$, CD, and TBARS formation were detected soon after irradiation, whereas they all increased following incubation. Pirenoxine inhibited such increases. Cytofluorimetric and histological observations gave coherent results. The experimental data indicate that oxidative and toxic effects are ascribable to ROS avalanches triggered by laser irradiation-induced photodissociation and are inhibited by pirenoxine.
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Keywords: Reactive oxygen species; UV-C radiation; Pirenoxine

1. Introduction

During the last two decades the basic principles and clinical concerns of photorefractive keratectomy (PRK) by Argon Fluoride (ArF) excimer laser have been thoroughly investigated by many authors [1–6]. Various surgical procedures for the treatment of myopia, hyperopia, and astigmatism have been successfully introduced into clinical practice in many countries. One of the most serious side effects is the development of subepithelial scarring, which exhibits a strong light diffusion (haze) and produces a regression of the curvature correction. The incidence and intensity increase in eyes treated for higher degrees of refractive correction [5,6]. The nature of this surgical follow-up is related to the complex dynamics of the wound healing response. It involves various events such as the debris removal from the margins of the wound surface, the repair of damaged structures, the production of extracellular matrix components [7,8], as well as the replacement of cellular systems, namely the repopulation of stromal keratocytes to fill apoptotic areas [9–12], and the proliferation of epithelium to cover the ablated surface [13,14].

Different basic mechanisms underlying the evolution of wound healing which leads to haze formation have

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doi:10.1016/j.jphotobiol.2004.09.005

been proposed [15–17]. Various authors mention the important role of organic free radicals and reactive oxygen species (ROS) generated by UV laser irradiation of the corneal tissue, which could invasively react with the lipid components of cell membranes, nucleic acids and sulfur-containing enzymes and then trigger keratocyte death [18–20].

6.4 eV Photon energy associated with ArF excimer laser wavelength (193 nm) is high enough to induce the cleavage of peptide bonds and adjacent carbon–carbon bonds, as well as to produce hydroxyl radicals by water photodissociation [21]. At the same time, ROS could also be produced by a surface temperature increase associated with laser irradiation [19,22], as well as by infiltration of polymorphonuclear cells within the ablated area [20,23,24]. However, the role played by the various contributions has not yet been completely clarified. Moreover, the direct laser oxidative effect needs further investigation to establish the respective weights of subablative fluence exposure and ablation fluid dynamics in ROS production.

Some authors indicate that morphological alteration and disorganization of anterior stromal collagen [25] or photokeratitis [26] may depend on a subablative dose of UV radiation wavelength longer than 193 nm, probably due to laser-induced fluorescence. On the other hand, it was found that free radical production by 193 nm laser subablative radiation is quite comparable to that of 213 nm, even if the latter is responsible for greater cell lethality [27].

In the present work, we have investigated if a laser irradiation at subablative fluence can induce ROS-dependent damage in rabbit corneas.

Since previous studies suggest the use of antioxidant enzymes and scavenger molecules to prevent the production of free radicals or neutralize their toxic action [28–31], the effectiveness of the scavenging molecule pirenoxine was tested contemporaneously.

Pirenoxine sodium salt (1-hydroxy-5H-pyrido-(3,2-a)-phenoxazin-5-one-3-carboxylic acid sodium salt) is a molecule with a chemical structure similar to that of xanthomatin(ommochrome found in the ocular pigment of several insects), known for many years for its therapeutic action on cataracts (Catalin[R]: Takeda, Japan) [32–34]. In our recent studies on this active molecule, we have proved that the scavenging activity of the compound is selectively directed to the neutralization of hydroxyl radicals and is therefore able to reject various induced oxidative attacks on the lenses in vitro, ex vivo and in vivo [35] and to protect corneal tissue exposed to UV-B light in vitro and ex vivo [36]. Moreover, we have demonstrated that the pirenoxine efficacy as scavenging molecule is at least comparable or in several tests higher than that exerted by other well known antioxidant compounds (i.e., vitamin E, melatonin and 21-aminosteroid U74500A; [35,36]). The demonstration of a similar protective effectiveness against corneal oxidation stress following ArF excimer laser irradiation could extend the use of this compound to the preventive treatment of the cornea before and after PRK surgical intervention.

2. Materials and methods

2.1. Tissue preparation

Laser-induced oxidative effects were investigated on New Zealand white rabbits weighing ≈2–2.5 kg. The experimentation was carried out in accordance with European Community rules for the care and use of laboratory animals. After their arrival the rabbits were acclimated in comfortable cages under controlled light exposition cycles between 8:00 AM and 8:00 PM and supplied with unlimited food and water for three days. They were then killed with an overdose of sodium pentothal[R] (100 mg/kg e.v). The removed eyes were placed in phosphate buffer solution (PBS: pH 7.4) at 0 °C for 1 h.

The eyes were extracted from the medium and placed on a suitable support to be irradiated by ArF excimer laser (193 nm). An irradiation spot of 6 mm was imaged on the corneal surface through an optical homogenizing system, a circular diaphragm, and a focusing lens. The total fluence dose was 200 mJ/cm^2 cornea, achieved by firing 20 pulses of 10 mJ/pulse, at a frequency of 1 Hz. These irradiation conditions, well below the ablation threshold (46 mJ/cm^2) [37] and at a low pulse repetition rate, generated a negligible temperature rise [38], hence the only possible direct damage was of a photochemical nature. All corneas were excised at the limbus, washed with PBS and processed for biochemical analysis and histological examination immediately or after incubation. This incubation was performed for 2 h in the presence or absence of 10^{-5} M pirenoxine and, after washing, for 18 h in PBS. Such a concentration of the active principle was selected on the basis of its proven effectiveness to inhibit lens homogenate lipid peroxidation and prevent corneal damage due to UV-B radiation [35,36]. Basal values were obtained by identical treatment of eyes without the ArF irradiation and without pirenoxine.

2.2. Cell preparation

All corneas, thinly divided with a pair of scissors, were directly processed for at least 40 sec with semiautomatic disintegrator (Medimachine, with 35 μm Ø Medicon: Becton Dickinson and Co, USA) [39]. The resulting material was fully recovered with 5 ml PBS and subjected to 800*g* centrifugation for 10 min, then the supernatant was eliminated while the obtained corneal cell pellets were suspended in 1 ml PBS. The

cellular suspension was evaluated for superoxide anion production or, after homogenization, assayed for conjugated diene (CD) and TBARS formation.

2.3. Superoxide assay

After the addition of 1 mg/ml cytochrome *c* solution, the cell suspension was incubated at 37 °C for 1 h in the presence or absence of superoxide dismutase (300 U/ml). The mixture reaction was centrifuged (800*g*) and the purple color which developed was spectrophotometrically measured at 550 nm. The superoxide quantity was expressed as nmol/10^6 cells/h using an extinction coefficient of 2.1 10^4 $M^{-1}\,cm^{-1}$. Viable cells were counted using a Bürker chamber as trypan blue excluding cells.

2.4. Conjugated diene assay

The cell suspensions were homogenized at 0 °C and aliquots (0.5 ml) were extracted with a 3 ml chloroform–methanol (2:1 v/v) mixture and centrifuged (1900*g* for 10 min at 0 °C). The chloroform phase was evaporated under argon stream and the dry extract dissolved with 1.5 ml cyclohexane. The absorbance spectrum was scanned between 220 and 350 nm (cyclohexane as blank) and CD were evaluated at 232 nm, after elimination of the background due to Rayleigh scattering [35]. The data were expressed as nmol/cornea using an extinction coefficient of 2.52×10^4 $M^{-1}\,cm^{-1}$.

2.5. Thiobarbituric acid reagent substance (TBARS) assay

Homogenized cell suspensions (0.5 ml) were mixed with 0.5 ml of thiobarbituric acid (1% w/v in 0.05 N NaOH) and 0.5 ml 25% (v/v) HCl. The mixture was boiled for 10 min and, after cooling, extracted with 3 ml of *n*-butanol. After liquid phase separation (centrifugation at 2000*g* for 10 min), the absorbance of the pink organic phase was spectrophotometrically evaluated at 532 nm and TBARS was expressed as nmoles malondialdehyde (MDA)/cornea using a curve with 1,1,3,3,tetramethoxypropane as the standard reference [40].

2.6. Flow cytometric analysis

Corneas were stained with propidium iodide (PI: 50 μg/ml, 30 min in darkness), in order to point out only killed corneal cells by laser radiation. The tissue was washed twice with PBS, fixed in formaldehyde (3.6%) and processed with Medimachine. The disaggregated cells were submitted to flow cytometric analysis.

The PI fluorescence of individual cells from laser-irradiated corneas was measured using a FACStar cell sorter (Becton Dickinson, Mountain View, CA, USA) equipped with an argon ion laser (Model Innova 90) operating at 500 mW output at 488 nm.

Chicken erythrocyte nuclei were used for optical and electronic alignment of the instrument. Red fluorescence due to PI staining of DNA was collected by a 620 nm long pass filter. Data from 20,000 cells were analyzed by recording forward and scattered light and red fluorescence.

2.7. Histological examination

Corneas were fixed in 4% paraformaldehyde immediately after irradiation (time 0) or after 20 h incubation in the absence (control) or presence of pirenoxine at room temperature. Corneas without excimer laser irradiation were treated likewise and fixed at time 0 or after incubation (basal). Specimen were processed using routine methods for paraffin sections and hematoxylin-eosin staining.

2.8. Statistical analysis

Data are reported as means ± SD. One-way analysis of variance (ANOVA) followed by Bonferroni's test was performed to estimate significant differences among groups. As indicated, comparisons were performed among the basal (unirradiated corneas), control (not pirenoxine-treated irradiated corneas) and treated (pirenoxine-treated irradiated corneas) groups. Statistical tests were performed using PRIMER®, version 3.02 software (McGraw-Hill Companies Inc.).

3. Results

3.1. Superoxide anion formation and lipid peroxidation

The superoxide anion production in cells obtained from unirradiated corneas (basal) was 6.3 ± 0.7 nmol/10^6 cells/h (mean ± SD), while in cells obtained from laser irradiated corneas (control) this value was increased up to 15.7 ± 5.4 nmol/10^6 cells/h ($p < 0.05$ vs. basal). As reported in Fig. 1, this significant rise in superoxide formation, was totally prevented by 10^{-5} M pirenoxine; indeed the result, obtained in the presence of pirenoxine, was 5.9 ± 0.8 nmol/10^6 cells/h ($P < 0.05$ vs. control), very close to the basal value. Similarly, the significant increase in CD (177% vs. 100% basal value) in the irradiated corneas was inhibited when pirenoxine was in the incubation solution (Fig. 2).

TBARS formation following laser exposure was 144% of the corneal basal value (100%), whereas the pirenoxine treatment very effectively stopped the oxidative degradation (Fig. 3).

It is noteworthy to underline that neither $O_2^{-\bullet}$ nor CD nor TBARS quantifiable increases were found in all

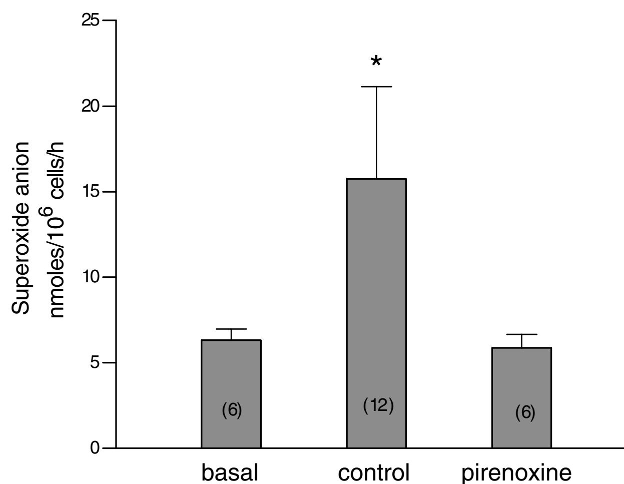

Fig. 1. In vitro formation of SOD inhibitable superoxide anion in cell suspensions obtained from rabbit corneas subjected to a cumulative ArF excimer laser irradiation dose of 200 mJ/cm^2 (10 mJ/cm^2 pulse) either in the absence (control) or presence of 10^{-5} M pirenoxine. Basal value is the superoxide anion production of cells obtained from unirradiated rabbit corneas. Data are means ± SD (the number of processed corneas is in brackets). The presence of SOD decreased laser-induced superoxide to basal value (6.3 ± 0.7 nmol/10^6 cells/h). * $p < 0.05$ vs. basal and pirenoxine.

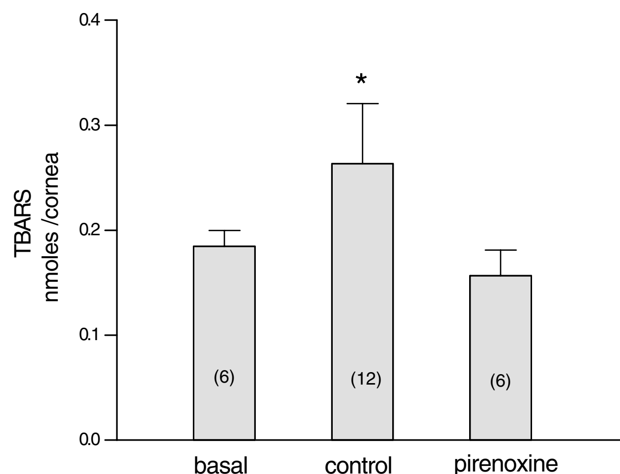

Fig. 3. TBARS formation in homogenized cells obtained from rabbit corneas subjected to a cumulative ArF excimer laser irradiation dose of 200 mJ/cm^2 (10 mJ/cm^2 pulse). Basal value (0.180.03 nmol/cornea) is the TBARS production obtained from unirradiated rabbit corneas. Data are means ± SD (the number of processed corneas is in brackets). * $p < 0.05$ vs. basal and pirenoxine.

specimens when the assays were performed soon after irradiation.

3.2. Cytofluorimetry

As shown in Fig. 4, the result of the cytofluorimetric assay indicated that necrotic cells increased significantly in control tissue, as compared to basal (unirradiated tissue) one (24.2 ± 0.8% vs. 10.51%). Pirenoxine improved cell viability of the laser-exposed corneal tissue, with a lesser increase in necrotic cells induced by laser treatment (16.3 ± 0.6%).

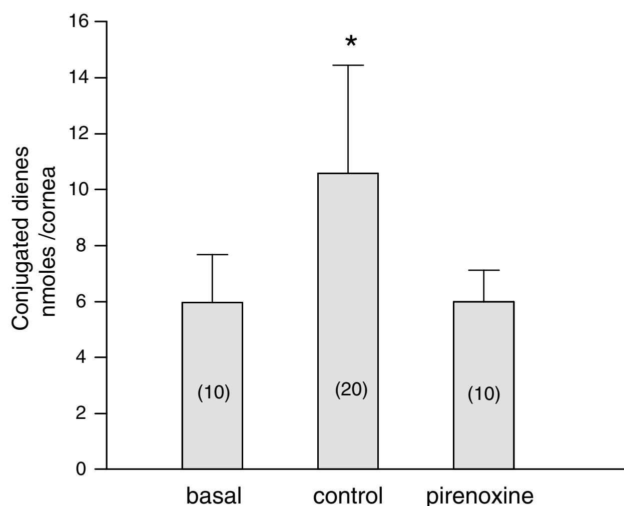

Fig. 2. Conjugated-diene production in homogenized cells obtained from rabbit corneas subjected to a cumulative ArF excimer laser irradiation dose of 200 mJ/cm^2 (10 mJ/cm^2 pulse) either in the absence (control) or presence of 10^{-5} M pirenoxine. Data are means ± SD (the number of processed corneas is in brackets). Unirradiated corneal tissue 5.9 ± 1.7 nmol/cornea. * $p < 0.001$ vs. basal and pirenoxine.

3.3. Histology

The results of the histological examinations are shown in Fig. 5. Fig. 5(a) shows basal corneal structure morphology. When specimen were fixed at time 0, no differences among basal (Fig. 5(a)), irradiated (Fig. 5(b)) or unirradiated (not shown) corneas were observed.

Pirenoxine did not modify tissue morphology in aforesaid conditions. Conversely, the histological sections of irradiated and incubated corneas (control) showed detachments of superficial epithelial cells and a vacuolization phenomenon with several large lacunae in the adjacent stromal tissue (Fig. 5(c)). Low magnification of these sections demonstrated that at deeper levels, far from the epithelium, the number and average size of these lacunae were gradually reduced. In the presence of pirenoxine (Fig. 5(d)) the epithelium appeared compact and homogeneous, likewise the stroma did not show substantial modifications.

4. Discussion

ArF excimer laser irradiation at subablative fluence induces strong ROS production, an increase in lipid peroxidation, cell viability reduction, and tissue mor-

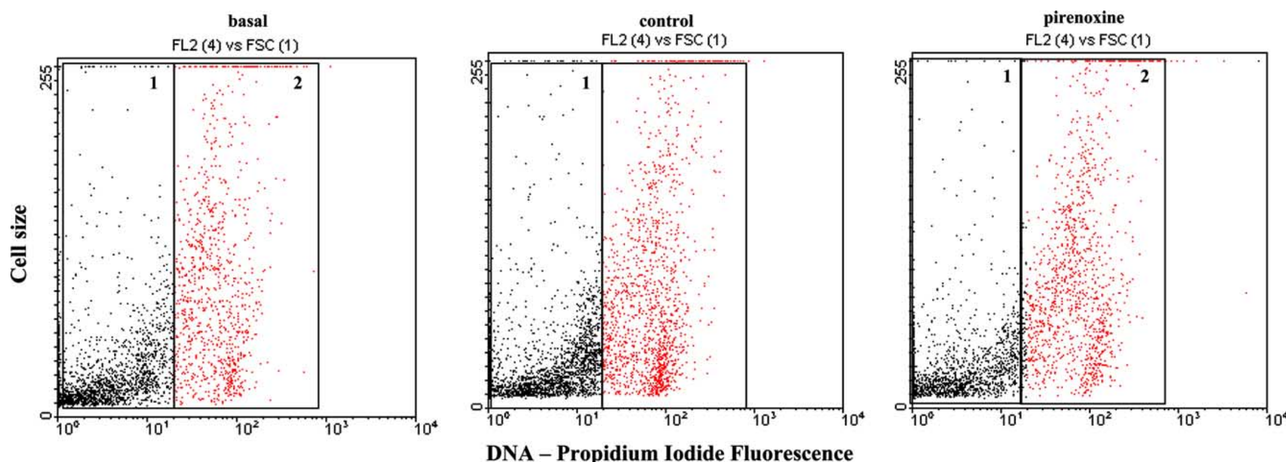

Fig. 4. Cell suspension density plot from unirradiated and irradiated corneas, in the absence or presence of pirenoxine (10^{-5} M) as indicated. The results are displayed by two parameters: DNA fluorescence (FL2, abscissa) and cell size (evaluated by forward light scattering, FSC, ordinate) both expressed as arbitrary units of fluorescence; alive cells are shown in box 1 and necrotic cells in box 2. Non-stained basal corneal cells were used to determine the FL2 cut-off (dot-plot not shown).

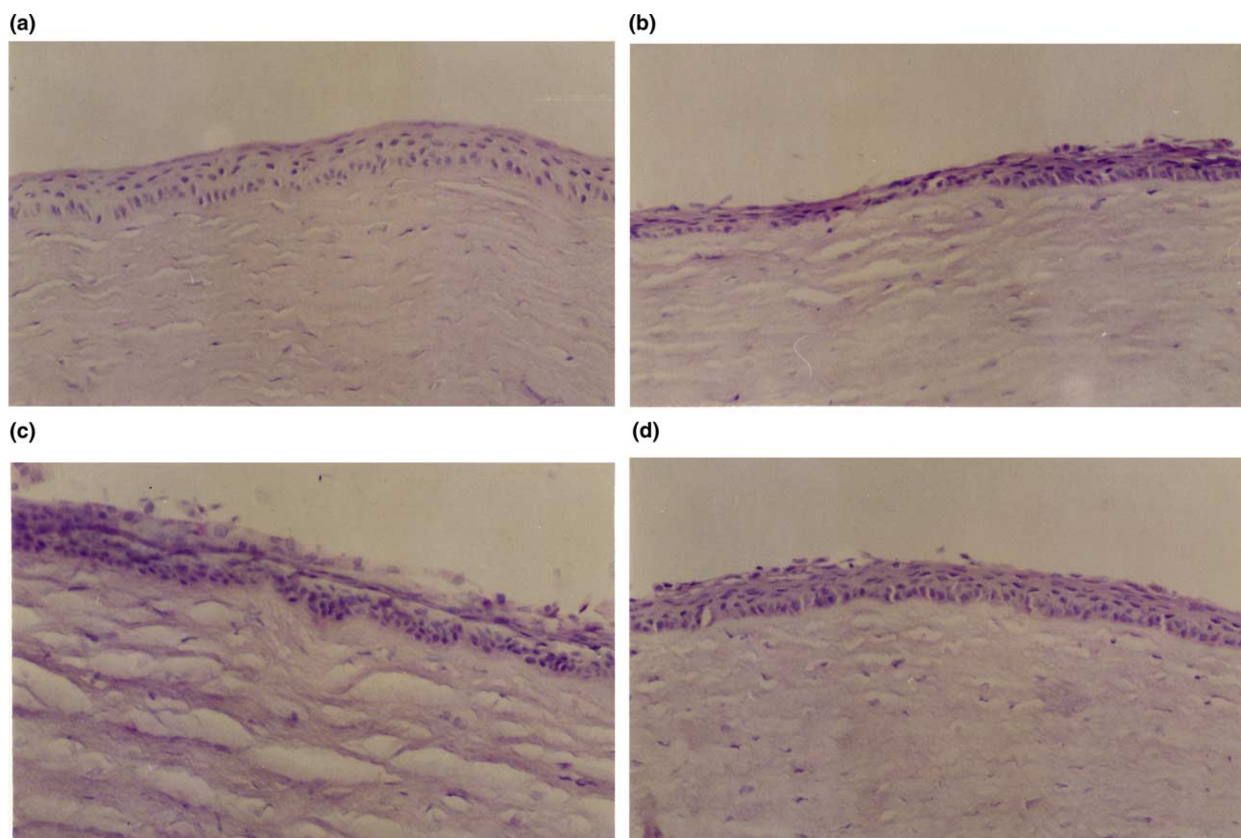

Fig. 5. Light microscopy (hematoxylin and eosin staining, ×200) of rabbit corneas subjected or not to a cumulative ArF excimer laser radiation dose of 200 mJ/cm^2 (10 mJ/cm^2 pulse). Epithelium and anterior stroma of: (a) Cornea unirradiated and fixed after 20 h of incubation (basal); (b) cornea immediately fixed after laser excimer irradiation (time 0); (c) cornea irradiated with laser excimer and incubated (20 h) in the absence (control) or (d) presence of 10^{-5} M pirenoxine.

phological modifications in rabbit corneas, as evaluated after incubation. On the other hand, $O_2^{-\bullet}$, CD and TBARS values obtained soon after laser exposure, as well as histological examinations of corneal sections immediately fixed, are not substantially different from those of unexposed corneas. Since all the damage indexes are evident after 20 h incubation, but not soon after laser exposure neither after 8 and 12 h (data not

shown), we suggest that the initial subablative photon flux acts as a trigger for more efficient ROS sources ($O_2^{-\bullet}$; H_2O_2; $OH^{\bullet}$). Actually, according to other authors, a direct breaking of various chemical bonds producing free radical generation is likely associated with photons in the vacuum ultraviolet (VUV) spectral region [18,21]. On this basis, it is probable that the superoxide increase, evaluated after incubation, indicates that there is functional damage to the electron transport chain of mitochondria [41] and/or an unbalance of the oxidoreductase to oxidase enzyme system [42,43] ascribable to initial radical stress produced by VUV laser irradiation. Epiphenomena, such as the increase in CD, which evidence early or moderate oxidative alterations [44], and TBARS formation, representing an index of a progressed polyunsaturated lipid decomposition [45], indicate that an ROS attack is present, which works during incubation. Furthermore, the hypothesis of damage produced by an ROS avalanche proliferation during incubation time, following the primary photochemical effect, is also supported by histological results, which show morphological alterations only 20 h after laser exposure (Fig. 5). Since at the subablative laser fluence and frequency employed (10 mJ/cm^2, 1 Hz) here, photothermal and photomechanical effects can be considered negligible [38,46]. The detachment of epithelial cells and the destruction of epithelial structure in irradiated and incubated corneas are directly consistent with the considerable lipid peroxidation and increase in necrotic cells. Given the short optical penetration of the VUV radiation into the corneal tissue, we think that the anterior stromal vacuolization phenomenon observed in laser-exposed corneas, which proportionally decreases at deeper layers, can also originate from a possible excimer laser fluorescent emission of longer and more penetrating UV wavelength, and, consequently, from ROS formation [25,26]. Moreover, such vacuolization may be due to the injury induced by ROS on proteoglycans, molecules with high water-binding capacity, and therefore an alteration of the matrix hydration.

All the experimental data coherently show a protective effectiveness of the pirenoxine, which prevents ROS overproduction and lipid peroxidation, increases cell viability and avoids morphological alteration of the corneal tissues. Since in our previous study we found that the mechanism of pirenoxine action is essentially based on its pronounced scavenging activity against the hydroxyl radical [35], we think that the compound is able to neutralize the initial photochemical free radical production occurring during the laser irradiation itself (not detectable with the present analytical approaches). In our hypothesis, the neutralization of these seeding events prevents the damage to mitochondrial apparatus and the unbalance of the cell oxidoreductase system, thus inhibiting the avalanche multiplication of ROS and their consequent effects. This is consistent with the protection provided by pirenoxine in preserving mitochondrial viability and scavenging capability of corneal cells exposed to 800 mJ/cm^2 UV-B [36]. Moreover, histological results show that the compound can improve antioxidant defenses, thus inhibiting stromal extracellular vacuolization and preserving natural stroma morphology.

In conclusion, the present study demonstrates that ArF excimer laser irradiation of rabbit corneas produces a cytotoxic pattern ascribable to massive ROS formation triggered by direct photochemical damage. Pirenoxine prevents ROS production and associated lipid peroxidation, thus defending cell viability and preserving the natural corneal tissue structures. Although we have described the basic mechanisms underlying the observed features, they still need further investigation, and especially in vivo experimentation with ArF laser in order to evaluate the protective effectiveness of pirenoxine in the evolution of wound healing of irradiated corneas.

Acknowledgements

This study was supported in part by grants from the University of Florence (ex MURST 60%) and Farmigea (Pisa, Italy).

We thank Professor Daniele Bani (Department of Anatomy, Histology and Forensic Medicine, University of Florence) for his helpful suggestions, Mary Forrest for editing the manuscript and Paolo Ceccatelli and Mauro Beni for their excellent technical assistance.

References

[1] S. Trokel, R. Srinivasan, B. Braren, Excimer laser surgery of the cornea, Am. J. Ophthalmol. 96 (1983) 710–715.
[2] R. Brancato, A. Tavola, F. Carones, A. Scialdone, G. Gallus, P. Garancini, G. Fontanella, Excimer laser photorefractive keratectomy for myopia: results in 1165 eyes, Refract. Corneal Surg. 9 (1993) 95–104.
[3] J.M. Krauss, C.A. Puliafito, Lasers in ophtalmology, Laser Surg. Med. 17 (1995) 102–159.
[4] P.J. McDonnell, Emergence of refractive surgery, Arch. Ophthalmol. 118 (2000) 1119–1120.
[5] D.S. Gartry, M.M.G. Kerr, J. Marshall, Excimer laser photorefractive keratectomy: 18-month follow-up, Ophthalmology 99 (1992) 1209–1219.
[6] M.C. Corbett, D.P.S. O'Brart, F.G. Warburton, J. Marshall, Biologic and environmental risk factors for regression after photorefractive keratectomy, Ophthalmology 103 (1996) 1381–1391.
[7] T. Larvala, K. Tervo, R. Mustonen, T. Tervo, Expression of cellular fibronectin and tenascin in the rabbit cornea after excimer laser photorefractive keratectomy, Br. J. Ophthalmol. 79 (1995) 65–69.

[8] C. Chen, B. Michelini-Norris, S. Stevens, J. Rowsey, X. Ren, M. Goldstein, G. Schultz, Measurement of mRNAs for TGFβ and extracellular matrix proteins in corneas of rats after PRK, Invest. Ophthalmol. Vis. Sci. 41 (2000) 4108–4116.
[9] S.J. Tuft, R.W. Zabel, J. Marshall, Corneal repair following keratectomy. A comparison between conventional and laser photoablation, Invest. Ophthalmol. Vis. Sci. 30 (1989) 31769–31777.
[10] W.R. Wee, B.A. Nassaralla, J. Garbus, P.J. McDonnell, Keratocyte-populated collagen gel as an in vitro model of excimer laser keratectomy, J. Refract. Surg. 12 (1996) 98–102.
[11] M.C. Corbett, J.I. Prydal, S. Verma, K.M. Oliver, M. Pande, J. Marshall, An in vivo investigation of the structures responsible for corneal haze after PRK and their effect on visual function, Ophthalmology 103 (1996) 1366–1380.
[12] J. Gao, T.A. Gelber-Schwalb, J.V. Addeo, M.E. Stern, Apoptosis in the rabbit cornea after photorefractive keratectomy, Cornea 16 (1997) 200–208.
[13] C. Hanna, Proliferation and migration of epithelial cells during corneal wound repair in the rabbit and the rat, Am. J. Ophthalmol. 61 (1966) 55–63.
[14] I. Ratkay-Traub, B. Hopp, Z.S. Bor, L. Dux, D. Becker, T. Krenacs, Regeneration of rabbit cornea following excimer laser photorefractive keratectomy: a study on gap junctions, epithelial junctions and epidermal growth factor receptor expression in correlation with cell proliferation, Exp. Eye Res. 73 (2001) 291–302.
[15] C.P. Lohmann, D.S. Gartry, M.G. Kerr Muir, G.T. Timberlake, F.W. Fitzke, J. Marshall, Corneal haze after excimer laser refractive surgery: objective measurements and functional implication, Eur. J. Ophthalmol. 1 (1991) 173–180.
[16] S.E. Wilson, Programmed cell death, wound healing and laser refractive surgical procedures: molecular biology for the corneal surgeon, J. Refract. Surg. 13 (1997) 171–175.
[17] W.J. Kim, S. Shah, S.E. Wilson, Difference in keratocyte apoptosis following transepithelial and laser scraper photorefractive keratectomy, J. Refract. Surg. 14 (1998) 526–533.
[18] R.J. Landry, G.H. Petit, D.W. Hahn, M.N. Ediger, G.C. Yang, Preliminary evidence of free radical formation during argon fluoride excimer laser irradiation of corneal tissue, Laser Light Ophthalmol. 6 (1994) 87–90.
[19] K. Bilgihan, A. Bilgihan, F. Akata, N. Turkozkan, B. Hasanreisoglu, Excimer laser corneal surgery and free oxygen radicals, Jpn. J. Ophthalmol. 40 (1996) 154–157.
[20] S. Hayashi, S. Ishimoto, G.S. Wu, W.R. Wee, N.A. Rao, P.J. MCDonnell, Oxygen free radical damage in the cornea after excimer laser therapy, Br. J. Ophthalmol. 81 (1997) 141–144.
[21] S. Shimmura, T. Masumizu, Y. Nakai, K. Urayama, J. Shimazaki, H. Bissen-Miyajima, M. Kohno, K. Tsubota, Excimer laser-induced hydroxyl radical formation and keratocyte death in vitro, Invest. Ophthalmol. Vis. Sci. 40 (1999) 1245–1249.
[22] T. Niizuma, S. Ito, M. Hayashi, M. Futemma, T. Utsumi, K. Ohashi, Cooling the cornea to prevent side effects of photorefractive keratectomy, J. Refract. Corneal Surg. 10 (1994) 262–266.
[23] Y.S. Choi, J.Y. Kim, W.R. Wee, J.H. Lee, Effect of the application of human amniotic membrane on rabbit corneal wound healing after excimer laser photorefractive keratectomy, Cornea 17 (1998) 389–395.
[24] W.C Park, C.G.S. Tseng, Modulation of acute inflammation and keratocyte death by suturing, blood and amniotic membrane in PRK, Invest. Ophthalmol. Vis. Sci. 41 (2000) 2906–2914.
[25] Z.Z. Nagy, P. Hiscott, B. Seitz, U. Schlotzer-Schrehardt, I. Suveges, G.O.H. Naumann, Clinical and morphological response to UV-B irradiation after excimer laser photorefractive keratectomy, Survey Ophthalmol. 42 (1997) S64–S76.
[26] R.R. Krueger, D.H. Sliney, S.L. Trokel, Photokeratitis from subablative 193-nanometer excimer laser radiation, Refract. Corneal Surg. 8 (1992) 274–279.
[27] M.N. Ediger, G.H. Pettit, L.S. Matchette, In vitro measurements of cytotoxic effects of 193 and 213 nm laser pulses at subablative fluences, Laser Surg. Med. 21 (1997) 88–93.
[28] S. Jain, T. Hahn, R.L. McCally, D.T. Azar, Antioxidants reduce corneal light scattering after excimer keratectomy in rabbits, Laser Surg. Med. 17 (1995) 160–165.
[29] N. Kasetsuwan, F.M. Wu, F. Hsieh, D. Sanchez, P.J. McDonnell, Effect of topical ascorbic acid on free radical tissue damage and inflammatory cell influx in the cornea after excimer laser corneal surgery, Arch. Ophthalmol. 117 (1999) 649–652.
[30] R. Brancato, N. Schiavone, S. Siano, A. Lapucci, L. Papucci, M. Donnini, L. Formigli, S. Zecchi Orlandini, G. Carella, F. Carones, S. Capaccioli, Prevention of corneal keratocyte apoptosis after argo fluoride excimer laser irradiation with the free radical scavenger ubiquinone Q10, Eur. J. Ophthalmol. 10 (2000) 32–38.
[31] K. Bilgihan, U. Adiguzel, S. Cern, G. Akyol, B. Hasanreisoglu, Effects of topical vitamin E on keratocyte apoptosis after traditional photorefractive keratectomy, Ophthalmologica 215 (2001) 192–196.
[32] K. Nishizaki, K. Inoue, Clinical experiments with high concentration Catalin ophthalmic solution, Folia Ophthalmol. Jpn. 26 (1975) 1087–1090.
[33] T. Okamoto, Catalin ophthalmic solution in treatment of senile cataract. An evaluation by transillumination photographic technique, Folia Ophthalmol. Jpn. 26 (1975) 1335–1345.
[34] T. Yuge, H. Takeda, K. Ozasa, Effects of Catalin eye drops on senile cataract, J. Eye 5 (1988) 1615–1618.
[35] M. Ciuffi, S. Neri, S. Franchi-Micheli, P. Failli, L. Zilletti, M.R. Moncelli, R. Guidelli, Protective effect of pirenoxine and U74389F on induced lipid peroxidation in mammalian lenses. An in vitro, ex vivo and in vivo study, Exp. Eye Res. 68 (1999) 347–359.
[36] M. Ciuffi, M. Pisanello, G. Pagliai, L. Raimondi, S. Franchi-Micheli, M. Cantore, L. Mazzetti, P. Failli, Antioxidant protection in cultured corneal cells and whole corneas submitted to UV-B exposure, J. Photochem. Photobiol. Biol. 71 (2003) 59–68.
[37] C.A. Puliafito, R.F. Steinert, T.F. Deutsch, F. Hillenkamp, A.J. Dehm, C.M. Adler, Excimer laser ablation of the cornea and lens: experimental studies, Ophthalmology 92 (1985) 741–748.
[38] M. Ishihara, T. Arai, S. Sato, Y. Morimoto, M. Obara, M. Kikuchi, Measurement of the surface temperature of the cornea during ArF excimer laser ablation by thermal radiometry with a 15 ns time response, Laser Surg. Med. 30 (2002) 54–59.
[39] G. Brockhoff, S. Fleischmann, A. Meier, F.P. Wachs, F. Hofstaedter, R. Knuechel, Use of a mechanical dissociation device to improve standardization of flow cytometric cytokeratin DNA measurements of colon carcinomas, Cytometry 38 (1999) 184–191.
[40] O.I. Aruoma, B. Halliwell, M.J. Laughton, G.J. Quinlan, J.M.C. Gutteridge, The mechanism of initiation of lipid peroxidation. Evidence against a requirement for an iron(II)–iron(III) complex, Biochem. J. 258 (1989) 617–620.
[41] S. Shimmura, K. Tsubota, Ultraviolet B-induced mitochondrial dysfunction is associated with decrease cell detachment of corneal epithelial cells in vitro, Invest. Ophthalmol. Vis. Sci. 38 (1997) 620–626.
[42] J. Cejkova, Z. Lojda, Histochemical study on xanthine oxidase activity in the normal rabbit cornea and lens and after repeated irradiation of the eye with UV-B rays, Acta Histochem. 98 (1996) 47–52.

[43] J. Ceikova, S. Stipek, J. Crkovska, T. Ardan, A. Milelfart, Reactive oxygen species generating oxidases in the normal rabbit cornea and their involvement in the cornel damage evoked by UV-B rays, Histol. Histopathol. 16 (2001) 523–533.
[44] R.A. Klein, The detection of oxidation in liposome preparation, Biochim. Biophys. Acta 210 (1970) 486–489.
[45] H.B. Demopoulos, E.S. Flamm, D. Pietronigro, M.L. Seligman, The free radical pathology and the microcirculation in the mayor central nervous system disorders, Acta Physiol. Scand. 492 (1980) 91–119.
[46] S. Siano, R. Pini, F. Rossi, R. Salimbeni, P.G. Gobbi, Acoustic focusing associated with excimer laser ablation of the cornea, Appl. Phys. Lett. 72 (1998) 647–649.