EXPERIMENTAL METHODS FOR CLINICAL PRACTICE

Antioxidant Activity of Tear Fluid in Experimental Alkali Eye Burns

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Antioxidant activity of the tear fluid in intact rabbits and after alkali burn of the cornea was evaluated by measuring chemiluminescence in a hemoglobin—hydrogen peroxide—luminol system. After alkali burn of the cornea antioxidant activity of tear fluid decreased starting from day 3 and reached its minimum on days 7-21. This decrease correlated with the depth of ulceration of the corneal stroma. This method allow to specify indications for antioxidant therapy in corneal disease and to choose the most effective antioxidants.

Key words: *tear fluid; antioxidant activity; chemiluminescence; alkali burn of the cornea*

Studies of tear fluid is a highly informative noninvasive method used in ophthalmology. Tears contain biologically active components and play an important role in the protection of anterior eye structures. Tear antioxidants are an essential part of this defensive system which protects the surface eye structures against free radical oxidation (FRO) under normal and pathological conditions. Published data suggest that, tear fluid contains a variety of antioxidants: ascorbic acid (3.9-23 mg/dl) [9], non-protein SH-groups, γ-glutamyltranspeptidase [6], glutathione, glutathione reductase, Cu, Zn superoxide dismutase (SOD) (103 ± 32 ng/ml soluble protein) [8], Ca^{2+} and lactoferrin [3], as well as urate, albumin, and ceruloplasmin.

Damage and inflammation in the cornea are accompanied by massive release of oxidants and free oxygen radicals into the tear fluid from corneal and conjunctival cells and activated neutrophils and macrophages migrating into the tear fluid during inflammation. Antioxidant activity (AOA) of tears is characterized by inhibition of FRO of various substrates. Natural and synthetic antioxidants are widely used for the treatment of various corneal diseases. However, little is known about antioxidation potency of tears under different pathological conditions. For instance, chemiluminescence (ChL) methods revealed reduced AOA of the tear fluid in patients with pathological myopia, diabetes, and open angle glaucoma.

ChL is a direct method for studying free radicals and relevant reactions [2]. It is widely used for evaluation of the functional state and production of active oxygen species by blood and tissue phagocytes responsible for their protective function. In the present study we investigated AOA of the tear fluid by the kinetics of luminol-activated chemoluminiscence in a hemoglobin—hydrogen peroxide—luminol system (Hb—H_2O_2—Lm) [4,5]. In our experimental model, chemoluminiscence is emitted during oxidation of luminol by free radicals generated in the presence of hemoglobin and hydrogen peroxide. The resultant AOA in the system of Hb—H_2O_2—Lm depends on contributions of different hydrophilic antioxidant components of the tear and their interactions.

Our aim was to study AOA of rabbit tear fluid in a Hb—H_2O_2—Lm system under normal conditions and in the dynamics of corneal burn diseases caused by alkali.

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MATERIALS AND METHODS

Experiments were performed on 10 Chinchilla rabbits (20 eyes) weighing 2.5-3.0 kg. Alkali burn of the cornea (stage III burn of standard area and depth) was produced by applying 7-mm cotton disks soaked with 10% NaOH on the central area of the cornea. After 40 sec the disks were removed, and the eye was rinsed with 20 ml physiological saline. Alkali burns were inflicted against the background of subcutaneous anesthesia with 2.5% chlorpromazine and 0.5% diazepam (1:1) in a volume of 0.5 ml/kg body weight and local instillation of 0.5% tetracaine.

Tear fluid was collected from intact animals and on days 1, 3, 7, 14, 21, and 28 after corneal injury. The fluid was collected with filter paper disks placed for 5 min into the conjunctival sac. Tear components were eluted with physiological saline and centrifuged to remove insoluble components, the supernatant was stored before tests.

Examination of rabbit eyes was performed on days 3, 7, 14, 21, and 28 after injury using a binocular microscope. Conjunctival (eyelid edema, hyperemia, and discharge) and corneal symptoms (injection and neovascularization assessed by the length and density of newly formed vessels penetrating the cornea, as well as infiltration, total area and depth of erosions) were scored. The presence of exudate in the anterior eye chamber was determined. The state of the cornea was assessed using 0.5% fluorescein which positively stains deepithelialized regions.

Tear AOA was determined by measuring ChL kinetics in the Hb—H_2O_2—Lm model system [5]. Reaction medium (500 µl) contained 0.3 µM Hb and 10 µM luminol in phosphate buffer (50 mM KH_2PO_4, 100 µM EDTA, pH 7.4). Luminol-dependent FRO was stimulated with hydrogen peroxide (30 µM). ChL was measured on a Biotoks-7 chemiluminometer (Energiya, Moscow) connected to a personal computer. The latency of ChL, i. e. the period from initiation of FRO to appearance of ChL, was determined. AOA of tear eluate was calculated as the ratio of ChL latencies determined in the presence of tear (T) and in the model system (T_0): $AOA=T/T_0$.

Hemoglobin (Sigma) and luminol (ICN) were used.

RESULTS

First, the effect of tear eluate on the kinetics of ChL in the Hb—H_2O_2—Lm system was studied. Typical ChL kinetic curves in the absence and presence of tear eluate (200 µl) are presented in Fig. 1. Addition of tear eluate into the system prolonged the latency of ChL compared with the control and this increase directly correlated with the volume of added eluate (Fig. 1,

insert). This effect of tears was similar to that of the serum and main serum antioxidants on this model system [4]. Therefore, the latency of ChL may be used as a characteristic of AOA in the tear fluid.

The cornea represents a vessel-free structure, which consists primarily of the connective tissue (stroma) with layers of collagen fibers. The distance between these fibers determines transparency of the cornea, while distortions can cause its opacity. The stroma is coated with Bowman's membrane with stratified epithelium (anterior) and Descemet's membrane (posterior) with single-layer epithelium (corneal endothelium). Alkali burn caused immediate deepithelialization of the injured area and corneal edema which decreases only on days 10-14 after injury. Within 2-3 days after injury the deepithelialized surface was usually covered with new epithelium and blood vessels grow into the cornea from the limb. Rejection of newly formed epithelium and ulceration of the corneal stroma led to deep ulcers, which were formed on days 14-21 after burn and sometimes reached the internal basement membrane culminating in descemetocele and perforation of the cornea. Twenty-one days after burn, a scar formed on the injured surface and the vessels were emptied. In most cases, the injured cornea was totally covered with epithelium by day 28. The state of the cornea (erosion area and depth of ulceration) was scored according to a 4-point scale.

We found that despite standard procedure of burn modeling, the dynamics of healing was different in different animals and even in different eyes of the
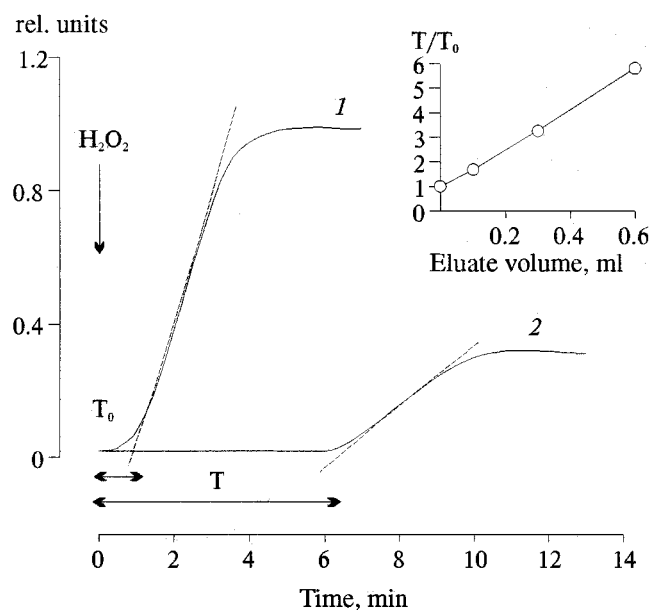

Fig. 1. Intensity of chemiluminescence in the absence (1) and presence (2) of tear eluate in the system of hemoglobin—hydrogen peroxide—luminol. Insert: chemiluminescence latency as a function of added volume of tear eluate.
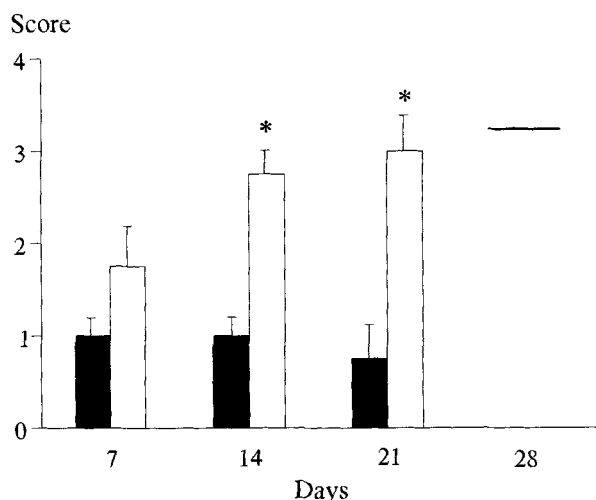
Score

Fig. 2. Depth of corneal ulceration during favorable (black bars) and unfavorable (open bars) course of burn disease. *$p<0.05$ compared with favorable course.
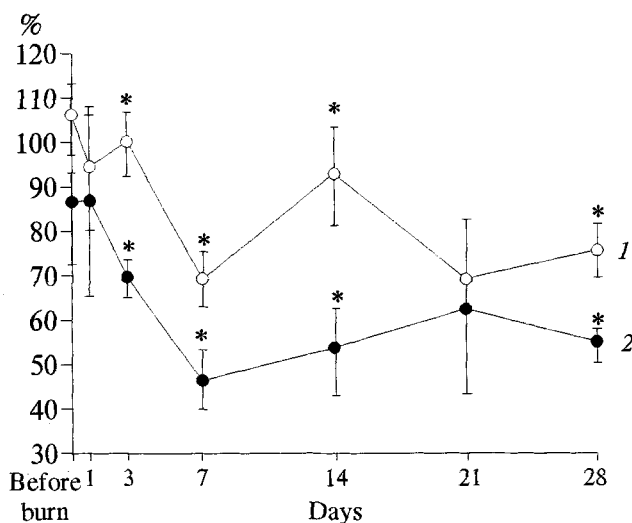

Fig. 3. Antioxidant activity in tear fluid after alkali burn of the cornea in groups with favorable (1) and unfavorable (2) course of eye burn disease. *$p<0.05$ compared with the control (100%).

velopment of deep ulcers. Minimum AOA in groups 1 and 2 (69.0 ± 0.5 and $46.3\pm6.7\%$, respectively) was observed on days 21 and 7, respectively. It should be noted that during reepithelization and formation of scar tissue (day 28) AOA in both groups remained at a low level ($75.5\pm7.0\%$ and $54.9\pm4.9\%$ in groups 1 and 2, respectively).

There was a positive correlation between AOA of tears and the severity of pathological process. Moreover, the marked drop of total AOA preceded deep destructive changes in the cornea.

When considering the mechanism responsible for reduced AOA of the tear fluid after injury one can assume that breakdown products of damaged collagen stimulate migration of polymorphonuclear leukocytes (PNL) into the limbic vessels [10] and then into the peripheral cornea and tear fluid. Migration of PNL into the inflammation focus can be due to the development of the delayed type hypersensitivity response 2-3 weeks after alkali burn. Cytokines released by T cells sensitized to eye antigens possess chemotactic properties and induce migration of neutrophils and macrophages [7]. Activation of PNL in the cornea and tear fluid is accompanied by their degranulation and release of proteolytic enzymes into the interstitial medium. First, PNL, the major cell fraction of inflammatory infiltrate, contain a complex of proteolytic enzymes (including collagenolytic) and play an important role in destruction of the connective tissue. Second, activation of neutrophils is accompanied by a respiratory burst, oxygen consumption sharply increased, superoxide anion radical is generated and induces the formation of various toxic derivatives (hydrogen peroxide, hydroxyl, and hypochlorite) [2]. These substances induce lysis of biological membranes and activate lipid peroxidation. Antioxidants of the tear fluid participate in neutralization of these active oxygen species. Third, oxidants produced by activated PNL can oxidize methionine-rich active centers of antiproteinase, in particular, of $\alpha 1$-proteinase inhibitor which controls proteolytic enzyme activity during inflammation [1]. Activation of proteolysis can aggravate corneal damage.

Thus, intensification of destructive processes and activation of FRO on week 2 after alkali burn of the cornea are accompanied by intense utilization of tear fluid antioxidants. The intensity of all these processes varies and can be different in eyes of the same animal, which emphasizes the role of local processes in the metabolism of the eye. Thus, for ophthalmopathological purposes tear fluid tests are more informative then routine blood serum tests.

same animal. We subdivided all examined eyes into two groups: with favorable (group 1) and unfavorable (group 2) course of the disease basing on the state of the cornea (Fig. 2). Thus, group 1 included eyes with moderate, while group 2 with deep corneal ulceration involving Descemet's membrane and eyes with corneal perforation.

In both groups antioxidant potency of tear fluid decreased after injury (Fig. 3). However, in group 1 (with favorable course of the process) AOA slightly decreases only on day 3 after injury and on day 7 reached $69.1\pm6.0\%$ of the control value. In the case of unfavorable dynamics (group 2), AOA of the tear fluid on days 3 and 7 after burn constituted $69.5\pm5.0\%$ and $46.3\pm6.7\%$ of the control, respectively. This more pronounced drop of AOA in group 2 preceded the de-

The proposed method allows to evaluate the degree of tear antioxidant deficiency in the tear fluid under different pathological conditions and its dynamics

during treatment, and improve the efficacy of antioxidant therapy by choosing the most potent antioxidants.

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