Effect of Topical Ascorbic Acid on Free Radical Tissue Damage and Inflammatory Cell Influx in the Cornea After Excimer Laser Corneal Surgery

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Objective: To evaluate the effect of topical ascorbic acid on oxygen free radical tissue damage and the inflammatory cell influx in the cornea after excimer laser keratectomy.

Methods: Five New Zealand white rabbits underwent bilateral phototherapeutic keratectomy with the 193-nm argon fluoride excimer laser. Following treatment, the right eye of each rabbit was treated with 10% ascorbic acid every 3 hours for 24 hours. The left eyes served as controls. After 24 hours, all animals were killed and their corneas were trephined and processed. Sections were stained with fast blue B and with hematoxylin-eosin. Oxidative tissue damage in the form of lipid peroxidation was detected by fluorescent peroxidized carbonyl compounds using a confocal laser scanning microscope. The quantity of these compounds was determined using the National Institutes of Health digital image analysis system. Statistical comparisons of lipid peroxidation and polymorphonuclear cell count between the ascorbic acid groups and the controls were performed using the Student *t* test.

Results: Lipid peroxidation and polymorphonuclear cell counts were significantly decreased in the superficial cornea of ascorbic acid–treated eyes compared with control eyes ($P < .03$ and $< .02$, respectively).

Conclusions: Topical ascorbic acid application decreased oxygen radical tissue damage following excimer keratectomy; moreover, topical application of ascorbic acid was shown to reduce the acute inflammatory reaction efficiently. This suggests that topical ascorbic acid could be considered a complementary treatment in the pharmacological modulation after excimer laser corneal surgery.

Clinical Relevance: Corneal opacity may complicate excimer keratectomy. The use of an antioxidant to reduce tissue damage could help minimize postoperative stromal opacification.

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THE USE of the 193-nm excimer laser has continued to generate wide interest for the treatment of myopia, astigmatism, and corneal scar.[1-5] As laser therapy has advanced, researchers have attempted to elucidate the pathological and microstructural changes that are present after excimer laser ablation. Recent studies of this laser-tissue interaction have demonstrated free radical formation and polymorphonuclear (PMN) cell infiltration of surface ablated cornea.[6-10] Free radicals, which are reactive species capable of independent existence, contain 1 or more unpaired electrons.[11] These free radicals can induce the peroxidation of fatty acids or of the lipids of cell membranes, a process known as lipid peroxidation. Hayashi et al[9] found free radical tissue damage in the form of lipid peroxidation in the superficial corneal stroma of excimer laser–treated corneas. The extent of tissue damage reflects the balance between the free radicals generated and the local antioxidation defense system. The termination of lipid peroxidation, that is, the interruption of the auto-oxidative chain reaction, is accomplished by free radical scavengers. One of the most extensively studied of these scavengers is ascorbic acid (vitamin C), the ubiquitous water-soluble sugar acid.[11] Moreover, Williams et al[12] found that ascorbic acid inhibits the activity of PMN leukocytes by impairing myeloperoxidase in inflamed ocular tissue.

Considering these observations, we performed histochemical and histopathologic analyses to determine the influence of topical ascorbic acid therapy after excimer laser ablation in a rabbit model.

RESULTS

Twenty-four hours after laser ablation, the corneas were grossly devoid of haze. There was no conjunctival injection, corneal edema, or thinning.

Oxygen free radical–induced tissue damage in the form of lipid peroxidation was demonstrated by the presence of posi-

MATERIALS AND METHODS

EXCIMER LASER ABLATION

Five New Zealand white rabbits, each weighing between 2.0 and 2.5 kg, were anesthetized with intramuscular injections of ketamine hydrochloride (40 mg/kg) and xylazine (7 mg/kg). After induction of anesthesia, topical proparacaine hydrochloride was applied to the rabbit corneas, and the 193-nm argon fluoride excimer laser (Twenty-Twenty; VISX Inc, Sunnyvale, Calif) was used to treat both eyes of each rabbit. The laser was programmed for an average fluence of 150 mJ/cm^2 with an ablation rate of 5 Hz and an ablation zone diameter of 6 mm. In the first step, 30 µm of epithelium was removed by laser ablation; thereafter, phototherapeutic keratectomy was performed on the corneal stroma to a depth of 70 µm.

DRUG TREATMENT AND REGIMEN

A 10% ascorbic acid solution was prepared using distilled water and adjusted to a pH of 7.2 with sodium hydroxide. The right eye of each rabbit was treated with 2 drops (50 µL per drop) of 10% ascorbic acid immediately after the laser photoablation and then every 3 hours thereafter; the left eye of each rabbit was treated with distilled water at the same frequency. Both eyes were used to control for differences in corneal wound healing among the 5 rabbits. Twenty-four hours after photoablation, all animals were examined with a slitlamp biomicroscope to assess for any signs of ocular inflammation, corneal clarity, and thinning. All animals were killed by intravenous injection of pentobarbital sodium 24 hours after photoablation. All procedures used were in full accord with the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research.

HISTOCHEMICAL STAINING OF PEROXIDIZED LIPIDS

The corneas were harvested for analysis by rapidly trephining a 7.5-mm corneal button, centered around the site of laser ablation. Peroxidized lipids were detected histochemically by the method previously described.[13] Briefly, each cornea was embedded in OCT compound and immediately snap frozen in liquid nitrogen. Ten-micrometer frozen sections were prepared, fixed in 5% trichloroacetic acid for 1 minute, then rinsed in saline (4 times for 5 minutes each time). The sections were allowed to react overnight at room temperature in a 0.1% wt/vol 3-hydroxy-2-naphthoic acid hydrazide (NAH) solution in saline, containing 10% vol/vol dimethyl sulfoxide and 0.095% wt/vol *p* -toluene–sulfonic acid as a catalyst. Sections were then washed (4 times for 5 minutes each time) in a 15% vol/vol dimethyl sulfoxide solution in 1-mmol/L hydrochloric acid and exposed for 20 minutes to a 0.1% wt/vol fast blue B (Sigma-Aldrich Corporation, Milwaukee, Wis) solution in 0.1-mol/L phosphate buffer, pH 6.5. After fast blue B coupling, sections were washed in running tap water for 5 minutes, then in saline for 5 minutes, before they were embedded in glycerol gelatin. The resultant fluorescent NAH-carbonyl compounds were detected by a confocal laser scanning microscope (Carl Zeiss, Thornwood, NY), using an excitation wavelength of 488 nm and an emission wavelength of 520 nm.

MEASUREMENT OF PEROXIDIZED LIPIDS

The 6-mm ablation zone in each corneal section was easily identified under the confocal microscope by the lack of epithelium and topographic changes on the corneal surface. The cell membranes that had flourescent staining were distributed evenly throughout the ablation zone of each corneal section. The areas closest to the center of the cornea were arbitrarily chosen for measurement. The amount of fluorescence present in the laser-treated corneal stroma was measured using a digital image analysis system based on a shareware computer program (NIH Image, V1.60; National Institutes of Health, Bethesda, Md) that permits the monochrome image at a pixel resolution of 0 to 255 and an 8-bit gray scale (256 gray levels per pixel). Corneal images from the laser confocal microscope were loaded into the shareware computer program for analysis. The program permitted us to outline the areas from each image that we wanted to analyze. The flourescence from the anterior corneal surface to a depth of 100 mm for each corneal image was measured. The total surface area measured was kept constant for each of the images to minimize measurement bias. Four sections from each of the 10 corneas were examined in this manner, and the results were averaged.

HISTOPATHOLOGIC EVALUATION

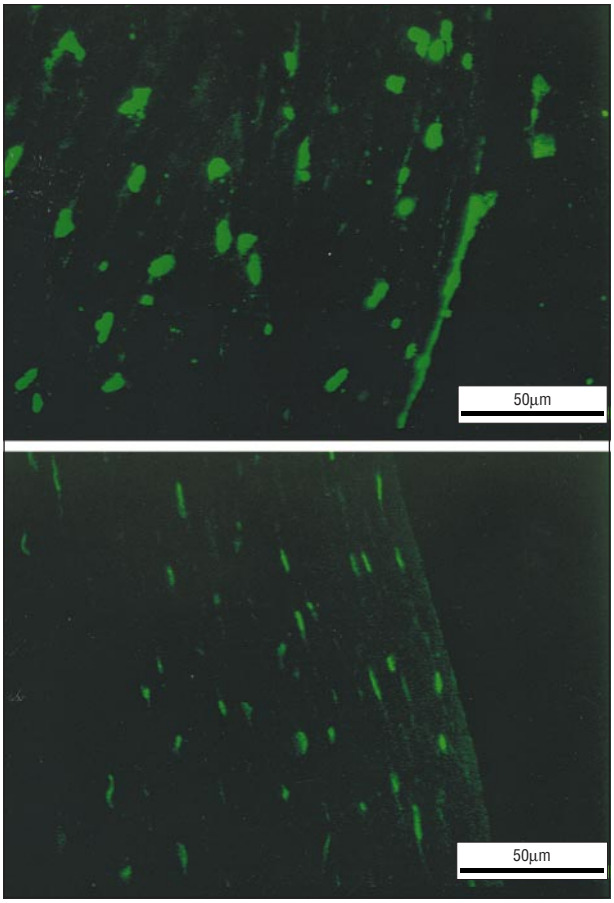
To evaluate the presence of PMN infiltration, 5-µm histological sections were cut and stained with hematoxylin-eosin. Two histological sections from the anterior half of each cornea were examined under ×40 high-power fields. The PMN cells from each section were counted, and the values were averaged.

STATISTICAL ANALYSIS

The paired 2-tailed Student *t* test was used to evaluate the differences between the groups.

tive fluorescent NAH-carbonyl compounds, as shown by fast blue B staining, in the laser-treated cornea, primarily near or at the surface of the ablated stroma (**Figure**). The intensity of fluorescence of NAH-carbonyl compounds from 4 sections of each cornea, expressed as mean ± SD in pixels per anterior half of laser-treated corneas, is shown in **Table 1**. A statistically significant decrease of fluorescence was found in the corneal stroma of the ascorbic acid–treated eyes compared with control eyes ($P < .03$).

Both the ascorbic acid–treated eyes and the control eyes revealed PMN cells infiltrating the ablated area, concentrated primarily near the laser-treated surface. The PMN cell count from 2 sections of each cornea is shown in **Table 2**. A statistically significant decrease of PMN cells was also found in the ascorbic



The corneal surfaces within the ablation zone after photorefractive keratectomy are represented. The anterior surfaces of the postablated corneas are to the right. These transverse corneal sections were reacted with the fast blue B stain, selectively causing products of lipid peroxidation to fluoresce. When compared with light microscopic images, the fluorescent areas corresponded to keratocyte cell membranes. Fluorescence in the ascorbic acid–treated cornea (top) is decreased in comparison to that of the control cornea (bottom) (original magnification ×40).

Table 1. Mean Fluorescence in 4 Sections of Each Cornea

Eye No.	Mean Fluorescence*							
	Right Eye				Left Eye			
	A	B	C	D	A	B	C	D
1	5.85	14.41	10.48	12.04	46.32	37.26	40.25	49.73
2	15.63	16.80	21.74	23.19	28.29	50.39	49.78	24.85
3	13.63	13.87	15.85	13.09	26.95	22.25	19.52	24.48
4	10.83	10.61	11.91	9.23	23.77	25.90	22.24	19.08
5	6.01	9.04	10.26	7.25	10.93	15.25	17.60	14.36

**The mean fluorescence is expressed as pixels per anterior half of laser-treated corneas. The right eyes were treated with ascorbic acid; left eyes, distilled water (controls). A, B, C, and D represent the 4 sections of each cornea. The mean ± SD fluorescence of the right eyes was 12.59 ± 4.33; left eyes, 28.46 ± 11.97 (P = .03).*

Table 2. Polymorphonuclear Cell Count in 2 Sections of Each Cornea

Eye No.	Polymorphonuclear Cell Count†			
	Right Eye		Left Eye	
	A	B	A	B
1	32	29	80	74
2	35	40	53	51
3	25	36	102	103
4	35	29	54	53
5	24	27	103	112

**The polymorphonuclear cell count is expressed as number of cells per anterior half of laser-treated corneas. The right eyes were treated with ascorbic acid; left eyes, distilled water (controls). A and B represent 2 sections of each cornea. The mean ± SD cell count of the right eyes was 31.2 ± 4.3; left eyes, 78.5 ± 26.2 (P = .02).*

acid–treated corneas compared with the control corneas ($P < .02$).

COMMENT

In the present study, we have demonstrated that topical ascorbic acid significantly decreases oxygen free radical tissue damage in the cornea following excimer laser photoablation. The presence of oxygen radical–induced tissue damage was indirectly demonstrated by the measurement of oxidized lipid fluorescence in the corneal tissue after fast blue B staining.

The presence of free oxygen radicals after excimer laser ablation has been demonstrated in many studies.[6-9] Landry et al[6] found that free radicals are formed during argon fluoride laser ablation of the cornea, but that these products remain only transient. Hayashi et al[9] also found oxygen free radical damage in the laser-ablated corneas after 24 hours by oxidation of the unsaturated fatty acids present in the cell membrane, primarily in keratocytes. Reactive oxygen species, such as superoxide and hydrogen peroxide, may injure the corneal tissues by degrading corneal stromal macromolecules (proteoglycans and collagen) either directly by scission of covalent bonds or indirectly by enhancing their susceptibility to hydrolytic enzymes.[14,15] Moreover, active oxygen species are proinflammatory because they activate prostaglandin H synthesis through the formation of hydroperoxides[15] and because they activate PMN collagenase.[16]

Polymorphonuclear cell infiltration of the corneal stroma after excimer laser photoablation has been reported.[9,10] Our study confirms these findings and demonstrates that the number of PMN cells was significantly decreased in the ascorbic acid–treated corneas compared with the controls.

The eye protects itself from radical injury by 2 major protective mechanisms: the endogenous antioxidant enzyme systems, such as superoxide dismutase, catalase, and glutathione peroxidase, and the free radical scavengers, such as ascorbate and vitamin E. The extent of corneal damage due to oxidation is ultimately limited by these systems. The presence of excessive amounts of free radicals that break through these defense systems leads to oxidative damage of the corneal tissue and further corneal inflammation through proinflammatory, modulatory, and toxic effects as well.[17] Corneal stromal wound healing follows excimer keratectomy and results in a change in collagen lamellar uniformity and scar formation. These events can cause visual disturbance from increased light scattering. Jain et al[18] found that intraoperative application of antioxidant, either 50% dimethyl sulfoxide or 1% superoxide dismutase, de-

creased light scattering following excimer keratectomy. Moreover, photo-oxidative stress in the lens can cause biochemical alterations that may increase the risk of cataractogenesis.[19,20] Therefore, there is considerable evidence to support the use of pharmacological agents, specifically those directed against the oxygen free radicals that occur after excimer photoablation.

In this study, the application of 10% ascorbic acid immediately after excimer laser photoablation and every 3 hours thereafter for 24 hours has been shown effective. Ascorbic acid has a small molecular weight and accumulates in ocular tissues at a concentration several times higher than the plasma level.[11,21,22] It is not synthesized by humans; therefore, dietary intake is essential. Ascorbic acid in the cornea could be supplied by the tears by transport across the corneal epithelium or by the aqueous humor by transport across the corneal endothelium. The protective role of ascorbic acid in the antioxidant defenses of the eye occurs in the termination step of lipid peroxidation. Lipid peroxidation can be defined as the free radical–induced, nonenzymatic oxidation of long-chain polyunsaturated fatty acids, most of which are present in cell membranes. Because of ascorbic acid's strong reducing properties, the molecule is likely to contribute an electron to any free radical species. This results in neutralization of the original free radical and formation of the ascorbate free radical. The ascorbate free radical is not particularly reactive. It can undergo a biologically irreversible reaction to form an inert product; alternatively, it can be reduced to useful ascorbic acid and disappear by second-order kinetics with a constant decay rate.[21,22] Ascorbic acid is essential for the synthesis of collagen. On a molecular level, proline and lysine must be taken up by fibroblasts and transported to the ribosomes by transfer RNA. Proline and lysine are incorporated into the collagen peptide chain as dictated by the messenger RNA sequence. During this ribosomal collagen synthesis, ascorbic acid is required as a cofactor in the hydroxylation process. Impairment of this process results in the production of unassociated pro–α chains, which fail to associate into the stable triple helical structure of collagen.[21,23] Such unstable collagen molecules are vulnerable to the action of proteolytic enzymes.[24] The lack of ascorbic acid in collagen synthesis has also been attributed to interference with its development and maturation, which will influence the healing process. Corneal wound healing is an important determinant of the clinical outcome of excimer laser surgery, with respect to corneal clarity and regression. Moreover, Williams et al[12] found that ascorbic acid appears to inhibit the functional activity of myeloperoxidase, the most abundant lysosomal enzyme in the PMN cells, thus preventing potential tissue damage by this enzyme when it is released during leukocyte degranulation in inflammation. The mechanism by which ascorbic acid impairs myeloperoxidase activity may involve direct inhibition of the enzyme and/or the scavenging of free radicals essential for peroxidative activity.

The concentration of ascorbic acid used in this study has been reported to be acceptable in terms of ocular irritation and reaction.[25]

In conclusion, the present study demonstrates that the immediate postoperative use of ascorbic acid significantly reduces oxygen radical–mediated tissue damage in the form of lipid peroxidation following excimer laser keratectomy. Further studies will be required to determine whether the reduction of oxidative damage will result in an improvement in postoperative corneal clarity. We believe that concurrent antioxidant therapy may influence corneal wound healing after excimer laser keratectomy. The applicability of these data from rabbits to the human cornea remains to be verified.

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