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The effect of ultraviolet radiation on the cornea – experimental study

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Abstract

Ultraviolet (UV) radiation in high doses may have harmful effects on the eye. The sources of UV radiation are the sun, as well as some artificial sources such as UV lamps or voltaic arcs. Chronic exposure to UV can cause damage to the anterior pole of the eye, ranging from minor (pterygium) to serious photokeratitis. In our study, we applied a UV dose of 6.5 J/cm^2 in the wavelength range of 290–400 nm, for five consecutive days per rat anterior pole of the eye. Seven days after the last dose of radiation, the animals were sacrificed, harvesting both the irradiated and the non-irradiated eye. Histological and immunohistochemical examination of the lesions revealed that the greatest damage to the epithelium was recorded prior to and 2/3 of the remaining corneal stroma. The epithelial lesions we found varied from pseudokeratosis and detachment of the Bowman epithelium membrane to deep epithelial necrosis. Within the corneal stroma, we observed the formation of interstitial edema with disruption of the collagen structure. We also noticed the presence of an inflammatory infiltrate composed mainly of lymphocytes and CD68+ and CD163+ macrophages, as well as the occurrence of vascular devices. These consisted of angiogenesis capillaries with structured wall composed mainly of endothelial CD34+ precursor cells and a basal membrane rich in collagen IV fibers.

Keywords: cornea, ultraviolet radiation, photokeratitis, corneal epithelium necrosis.

Introduction

Optical radiation in the electromagnetic spectrum includes ultraviolet (UV), visible light and infrared radiation.

UV spectrum is located between the X-ray and visible area, including electromagnetic radiation with wavelengths in the range 100–400 nm. This spectrum is subdivided in three groups: UV-A, containing wavelength 400–320 nm, UV-B with wavelength between 320–280 nm and UV-C with wavelength ranging between 280–100 nm.

Solar radiation represents the main source of UV. From solar UV spectrum, only UV-B and a small amount of UV-A reach Earth surface, and they are responsible for some diseases, like erythema, skin cancer, immunodeficiency and skin aging [1]. However, the human body also benefits from the UV-B, due to vitamin D synthesis at the skin level.

Beside natural UV, humans are exposed to some artificial sources produced by fluorescent lamps in the voltaic arc welders, incandescent mercury vapor, UV lamps used for sterilization in surgery rooms or areas for small children and infants.

UV exposure can be acute or chronic. The most harmful UV effect that affects anterior pole of the eye seems to be that generated by voltaic arc of welding devices. This can explain the development of some ocular diseases at welders [2].

One of the most common ocular diseases induced by chronic UV exposure is pterygium [3–5].

In our study, we evaluated the effects of UV on cornea after repetitive high doses exposure, particularly if this can induce pterygium on animal model.

Materials and Methods

After approval of Ethics Committee of University of Medicine and Pharmacy of Craiova, Romania, we have selected 20 Wistar rats, 10 females and 10 males, weighing between 260–290 g from the Animal Facility of the University of Medicine and Pharmacy of Craiova, which have been anesthetized with 10% Ketamine (60 mg/kg body weight) and 2% Xylazine (10 mg/kg body weight). Afterwards, the left eyes of the animals have been exposed to an ultraviolet source, emitted by a pump that was made with LS-1 tungsten halogen lamp (spectral range 290–400 nm) providing a 6.5 W output power (over the entire spectral range).

During the experiment, the rats were kept at room temperature and received food and water *ad libitum*. Artificial lighting provided a 12 hours light/dark cycle. Special environmental conditions (temperature, humidity, air changes) were provided by a climate station.

A week after the last day of irradiation, all 20 rats were sacrificed after intravenous Thiopental anesthesia (50 mg/kg body weight), and then both left (irradiated) and right eyes were collected. We kept the eyes in a 10% formalin solution for three days.

After sampling and orientation, the biological material was embedded in paraffin. There were made serial sections of 3 μm using a Microm HM350 microtome fitted with a special transfer section (Section Transfer System STS) in a water bath. Then, the slides were stained with Hematoxylin–Eosin and light green trichromic Goldner–Szekely.

Afterwards, the sections were passed through 2% skimmed milk for 30 minutes at room temperature to block non-specific sites.

Finally, the sections were incubated with primary antibodies overnight at 4^0C (for 18 hours). After washing 3×5 minutes in 1% PBS biotinylated secondary antibody was applied for 30 minutes at room temperature.

The slides were washed in 1% PBS solution, and then Streptavidin (1:200) was applied for 30 minutes at room temperature.

After washing in PBS, 3,3'-Diaminobenzidine (DAB) (Dako) was applied, and then, the contrasting with Mayer's Hematoxylin was performed. After this, the protocol was completed with dehydration, clearing and mounting with DPX (Fluka).

For the IHC study, we used the following antibodies:

- CD34 to highlight vessels angiogenesis;
- CD68 to study the reaction of macrophages;
- CD163 to study the macrophages involved in angiogenesis;
- Collagen IV to study collagen synthesis membrane.

Results

To compare the lesions done by UV exposure, we have processed and stained normal corneas from non-irradiated eyes from each animal. As it can be seen (Figure 1), the cornea is composed of an anterior epithelium (stratified, squamous tissue, without keratinization, sitting on a basement membrane – Bowman membrane), stroma (which is the most developed) and a posterior epithelium (squamous tissue, lying on a thick basement membrane – Descemet membrane).

The irradiation of the anterior pole of the eye with UV caused significantly microscopic changes in all histological structures of the eye.

The first aspect observed by us was the irregular thickening and the distortion of irradiated corneas, mainly in center, where the spotlight was higher. Growth in the cornea thickness was determined mainly by the fluid swelling in stroma, which led further to fibrillar collagen disorganization at this level. Thus, collagen fibers appeared disrupted, occasionally broken and weakly stained. Also, in addition to fluid swelling, we observed an accumulation of inflammatory cells and angiogenesis blood vessels at the stroma level, which also contributed to the thickness of the cornea (Figure 2).

Under ultraviolet light, in some places, the anterior epithelium of the cornea appeared detached by Bowman membrane, due to edema liquid storage between epithelium and its basement membrane (Figure 3).

Superficial cells of the epithelium tend to exhibit pseudo-keratinization (Figure 4), while intermediate cells appeared polyhedral, with enlargement of intercellular spaces and desmosomes exhibition. In the central of the cornea, where the spotlight was most intensive, anterior epithelium showed extensive and deep necrotic areas, with lymphatic cells infiltration and overall denudation of Bowman membrane.

Bowman membrane appeared thickened, wavy, with irregular aspect and discontinuous areas, allowing the occurrence of local micro-bleedings (Figures 5 and 6).

Corneal stroma appeared strongly infiltrated with lymphatic and macrophages mononuclear cells. The inflammatory infiltrate was more intensive in anterior third of the corneal stroma and has been correlated with the intensity of the epithelial lesions, in the sense that where the epithelial lesions were at full intensity, the inflammatory infiltrate was very abundant (Figures 7 and 8).

Several vessels of neoformation with size of 4 to 50 μm appeared as well. The vessels were arranged mainly in anterior half of stroma. The blood vessels had their own wall, formed by flattened young epithelial cells, with large oval nucleus, junction each other, arranged on a thin basement membrane. Due to vascular wall disruption, located especially under anterior epithelium, red blood cells extravasation was possible.

To better highlight the newly formed vasculature, we used immunoblotting with CD34 antibody. CD34 is a surface glycophosphoprotein expressed on endothelial progenitor cells. As it can be noticed from our images, the angiogenesis process has been correlated with the intensity of inflammatory infiltrate and epithelial lesions. Neoformation vessels were more numerous where the inflammatory infiltrate was more abundant (Figure 9).

Endothelial cells CD34 are able to synthesize collagen IV, and so new blood vessels wall appeared bounded by a well-defined basement membrane, formed by collagen IV (Figure 10).

Macrophages from corneal inflammatory infiltrate were evaluated using two specific antibodies: CD68 and CD163.

It is known that macrophages complex is formed by a heterogeneous cell population, originating at red bone marrow, from which, by means of blood it is distributed as macrophages in the tissues. Until now, two types of macrophages have been described: CD68, which has preponderant phagocytic activity, and CD163 that elaborate proangiogenic factors. In our study, both CD68 and CD163 had homogenous distribution in stromal cornea (Figures 11 and 12).

It should be noted that the number of CD68+ was much higher than that of macrophages CD163+.

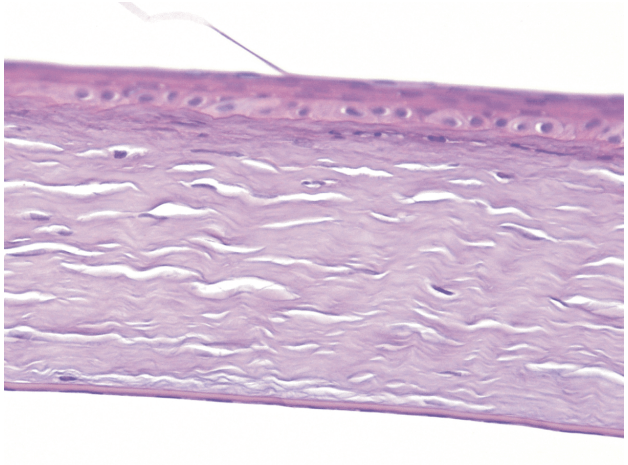

Figure 1 – *Microscopic image of normal cornea (non-irradiated). HE staining, ×200.*

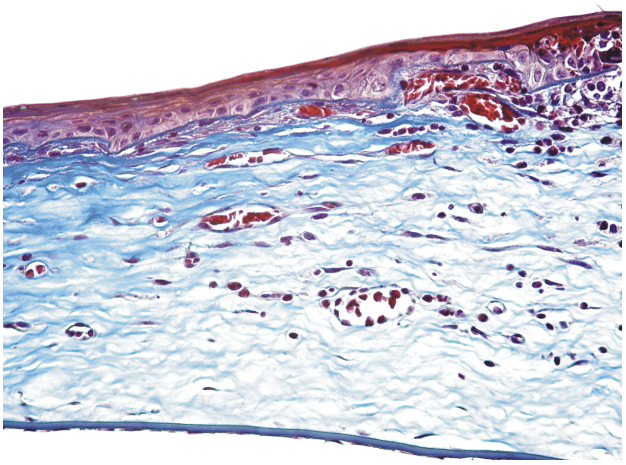

Figure 2 – *Histological image of irradiated cornea showing increase in thickness of the cornea due to accumulation of edema fluid in the stroma, disruption of the collagen fibers, appearance of rounded mononuclear inflammatory cells and angiogenic blood vessels. Goldner–Szekely trichromic staining, ×200.*

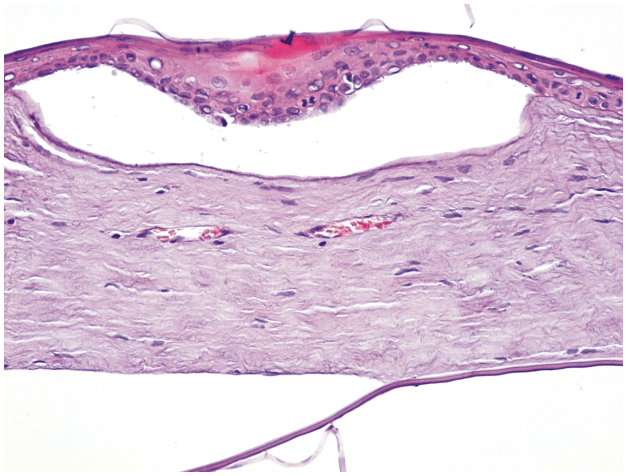

Figure 3 – *Detachment of the Bowman and Descemet membranes. HE staining, ×200.*

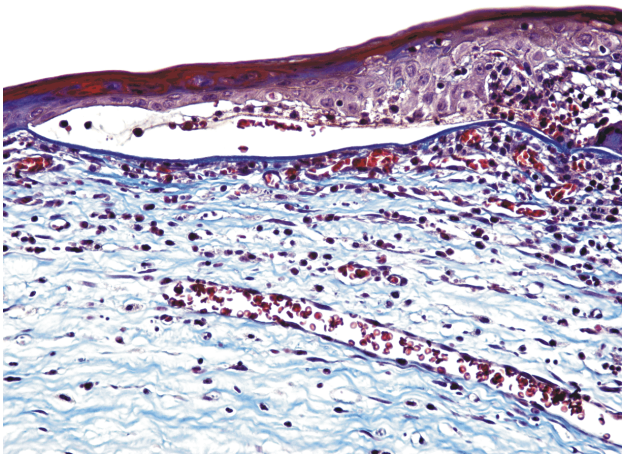

Figure 4 – *Anterior corneal epithelium that tend to pseudokeratinization, separated by a basal membrane. Goldner–Szekely trichromic staining, ×200.*

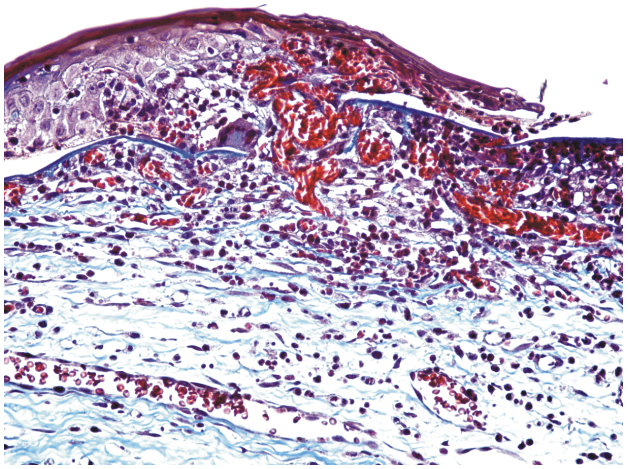

Figure 5 – *Area of deep necrosis, located in anterior corneal epithelium. Goldner–Szekely trichromic staining, ×200.*

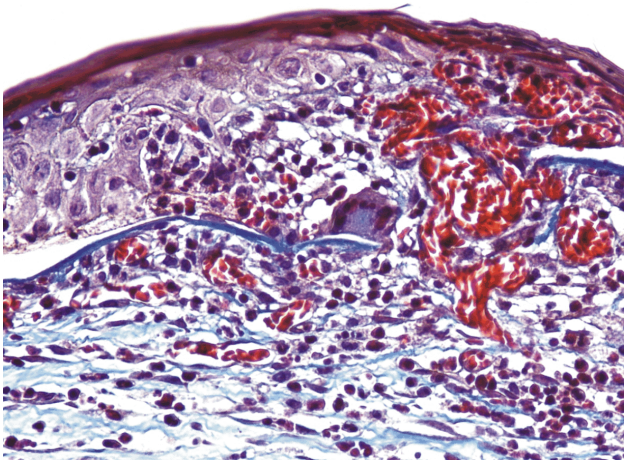

Figure 6 – *Detail from the previous figure, in which one can observe necrosis of the epithelial cells, enlargement of the intracellular spaces, hematic infiltrate and lymphocyte-type cells, disruption of the Bowman membrane with the emergence of local microhemorrhages. Goldner–Szekely trichromic staining, ×400.*

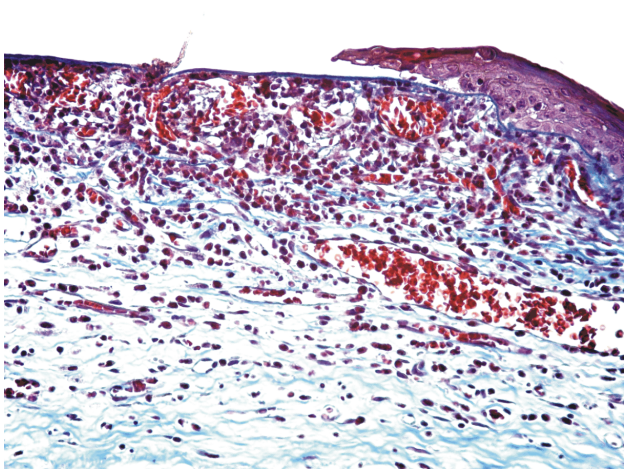

Figure 7 – ***Intensely vascularized stroma of the cornea, infiltrated with mononuclear round cells of lymphocytic and macrophage types. Goldner–Szekely trichromic staining, ×200.***

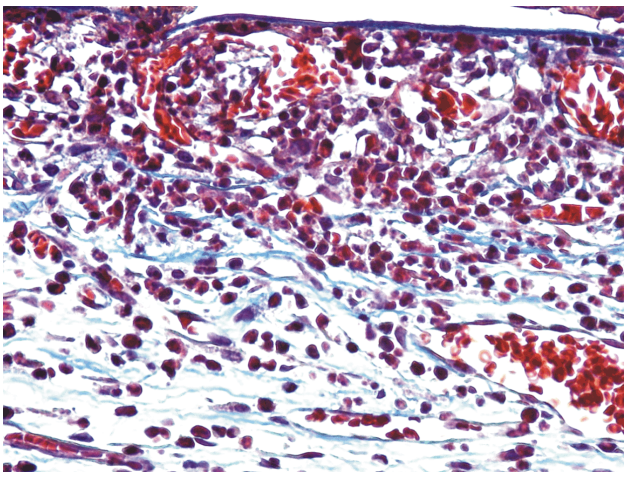

Figure 8 – ***Abundant inflammatory infiltrate of the anterior third of the cornea stroma, where the anterior epithelium was completely necrotized under the UV action (detail from the previous figure). Goldner–Szekely trichromic staining, ×400.***

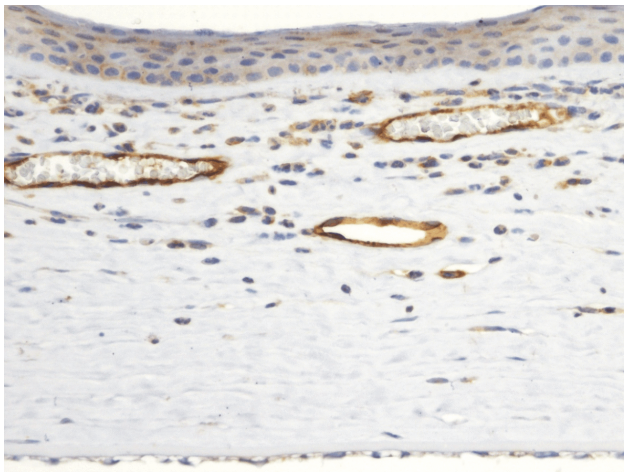

Figure 9 – ***Neoangiogenesis vessels in the anterior half of the cornea stroma, at the periphery of the irradiation area. CD34 immunostaining, ×200.***

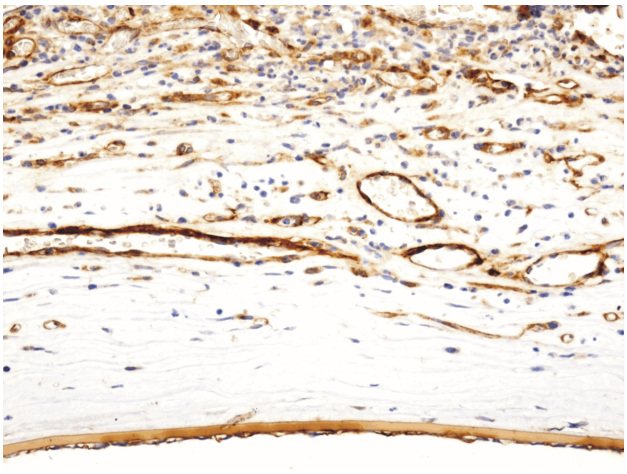

Figure 10 – ***Neoangiogenesis vessels with a well-formed basal membrane formed by collagen IV. Collagen IV immunostaining, ×100.***

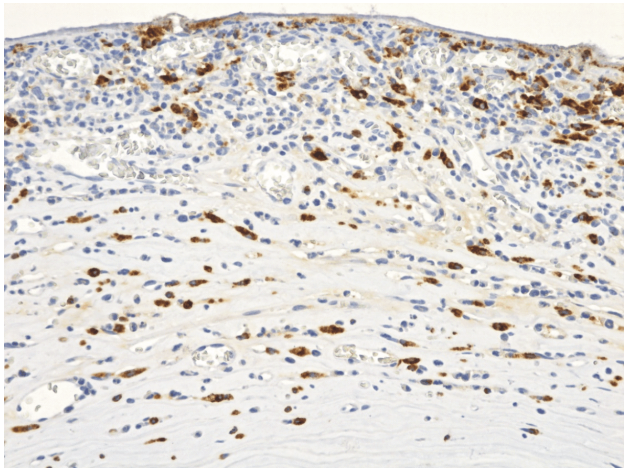

Figure 11 – ***Cornea stroma with cellular infiltrate rich in macrophages. CD68 immunostaining, ×200.***

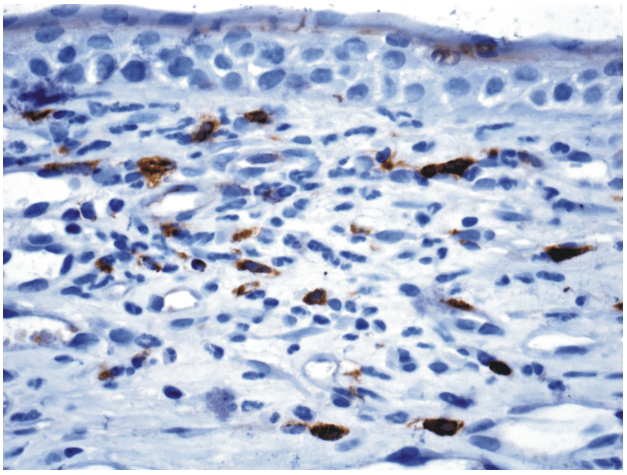

Figure 12 – ***CD163-positive macrophages uniformly distributed within the stroma of the cornea. CD163 immunostaining, ×400.***

Discussion

The cornea is the transparent and avascular structure, which allows the transmission of incident light to posterior ocular structures. It is a structure constantly exposed to a wide spectrum of radiation including UV light [6]. According to some studies, the adverse effects

of UV radiation include corneal stromal thinning, keratoconus, corneal vascularization, fibrosis and keratosis [7, 8].

The best-known effect of acute exposure to UV radiation is photokeratitis, characterized by enhanced apoptosis and exfoliation of the corneal epithelium, the appearance of ulceration, inflammation and edema of the corneal stromal structure, giving a sensation of ocular discomfort. Pathology caused by chronic exposure is varied, comprising numerous corneal and conjunctiva ailments such as pterygium and keratopathy [9–11].

In our study, repeated application of UV radiation in the wavelength range between 290–400 nm, determined necrosis of the anterior basal epithelium membrane and even erosion of the Bowman membrane, corresponding to point ulcerations. In some areas, we found the detachment of the Bowman membrane epithelium with accumulation of edema fluid between the two structures, indicating destruction of the junctional devices between the epithelium and its membrane. The Bowman membrane appeared wavy, thickened, sometimes discontinuous, allowing the migration of lymphocytes and macrophages in the anterior epithelium structure.

Previous studies reported that damage caused by exposure to UV radiation depends on numerous factors, such as the wavelength and the exposure time. Wavelengths below 290 nm are almost completely absorbed by the corneal epithelium and come to the structures located on a more profound layer, while the average UV wavelength between 300–320 nm are absorbed by the cornea and crystalline stroma [12], causing damage to these various structures.

UV rays act directly by phototoxicity or indirectly through free radicals. Among the structures of the eye, the cornea is the most exposed to oxidative stress because it absorbs the largest amount of radiation [13].

Experimental studies have shown that UV radiation and resulted reactive oxygen species produce large morphological changes in the cornea. A single exposure to UV-B radiation, cornea was sufficient to block proliferation of endothelial cells. Higher doses of UV-B lead to a significant reduction in the thickness of the epithelium [14]. Experimental data demonstrated that after exposure to UV, the rate of removal of oxygen from the cornea decreases and the intra-cornean glucose transmission system is inhibited [15, 16]. Following these biochemical changes, exposure to UV causes damage both in the epithelium of the anterior corneal stroma and the endothelium. Microscopic evaluations during an acute photokeratitis showed an immediate reaction of this structure [17]. These data correspond with our observations in which we observed detachment of both the anterior epithelium and the endothelium under the action of UV radiation.

Although being intensively investigated in the last decade, cellular and molecular mechanisms underlying UV-induced lesions [18, 19], are still far from being completely discovered. It appears that UV activates a number of pro-inflammatory molecules such as various interleukins, cytokines and matrix metalloproteinases that are responsible for cell injury. Several studies [20, 21] showed that the UV-B radiation produce a rapid activation of the nuclear factor kappa B (NF-kB), which in turn causes synthesis of cyclooxygenase-2 (COX-2) and nitric oxide synthase (iNOS), key mediators in the recruitment of inflammatory cells and angiogenesis processes [22]. At the level of the irradiated cornea, reactive oxygen species are formed along with NO and other free radicals, being responsible for the molecular and cellular changes [23].

In our study, besides the anterior epithelial lesions, we observed at the level of the stroma the appearance of an intense inflammatory reaction, with numerous lymphocytes and macrophages, increased in anterior part of the corneal stroma. This microscopic aspect shows that most of the UV energy is absorbed by the anterior layers of the cornea. The intensity of the inflammatory response was correlated with the changes in the epithelium, in the sense that where the anterior epithelium was destroyed, inflammation was maximal. Unlike our results, Chen BY *et al.* [22] found in the stroma and in the aqueous humor a number of polymorphonuclear leukocytes, some attached to the endothelium of the cornea of mice irradiated with UV.

Using immunohistochemical techniques, we identified two types of macrophage inflammatory infiltrate: CD68, which plays a major role in phagocytosis, and CD163, with a role in angiogenesis.

The fibrillar device of the stroma of the cornea appeared in most part to be edematous, disorganized, with fragmented collagen fibers and low dyeability, which indicated a change in the biochemical structure of collagen. These changes may arise from the stromal reactive oxygen species, in particular H_2O_2, producing changes in the stromal cell proteins and lipids [24]. Both cells from the anterior, epithelium and those from the stroma undergo degenerative phenomena due to intense oxidative stress and apoptosis caused by UV [25]. These phenomena increase in intensity with the UV dose and time [26].

Like other researchers, we have observed the emergence of angiogenic vessels in the corneal stroma, in particular arranged in the anterior part of the structure. The occurrence of corneal angiogenesis vessels is a matter of debate. There are some studies that state that inflammation is the main factor for the occurrence of angiogenesis stimulator corneal while others disagree, arguing that when injected with bFGF pellets, specific growth factor for angiogenesis, the inflammatory reaction on the histological products is minimal, despite the fact that the newly formed vessels were represented [27, 28]. In our study using the CD34 antibody, blood vessels appeared well defined and were more abundant where the inflammatory reaction was more intense. The vessels were capillary-type, with walls consisting of immature CD34-positive endothelial cells placed on a basement membrane rich in type IV collagen.

The mechanisms involved in corneal vascularization remain unknown. Some hypotheses claim that angiogenesis clearly occurs after episodes of hypoxia by decreasing the oxygen supply. This mechanism explains the appearance of vessels angiogenesis in contact lenses wearers, which prevents oxygen from reaching the corneal epithelium by the mechanical barriers they create. Other theories involve elevated levels of lactic acid, which has the ability to stimulate macrophages, and these in turn, secrete angiogenic factors [29]. In our study, we noted a strong relationship between microvascular density and the intensity of the inflammatory infiltrate.

Conclusions

Ultraviolet radiation causes damage in all of the various corneal structures, depending on the wavelength and exposure time. Upon the anterior epithelium, we recorded pseudokeratinisation ranging from serious injury to its total necrosis, lymphocytic infiltrations and even detachment of the underlying membrane. Bowman membrane appeared thickened, with curled areas of discontinuity. The corneal stroma showed evidence of an inflammatory infiltrate consisting of lymphocytes and macrophage-type cells, the most abundant on the front associated with a number of angiogenic vessels with a structured wall of CD34-positive cells placed on a basal membrane made of collagen IV. We also noted a close relationship between the intensity of inflammatory angiogenesis and vessel density.

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