Study of Wound-Healing Activity of Bioregulators Isolated from Eye Tissues and Bovine Serum in the Model of Experimental Corneal Injury in Rabbits *In Vivo*

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 153, No. 2, pp. 177-182, February, 2012
Original article submitted October 25, 2010

We compared wound-healing activity of bioregulators isolated from cattle cornea, serum, and retinal pigment epithelium on *in vivo* model of experimental corneal injury in rabbits. Bioregulators were instilled into the eye as solutions at a concentration corresponding to 10^{-12} mg protein/ml. The animals were sacrificed on day 21 after injury and the corneas were examined histologically. The best wound-healing effect was produced by bioregulators isolated from the cornea and serum and instilled successively into rabbit eyes with an interval of 15-20 min twice a day: multicellular epithelium was observed in the wound, and slight inflammation, in the stroma.

Key Words: *bioregulators; injury; cornea; rabbit; wound healing*

Treatment of corneal injuries in humans and animals is still a pressing problem. Some ophthalmic drugs recommended as wound-healing remedies in corneal injuries are little effective, especially in lesions involving deep stroma. Bioregulators belong to a new group of bioactive substances previously found in animal tissues [6,7,10,12,14,15]. Bioregulators of this group are localized extracellularly, have a complex structure, and contain proteins, carbohydrates, and lipids [6]. Their activity is determined by the protein component [11]. Bioregulators of this group modulate adhesion, migration, differentiation, and cell proliferation in the original tissues. In low doses, bioregulators promote tissue recovery and repair [12,14,15]. It was shown that bioregulator isolated from bovine cornea exerted a pronounced protective effect on *in vitro* cultured vertebrate cornea via additional activation of cellular regeneration sources [3,4,9,12]. Bioregulator isolated from the serum stimulated wound healing in rabbit cornea in *in vivo* experiment [1] as well as in humans with posttraumatic recurrent corneal erosions and in corneal burn [5]. Bioregulator isolated from bovine retinal pigment epithelium promoted stability of differentiation of pigment epithelial cells and protected Muller and bipolar cells in organotypic culture of eye tissues *in vitro* [2,8].

Here we compared the effects of bioregulators isolated from cornea and pigment epithelium of the eye and blood serum of cattle on healing of experimental corneal wounds in rabbits *in vivo*.

MATERIALS AND METHODS

The study was conducted on 21 male Chinchilla rabbits (body weight ~1.5 kg) kept under standard vivarium conditions (Institute of Developmental Biology).

Serum bioregulator was isolated from sterile inactivated cattle blood serum for cell culturing (M. P. Chumakov Institute of Poliomyelitis and Viral Encephalitides, Russian Academy of Medical Sciences).

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To isolate the bioregulators from eye tissues, freshly enucleated eyes of young bulls were used (slaughterhouse material of meat processing plants in Moscow and Moscow region).

Bioregulators were isolated using a previously developed isolation and purification procedure consisting of obtaining tissue extract, precipitation of protein impurities by saturated ammonium sulfate solution, and isoelectric focusing of supernatant fraction in a sucrose gradient in pH range of 3.5-10.0 [10]. We studied fractions of acid proteins of the serum and cornea collected at pH 1.5-3.0 and alkaline fraction of pigment epithelium collected at pH 8.5-9.5 [2,12].

During purification, the presence of bioregulators in fractions was verified by the adhesiometric method based on assessment of their membranotropic activity [10]. Bioregulators were tested in a concentration corresponding to 10^{-12} mg of protein/ml obtained by successive 10-fold dilutions of the stock solution. Protein concentration in the initial fractions of bioregulators was determined by the colorimetric method [13].

Physiological saline and Baralpan-N (eye drops used for the treatment of posttraumatic and surgical corneal lesions, Eye Microsurgery Reseach and Technology Complex) were used as additional controls.

The rabbits divided into groups (3 animals per group) received bioregulators isolated from bovine serum (group 1), bovine cornea (group 2), bovine pigment epithelium (group 3), mixture of bioregulators from bovine cornea and serum (group 4), successive instillations of bioregulators from bovine cornea and serum (time interval 15-20 min, group 5), and Baralpan-N (group 6).

In each animal, one eye (experimental) was treated with the test preparation and the other served as the control (saline instillation).

All preparations and saline were instilled for 21 days: two drops in each eye two times a day with 8-h interval.

The animals were intranasally anesthetized with Zoletil 5 mg/kg and Xylazine 2 mg/kg. Epibulbar anesthesia was also carried out with three-fold instillation of inocaine solution (0.4% oxybuprocaine). The lesion (diameter 8.5 mm, depth 0.8 mm) was made with a trephine: incision was made, and then the edge was pulled out with forceps. The cornea was delaminated with scissors and cut circularly. Instillations were started immediately after the operation.

Rabbit eyes were clinically examined on day 10 after experimental injury. The duration of examination was 60 sec.

Corneal epithelization was evaluated by fluorescein test using sterile disposable paper strips with fluorescein (Haag-Streit AG).

Tear production in the postoperative period was assessed by the Schirmer test: tear production over 60 sec was measured using a special disposable test strips (Buch and Lomb) placed under rabbit's eyelid (the length of soaked strip is normally 7 mm).

The intraocular pressure was measured with an automatic tonometer (Tonovet; Tiolat).

The rabbits were sacrificed by air embolism on day 21 after injury. For histological studies, the cornea was isolated, fixed in Bouin's fixative and 4% formalin, and embedded in paraffin; sections (7 μ) were stained with hematoxylin and eosin.

RESULTS

Clinical picture of corneal healing in rabbits. On the 10th postoperative day, the following results were obtained.

In group 1 rabbits receiving bioregulator isolated from the serum, the pupil was contracted and significant stromal corneal edema and pronounced pericorneal vascular injection in the hyperemic conjunctiva were seen. Map-like area of corneal erosion (3.5-5 mm) was diffusely stained with fluorescein. Uveitis, corneomalacia foci, and early epithelialization were observed; anterior chamber was edematous.

In group 2 animals receiving bioregulator isolated from bovine cornea, less pronounced inflammation was observed. The conjunctiva was pale pink, the cornea in the wound area was clear, bright, without pronounced edema. A slight opacity in the form of veil was seen in the wound; astigmatism was not expressed. Punctate staining of the cornea was observed; epithelialization was almost completed, with sites of pathological epithelialization <0.5 mm.

In group 3 rabbits receiving bioregulator isolated from pigment epithelium, the conjunctiva was pale pink, the cornea was transparent with weak diffuse edema, a scar of medium intensity and areas of impaired epithelialization (3-4 mm) and mixed vascularity were seen in the wound area.

In group 4 rabbits receiving the mixture of bioregulators, significant stromal edema of the cornea stretching beyond the wound was observed; the conjunctiva was pale pink; the cornea was transparent. Slight opacity in the form of a cloud, superficial vascularization of the cornea, incomplete epithelialization of the cornea (multiple separate fluorescein-stained areas <1-1.5 mm) were noted.

In group 5 rabbits receiving successive instillations of bioregulators isolated from bovine cornea and serum, moderate diffuse corneal stromal edema was observed; the conjunctiva was pale pink. A scar of medium thickness and punctate fluorescein spots were seen.

In group 6 rabbits receiving Balarpan-N, pericorneal vascular injection in the conjunctiva, local swelling of the cornea in the wound, and pronounced scar in center of the wound were detected. The cornea was transparent, superficially stained with fluorescein along the defect perimeter.

All eyes treated with saline (control eyes) had pale pink conjunctiva and slightly transparent cornea with pronounced swelling. In some cases, a pronounced scar in the center of the wound, stromal edema in the inner part of the wound, pericorneal vascular injection, superficial vascularization of the cornea, and foci of corneomalacia (purulent destruction of the cornea, <1 mm) were noted. Considerable fluorescein staining of the cornea (5-6 mm) and small amounts of pathological epithelium were recorded.

The results of evaluation of tear production (Schirmer's test) before the operation and on experimental day 10 were similar (5.5±2.0 mm/min) and corresponded to normal.

The test drugs had no effect on intraocular pressure; it was 10±2.3 mm Hg and corresponded to normal.

Results of histological assay. In eyes treated with saline (control eyes), detachment of the epithelium, sometimes its total absence due to degradation, and inflammation of the stroma were observed in the cornea. In some cases, significant accumulation of inflammatory cells in the stroma was seen (Fig. 1, *a*).

Application of bioregulator isolated from the serum (group 1) led to slight thickening of the cornea, well-defined stratified epithelium was seen; somewhere in the wound area exfoliation of the epithelium, accumulation of inflammatory cells in the stroma, and vascularization were noted (Fig. 1, *b*).

Under the influence of the bioregulator isolated from bovine cornea (group 2), restoration of the epithelium, slight inflammation under the epithelium in the wound area, and impairment of epithelial adhesion to the stroma were observed (Fig. 1, *c*).

In eyes treated with bioregulator isolated from pigment epithelium (group 3), severe inflammation and thickening of the stroma and degradation of the epithelium in the wound area were seen (Fig. 1, *d*).

In eyes treated with the mixture of bioregulators isolated from the cornea and serum (group 4), degradation and epithelial detachment in the wound area occurred. The stroma was inflamed and vascularized. Considerable impairment of adhesive interactions between the cells and layers of the cornea is worthy of note (Fig. 1, *e*).

The eyes treated by successive instillation bioregulators isolated from bovine cornea and serum (group 5) had normal stratified epithelium. Minor inflammation of the stroma was observed only in the wound area. Small foci of epithelium detachment were somewhere seen. Tissue structure in the area of injury was restored, which manifested in not only formation of all corneal layers, but also adhesive interactions between them (Fig. 1, *f*).

In eyes treated with Baralpan-N (group 6), epithelium detachment and inflammation in the injured stroma were detected. Adhesive interactions between the corneal cells and layers were not fully restored (Fig. 1, *g*).

The results obtained in this study suggest that bioregulators isolated from bovine eye tissues and blood serum can promote healing of corneal wound in rabbits *in vivo*. However, the wound-healing effect of bioregulators is clearly different.

In group 2, small stromal inflammation was observed; epithelium was well developed despite loosening of its adhesion to the stroma. These data are consistent with the results of studies of this regulator on organotypic culture of vertebrate cornea *in vitro* demonstrating a protective effect of the regulator on the tissue: it modulated migration and proliferation of epithelial cells and prevented hydration and swelling of the stroma [12].

In group 1, severe inflammation of the stroma and preserved epithelium and epithelial-stromal interactions (lack of detachment) were noted. This bioregulator apparently exhibits properties of an adhesion factor. These data are consistent with the results of earlier studies, where the capacity of this bioregulator to modulate adhesive interactions between cells, especially corneal endothelium was demonstrated [1]. Both bioregulators stimulate corneal wound healing more effectively than Baralpan-N.

Principally different results obtained by combined application of these two bioregulators are worthy of note. Bioregulator mixture produced no wound-healing effects. The state of the cornea in this group did not differ from that that in the control. At the same time, successive instillation of these bioregulators had the most pronounced effect on wound healing. Such a difference in the biological action of two bioregulators (each of them stimulated wound healing in the cornea in individual application) can be explained by changes in nanosized state of bioregulators in the solution caused by their interaction [11]. It has been previously shown that activity of bioregulators of this group was determined by their nanosize condition in solutions [11]. It can be hypothesized that bioregulators after mixing formed nanoparticles with new properties. In case of successive instillation of bioregulators, their effects somewhat complemented each other. Bioregulator isolated from the cornea stimulated epithelial cells and bioregulator isolated from the serum affected the endothelium and

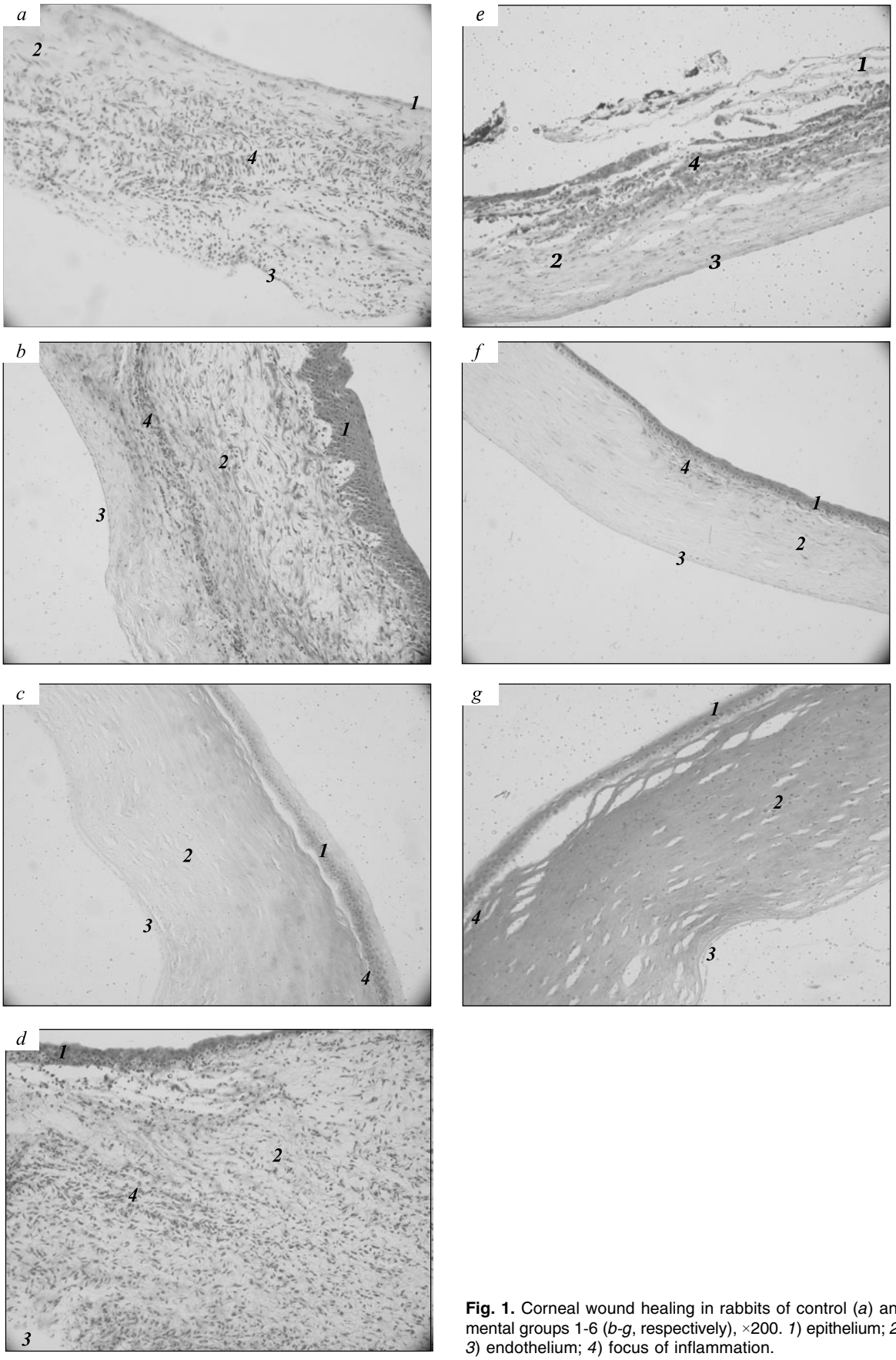

Fig. 1. Corneal wound healing in rabbits of control (*a*) and experimental groups 1-6 (*b-g*, respectively), ×200. *1*) epithelium; *2*) stroma; *3*) endothelium; *4*) focus of inflammation.

cell elements of the stroma and modulated adhesion between the corneal layers [5,12].

Bioregulator isolated from bovine pigment epithelium showed no pronounced wound-healing effects comparison with the two others. This can be obviously explained by the fact that the effect of this group of bioregulators is tissue-specific [2]. These results are of great importance for the development of pharmacological agents based on bioregulators of this group, in particular, those isolated from bovine cornea and serum.

Bioregulators isolated from bovine eye tissues and serum influenced corneal wound healing in rabbits *in vivo*. Successive instillations of preparations isolated from cornea and serum produced the most pronounced effect. This was confirmed by clinical examination on day 10 (fluorescein test) and histological examination of cornea on day 21 after injury. In this case, restoration of the corneal structure was observed in the experimental wounds.

REFERENCES

1. R. A. Gundorova, I. P. Khoroshilova-Maslova, E. V. Chentsova, *et al*., *Vopr. Oftal'mol*., **113**, No. 2, 12-15 (1997).
2. M. S. Krasnov, E. N. Grigoryan, and V. P. Yamskova, *Izv. Ross. Akad. Nauk. Ser. Biol*., No. 1, 22-36 (2003).
3. D. V. Margasyuk, M. S. Krasnov, V. P. Yamskova, *et al*., *Oftal'mologiya*, **2**, No. 3, 81-87 (2005).
4. D. V. Margasyuk, M. S. Krasnov, I. A. Yamskov, and V. P. Yamskova, *Izv. Ross. Akad. Nauk. Ser. Biol*., No. 6, 736-745 (2008).
5. I. Yu. Romanova, R. A. Gundorova, E. V. Chentsova, and V. P. Yamskova, *Byull. Eksp. Biol. Med*., **138**, No. 11, 505-507 (2004).
6. I. A. Yamskov, I. V. Blagodatskikh, M. S. Krasnov, *et al*., *Izv. Ross. Akad. Nauk. Ser. Khim*., No. 3, 623-628 (2009).
7 I. A. Yamskov, V. P. Yamskova, A. N. Danilenko, *et al*., *Ros. Khim. Zh.*, **43**, No. 5, 34-39 (1999).
8 V. P. Yamskova, M. S. Krasnov, D. V. Margasyuk, and I. A. Yamskov, *Izv. Ross. Akad. Nauk. Ser. Biol*., No. 1, 11-17 (2009).
9. V. P. Yamskova, M. S. Krasnov, and I. A. Yamskov, *Klet. Tekhn. Biol. Med.,* No. 1, 32-35 (2010).
10. V. P. Yamskova and M. M. Reznikova, *Zh. Obshch. Biol*., **52**, No. 2, 181-191 (1991).
11. V. P. Yamskova, V. S. Skripnikova, A. A. Molyavka, *et al*., *Biokhimiya*, **74**, No. 9, 1195-1203 (2009).
12. D. V. Margasyuk, M. S. Krasnov, I. V. Blagodatskikh, *et al*., *Biochemical Physics Frontal Research*, Eds. S. D. Varfolomeev, *et al*., New York (2007), pp. 47-59.
13. P. K. Smith, R. I. Krohn, G. T. Hermanson, *et al*., *Biochem*., **150**, 76-85 (1985).
14. V. P. Yamskova, M. S. Krasnov, E.Yu. Rybakova, *et al*., *Biochemical Physics Frontal Research*, Eds. S. D. Varfolomeev *et al*., New York (2007), pp. 71-78.
15. V. P. Yamskova, E. Yu. Rybakova, V. V. Vecherkin, *et al*., *Biochemical Physics Frontal Research*, Eds. S. D. Varfolomeev *et al*., New York (2007), pp. 61-69.