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Effectiveness of Photodynamic Therapy in the Healing of Corneal Alkali Burn in Rats

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Abstract

In this study, we investigated the effect of photodynamic therapy (PDT) on the healing of corneal alkali burns in rats. The experiment was performed on 50 adult non-linear rats. Depending on the intervention, the animals were divided into 5 equal groups with 10 animals in each group: Group 1 included rats with intact eyes (the control group) and Groups 2 through 5 were experimental groups with experimental alkali burn (EAB). Group 2 consisted of rats subjected to instillation of 0.25% chloramphenicol solution; Group 3 consisted of rats subjected to photodynamic irradiation according to our scheme: 300 mJ (630 nm) for 3 minutes; Group 4 consisted of rats subjected to instillation of methylene blue (MB); Group 5 consisted of rats subjected to instillation of MB with subsequent photodynamic irradiation according to the described scheme.

During all periods of observation, the infiltration of the subcorneal zone was less pronounced in Group 5 than in the other groups and was represented mainly by round cells in the anterior chamber, iris, retina, and ciliary zone. The instillation of MB with subsequent photodynamic irradiation was the most effective in reducing the bacterial contamination

Thus, PDT with the photosensitizer methylene blue, in accordance with the designed exposure mode, provided the epithelialization and bacteriostatic effect during corneal repair after EAB. In conclusion, PDT improves a wound's healing process, which is expressed in the reduction of inflammatory infiltration and the promotion of corneal epithelialization. **(Int J Biomed. 2016;6(2):124-127.)**.

Key Words: ocular surface • experimental alkali burn • photodynamic therapy • methylene blue

Introduction

Ocular chemical burns are the most urgent ophthalmic emergencies. Serious eye burns may destroy the entire corneal epithelium and extend into all eye structures, including conjunctiva, sclera, and vascular tract, which often results in a number of serious complications and adverse outcomes, despite active therapy.[1]

The ocular surface is an integrated system[2] with special features of regeneration after chemical burns; these features are not fully investigated and require experimental and histological studies.[3-5] Despite the variety of therapeutic and surgical treatments, achieving stable epithelialization and preventing scar tissue developing at the site of an injury is often not possible, which leads to disruption of corneal transparency. In this connection, the search for new therapies designed to stimulate cornea restoration and prevent the formation of scar tissue and aglia, leading to blindness, is an urgent task of ophthalmology.[6,7]

In recent years, several studies have demonstrated the effects of PDT in ophthalmology.[3,8-13] PDT employs the different light-sensitizing drugs, termed a photosensitizer, and low intensity visible light which, in the presence of oxygen, combine to produce cytotoxic species. PDT has the advantage of dual selectivity, in that the photosensitizer can be targeted to its destination cell or tissue and, in addition, the illumination can be spatially directed to the lesion. PDT has previously been used to kill pathogenic microorganisms in *vitro*, but its use to treat infections in animal models or patients has not yet been much developed.[14]

In Uzbekistan, a new device for ALT (Vostok) working in a range of 630 nm with a power of 5 watts has been developed and used in surgery, dentistry, and dermatology.[15] Our early experimental studies revealed the optimal and safe dose (300 mJ [630 nm for 3 min]) for PDT in ophthalmology, which did not cause endogenous intoxication.[4,5]

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The objective of this study was to investigate the impact of the developed PDT doses on the processes of corneal epithelialization under an experimental alkali burn (EAB).

Materials and Methods

The experiment was performed on 50 adult non-linear rats weighing about 150 grams, contained in standard vivarium conditions. Eye examinations were conducted on all rats; rats were also subjected to bacterioscopic and histopathological examination.

A corneal alkali burn was generated in the right eye of each anesthetized rat. A piece of filter paper (5-mm diameter) soaked with 2.5% NaOH was applied to the center of the cornea for 5 seconds.[7,16] As photosensitizer was used a 1% methylene blue (MB) aqueous solution (0.2 ml) applied just before irradiation. PDT was performed daily for 7 days using ALT equipment Vostok. Animal condition was monitored three times every day during 7 days post-injury.

Depending on the intervention, the animals were divided into 5 equal groups with 10 animals in each group: Group 1 included rats with intact eyes (the control group) and Groups 2 through 5 were experimental groups with EAB. Group 2 consisted of rats subjected to instillation of 0.25% chloramphenicol solution (2 drops 6 times per day); Group 3 consisted of rats subjected to photodynamic irradiation according to our scheme: 300 mJ (630 nm) for 3 minutes; Group 4 consisted of rats subjected to instillation of MB (2 drops 3 times per day); Group 5 consisted of rats subjected to instillation of MB (2 drops 3 times per day) with subsequent photodynamic irradiation according to the described scheme (300 mJ (630 nm) for 3 minutes). Rats of the control group underwent application of a 0.9% NaCl solution (2 drops 6 times per day) during 7 days.

The study was conducted in accordance with the principles of ARVO Statement for the use of animals in ophthalmic and visual research. Procedures for euthanasia (instant decapitation at the 3rd and 7th day of experiment) were performed according to MMRI Policy for the Humane Care and Use of Laboratory Animals in a manner consistent with the recommendations of the American Veterinary Medical Association (AVMA) Panel on Euthanasia.

Tissues of eye and orbit were fixed in 10% buffered formalin according to Lilly and embedded in paraffin. Sections 4 to 5 microns thick were stained with hematoxylin-eosin. For fluorescence microscopy, the dewaxed sections were stained with a 1% solution of acridine orange prepared on a base of phosphate isotonic buffer.[17]

To study the bacterial flora, the test material was taken with a sterile cotton swab and transported to the laboratory within an hour. The technique of bacteriological examination has been described [18] in the methodological recommendations. All isolated facultative-anaerobic microorganisms were identified up to the genus or species based on cultural, tinctorial, morphological, and biochemical properties. After corneal alkali burn was generated, the studied groups were not different in the number of eyes with bacterial infection (10 eyes in each group). Before treatment, the bacteriological examination revealed the presence the following bacterial pathogens: *St. hemolyticus*, *St. epidermidis*, and *St. aureus*. *St. hemolyticus* was found in most cases (28%).

Light and fluorescence microscopy and photomicrography were performed with a BIOLAM I2 microscope with an MFI-5 photo-attachment and digital camera Canon 300D. Morphometry was performed using AxioVision 4.8.2 software.

Results were statistically processed using the *software* package «Biostatistics» for Windows (version 4.03) and Microsoft Office Excel 2007. Group comparisons with respect to categorical variables are performed using chi-square tests with the Yates' correction or, alternatively, Fisher's exact test when expected cell counts were less than 5. A probability value of $P<0.05$ was considered statistically significant.

Results and Discussion

A day after EAB, all groups exhibited specific changes, which were manifested in a sharp edema of tissues around the wound. The burn zone was represented by detrital mass and infiltrated by neutrophilic leukocytes. Around the burn zone, we observed significant disturbances in the corneal structure: edema, infiltration, activation of keratocytes and neovasculogenesis. A moderate inflammatory response had developed in the sclera; the purulent-fibrinous exudate and polymorphocellular infiltration with a predominance of neutrophils were detected in the anterior chamber of the eye. Dramatically expanded vessels and polymorphocellular infiltration were identified in the ciliary body. On the surface of the retina, we observed a marked purulent-fibrinous exudate; the choroidal zone was also infiltrated by neutrophils. Moderate inflammation (swelling and infiltration) was observed in the conjunctival part of the eyelid. Moderate activation of secretory activity was identified in the lacrimal glands. Clinical manifestations in the form of redness, hypopyon, and edema of the ciliary body, iris and retina decreased in all groups under dynamic observation by the third day and especially by the seventh day, but the moderate leukocyte infiltration remained in Groups 2 and 4.

On the third day after EAB, in Groups 2 and 4, the cornea around the wound was infiltrated by neutrophilic leukocytes, and a bacterioscopic examination revealed the presence of a large number of coccal microflora (Table 1).

Table 1.

The results of bacteriological examination after intervention (n - the number of eyes/%)

Bacterial flora	Group 1	Group 2	Group 3	Group 4	Group 5	Total (n=50)	*P-value*
St.hemolyticus	2/14.3	4/28.6	2/14.3	5/35.8	1/7.1	14/28	0.6261
St.epidermidis	2/18.2	3/27.3	2/18.2	4/36.3	-	11/22	0.5874
St.aureus	1/14.3	3/42.8	1/14.3	2/28.6	-	7/14	0.7902
Mixed flora	3/16.7	5/27.8	3/16.7	5/27.8	2/11.1	18/36	0.8719
Number of eyes with bacterial infections							
n/%	8/47	10/25.6	8/20.5	10/25.6	3/7.7*	39/100	0.0065

*Fisher's exact test: *P-value = 0.003* between Gr. 5 and Gr. 2 and 4

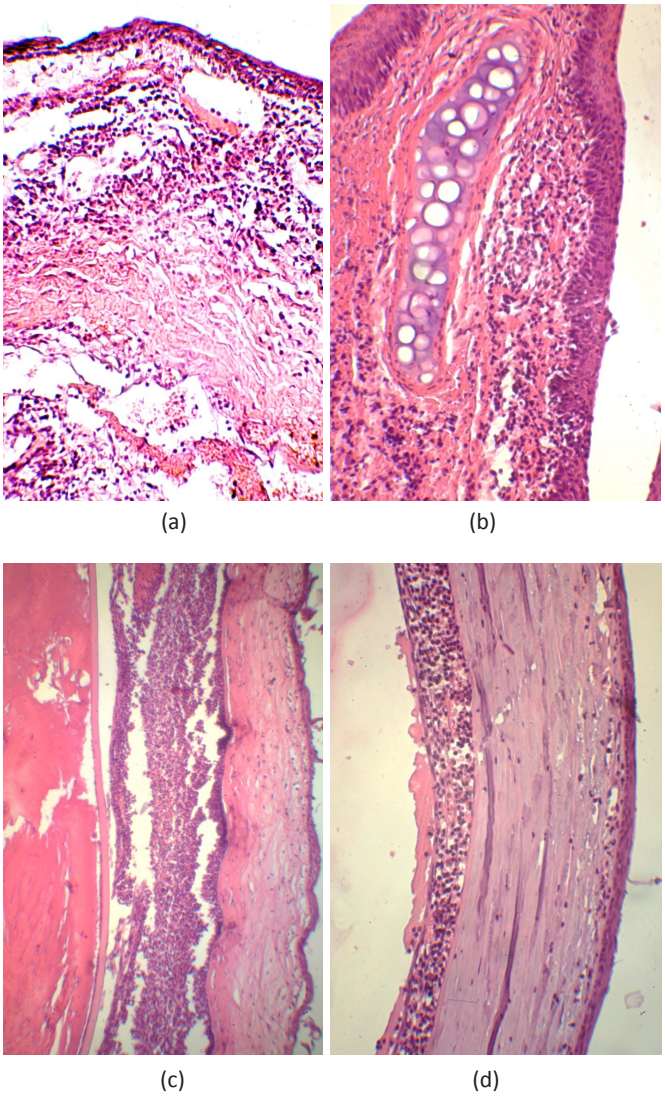

Fig. 1. Effect of PDT on the cornea and the eyelid after EAB in rats *(H&E, 10x20)*

a) Severe edema and infiltration of the cornea in the affected zone (Group 2, the 3rd day)
b) Moderate infiltration of the internal surface of the eyelid (Group 3, the 3rd day)
c) Massive subcorneal infiltration and corneal edema (Group 4, the 7th day)
d) Moderate subcorneal neutrophil infiltration (Group 5, the 7th day)

Polymorphocellular infiltration of the ocular structures, especially a massive accumulation of neutrophils, was observed in the area between the sclera and the vitreous body, as well as on the retina of most animals of these groups. In Group 5, starting from the third day of the experiment we observed a significant decrease in the number of detrital masses and a strong neutrophil infiltration in the affected zone. Around the burn zone on the cornea, as in the other groups, edema and disorders of the general architectonics were defined. However, activation of proliferative processes in the cornea and the degree of epithelialization of the wound surface were more intense in Group 5 (Fig.1).

By the seventh day after EAB, the phenomenon of neutrophil infiltration and bacterial contamination in all groups was significantly lower, but the pronounced edema remained in Groups 2 and 4. According to bacterioscopic examination, a reduction in the degree of bacterial contamination was found in each group with varying degrees of effectiveness. The instillation of MB with subsequent photodynamic irradiation was the most effective in reducing the bacterial contamination (Table 1, Fig.2).

During all periods of observation, the infiltration of the subcorneal zone was less pronounced in Group 5 than in the other groups and was represented mainly by round cells in the anterior chamber, iris, retina, and ciliary zone (Fig.1 and 2).

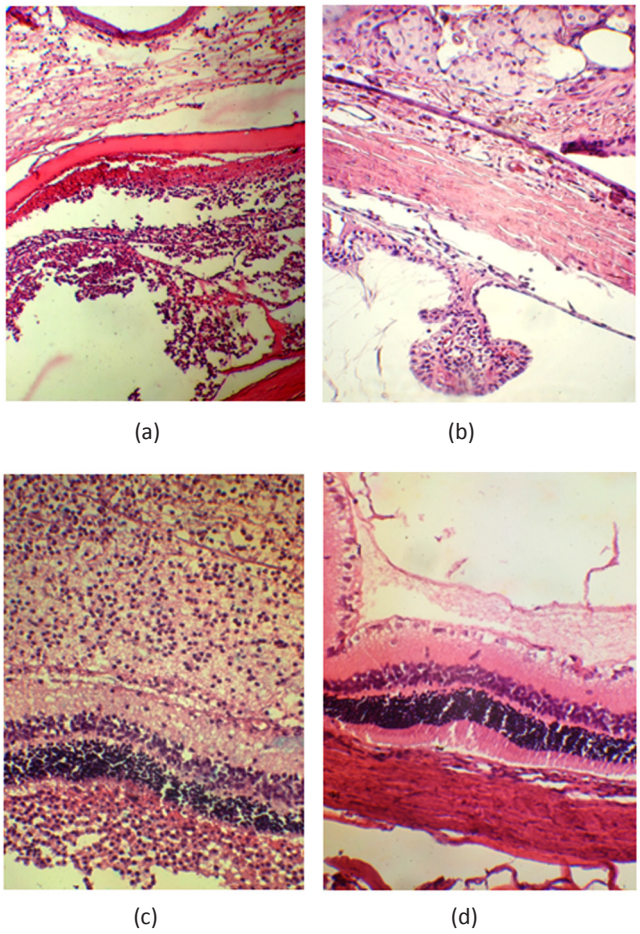

Fig. 2. Effect of PDT on the ciliary body and retina after EAB in rats *(H&E, 10x20)*

a) Severe edema and leukocyte infiltration in the region of the ciliary body (Group 2, the 2nd day)
b) Moderate hyperemia in the region of the ciliary body (Group 2, the 7th day)
c) Total leukocyte infiltration of the retina (Group 4, the 7th day)
d) Leukocyte infiltration is absent (Group 5, the 7th day)

It has been known since the first days of PDT, early in the last century, that certain microorganisms can be killed by the combination of dyes and light *in vitro*[19] In the 1990s, it was observed that there was a fundamental difference in susceptibility to PDT between Gram-positive and Gram-negative bacteria. It was found that, in general, neutral or anionic photosensitizer molecules are efficiently bound to and

photodynamically inactivate Gram-positive bacteria, whereas they are bound, to a greater or lesser extent, only to the outer membrane of Gram-negative cells, but do not inactivate them after illumination. The high susceptibility of Gram-positive species is explained by their physiology.[20]

In recent years interest in the antimicrobial effects of PDT has revived and it has been proposed as a therapy for a large variety of localized infections.[20,21] Burns are particularly susceptible to infection due to the destruction of the cutaneous barrier and the fact that coagulated and denatured proteins present in the burn provide a nutritious environment for bacterial growth. Gram-positive bacteria and in particular *S. aureus* are early colonizers of burn wounds which makes the occurrence of multidrug resistant *S. aureus* a worrisome development.[22,23] Superficial wound infections are potentially suitable for treatment by PDT because of the ready accessibility of these wounds for both topical delivery of the photosensitizer and light. Advantages of PDT include equal killing effectiveness regardless of antibiotic resistance, and a lack of induction of PDT resistance.

Conclusion

PDT with the photosensitizer methylene blue, in accordance with the designed exposure mode, provided the epithelialization and bacteriostatic effect during corneal repair after EAB. PDT improves a wound's healing process, which is expressed in the reduction of inflammatory infiltration and the promotion of corneal epithelialization.

Competing interests

The authors declare that they have no competing interests.

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