Acta Veterinaria (Beograd), Vol. 56, No. 1, 91-100, 2006.

UDK 619:617.711:617.7-089.243

PREVENTION OF STROMAL CELL LOSS AFTER SUPERFITIAL CORNEAL INJURY IN RABBITS

MISAILOVIĆ KATARINA, RISOVIĆ DUŠICA and DUJIĆ MIRJANA

Clinical Centre Zvezdara, Beograd

(Received 25. April 2005)

For the study of the effects of trauma on the cornea, usually a rabbit eye is used as a model. The aim of this experimental study of corneal surface injury was to evaluate stromal cell loss after epithelial scraping and to indicate the possibilities of preventing it by applying contact lenses or topical heparin.

The study was conducted on 21 Chinchilla gray rabbits, which were divided into five groups. The central portion measuring 6mm of corneal epithelium was removed from one eye of 18 experimental animals. Three rabbits in the fifth group, with intact corneas, served as controls. Postoperatively, the first experimental group (n=3) was treated for 24 hours with topically applied heparin. The second group (n=3) was treated for seven days with subconjunctivally applied heparin. In the third group, (n=6) soft contact lenses were placed for five days. The fourth group of (n=6), has not been treated after injury.

During the first postoperative day, anterior stromal keratocytes were lost after epithelial removal, but the cell repopulation was completed within two weeks. Twenty four hours after deepithelization the corneas showed decreased density of keratocytes within the anterior stroma compared with the posterior storma.

Topically applied heparin for 24 hours after deepithelization prevented keratocyte loss (p<0.05). Subconjunctivally applied heparin for 7 days after deepithelization did not show a statistically significant influence on the number of keratocytes (p>0.05). The eyes treated with contact lenses had a significantly higher number of keratocytes than the control corneas and corneas treated with heparin.

We were able to conclude that topical application of heparin during the first day and contact lens application during the first week, can minimize keratocyte loss and may thus be beneficial in the healing process.

Key words: cornea, keratocyte, heparin, contact lens

INTRODUCTION

The rabbit eye is the most convenient animal model for studying the effects of trauma on the corneal epithelium because it is easy to manage.

The cornea consists of three distinct cell types (epithelium, keratocytes, and endothelium) and an extracellular matrix comprised of collagen and glycosaminoglycans.

After full thickness wounding of the epithelium, glycoprotein fibronectin is deposited on the denuded area and polimorphonuclear neutrophils begin to debride cellular remnants. After approximately 6 hours the adjacent epithelium slides to cover the defect. When the defect is closed, cell movement stops and proliferation begins, reaching the peak between 24 and 48 hours after wounding. New basement membrane complex reforms 5 to 7 days after injury (Campos *et al*., 1992, Dua *et al*., 1994).

Initially, after deepithelization, the anterior stroma beneath the injured area is hypocellular due to keratocyte loss, with fewer than normal anterior stromal keratocytes after 24 hours (Song and Joo, 2004). Within a few days, following the closure of the epithelial defect, keratocytes begin to transforme into activated fibroblasts, and migrate into the treated region which becomes hypercellular. These activated keratocytes synthetise new collagen and extracellular matrix, both of which contribute to corneal haze (Campos *et al*., 1994).

An important aspect of corneal epithelial wound healing is the effect of hormones and pharmacological agents (Yanagishita, 1993, Nishida, 1993).

Recent research has been focused on the control of corneal cellular response to superficial injury using drugs that may alter fibroblast activity (Nassaralla *et al*., 1995, Podskochy and Fagerholm, 2001, Lai *et al*., 2004). Animal studies have suggested that the use of corticosteroids, nonsteroidal anti-inflammatory drugs, cytostatic agents mitomycin C or interferon alpha 2b, limit subepithelial keratocyte activity and deposition of new collagen (Miller *et al*., 1981, Hersh *et al*., 1990, Kulkarni and Srinivasan, 1985).

The purpose of this study was to determine if keratocyte loss can be prevented, after the deepithelization, by topical application of heparine or by using soft contact lenses.

MATERIAL AND METHODS

Twenty-one Chinchilla grey rabbits of both sexes, from the colony of the Medical Military Academy, Belgrade, were used in this study. The experiment was performed at the clinic of the Faculty of the Veterinary Medicine, Belgrade.

The animals were divided into five groups. All procedures were performed on anaesthetised rabbits (ketamine hydrochloride, 40 mg/kg). The central 6 mm of corneal epithelium was removed from one eye of each of the 18 animals. Three untreated rabbits served as controls.

Method of deepithelization: rabbits were selected in a randomised manner, the central cornea of one eye of each animal was marked with a 6-mm trephine. The demarcated epithelium was then scraped using a Bard Parker scalpel. After surgery the eyes were examined with the biomicrosope and the abraded area was coloured by fluoresceine.

Postoperatively in the first group (n=3), the eyes were treated every 3 hours for 24 hours with heparin drops (Heparin, ICN, water solution 5000 IJ/ml). The animals were killed 24 hours after corneal injury.

In the second group (n=3), the eyes were treated with subconjunctival heparin injections (5000 IJ/ml) every 2 days, for 7 days. The animals were killed 7 days after surgery.

In the third group (n=6), we placed the therapeutic soft contact lens (Focus Visitint soft contact lens, CIBA Vision) in one eye for 5 days, after that animals were sacrificed.

In the fourth group, which didn't receive any therapy after deepithelization, six rabbits were observed. Three of them were killed 24 hours after epithelium scraping, and the other three 7 days after injury.

The control group was consisted of three rabbits with intact corneas.

The animals were killed by an over dose of anaesthetic, the eyes were enucleated and the cornea were excised at the limbus, and immediately fixed in 10% formaldehyde. After 48 hours of fixation, the cornea underwent a serial alcohol dehydration and after routine preparation 5 m histologic sections were stained with hematoxylin-eosin.

To quantify keratocyte loss we placed a reticule A 100 (Graticules LTD., Tonbridge Kent, UK) in one of the oculars of a light microscope (Carl Zeiss). We counted keratocyte nuclei in six consecutive high – power fields with a magnification of x40 within the anterior and posterior half of the central and peripheral areas. We examined three histolgic sections of each cornea. Data presented are the average values (±SD).

Statistical methods: PHARM/PCS – Version 4 , Measurements of variance (ANOVA I) were used to compare the results obtained in different experimental groups. Pair-wise comparisons were made using Student Newman Keuls test; $p<0,05$ was considered to be statistically significant.

RESULTS

Normal unoperated cornea (Table 1) had a higher number of keratocytes within the anterior half of the stroma than in the posterior half. Twenty four hours after deepithelization complete absence of central epithelium was revealed, no signs of infection, and keratocyte nuclei were less numerous within the anterior stroma (Table 2). Compared with unoperated cornea the loss of anterior stromal cells was statistically significant ($p<0,01$). Unlike the anterior stroma, the number of keratocyte nuclei within the posterior stroma was not affected.

Seven days after deepithelization, the wound defect was completely covered by newly regenerated epithelium followed by repopulation of keratocytes in the anterior stroma. Number of keratocytes in the anterior half of the corneal stroma was not statistically different compared with the unoperated cornea ($p>0,05$, Table 3).

Heparin drops, applied every 3 hours, have prevented keratocyte loss in the anterior stroma, compared with eyes that had no treatment ($p<0,01$, Table 4). Heparin applied subconjunctivally, every 2 days, for seven days, did not

significantly influence the number of keratocytes within the anterior stroma (p>0,05, Table 4).

Table 1. Number of keratocytes (X±SD) in anterior and posterior stroma of the intact rabbit cornea

No rabbit	Central part		Peripheral part	
	Anterior stroma	posterior stroma	anterior stroma	posterior stroma
1	32.04±7.13	26.34±3.94	35.21±6.23	34.92±4.17
1	34.50±6.76	30.92±6.12	40.17±4.42	37.67±6.24
2	27.35±3.86	22.79±4.23	31.08±5.19	26.78±3.85
2	35.49±5.47	27.56±5.76	36.94±7.51	32.86±4.27
3	35.67±4.87	31.51±5.97	42.37±4.96	39.19±6.56
3	31.65±5.16	26.60±4.88	38.66±6.14	33.52±5.47

Table 2. Number of keratocytes (X±SD) in anterior and posterior stroma of the rabbit cornea 24 hours after deepithelization

No rabbit	Central part		Peripheral part	
	Anterior stroma	Posterior stroma	Anterior stroma	Posterior stroma
1	19.78±5.76	34.89±7.72	39.44±8.47	40.33±7.15
1	17.56±4.00	30.11±5.78	41.00±5.07	38.11±4.62
2	20.11±2.26	29.22±6.59	38.29±3.89	39.00±7.79
2	22.34±4.42	33.67±5.22	40.44±5.07	35.49±6.71
3	23.76±4.27	34.43±6.23	40.11±6.12	37.14±4.82
3	24.62±3.76	35.94±6.17	43.78±4.17	39.46±5.02
	P<0.01	p<0.05		

Table 3. Number of keratocytes (X±SD) in anterior and posterior stroma 7 days after deepithelization

No rabbit	Central part		Peripheral part	
	Anterior stroma	Posterior stroma	Anterior stroma	Posterior stroma
1	32.22±8.12	23.72±7.09	36.78±7.57	25.33±4.77
1	26.50±3.60	23.72±5.78	30.83±3.99	29.44±5.49
2	24.56±3.68	28.67±3.73	38.28±4.48	33.56±2.71
2	29.06±3.42	26.06±4.51	35.22±7.10	31.22±5.92
3	25.89±4.76	20.72±5.20	29.72±6.52	27.22±5.54
3	28.00±5.41	23.33±4.43	31.89±7.65	27.56±6.05
	p>0.05	p>0.05		

Table 4. Number of keratocytes (X±SD) in anterior and posterior stroma treated with heparin

	No rabbit	Central part		Peripheral part	
		Anterior stroma	Posterior stroma	Anterior stroma	Posterior stroma
24 hours	1	42.67±5.98	52.33±11.60	49.50±7.97	31.56±3.71
	2	38.44±8.31	54.89±9.60	46.56±4.85	35.11±3.33
	3	41.78±4.49	51.00±9.21	45.44±4.75	34.78±3.49
		P<0,01	p<0,01		
7 days	1	30.50±5.94	28.17±6.14	32.89±3.46	26.56±3.68
	2	33.56±4.22	21.78±5.35	34.72±7.45	28.77±5.89
	3	34.06±4.54	22.67±4.59	31.89±6.69	29.39±5.15
		P>0.05	p>0.05		

Contact lenses, used as surface ocular bandage, appears to be effective not only in epithelial healing, but also improve the repopulation of keratocyte cells in the anterior stroma. Compared to untreated corneas, the eyes that had worn after injury contact lenses for 5 days, have shown a significantly higher number of keratocyte nuclei in the anterior and posterior stroma ($p<0,01$, Table 5).

Table 5. Number of keratocytes (X±SD) in anterior and posterior stroma treated with contact lenses 7 days after deepithelization

No rabbit	Central part		Peripheral part	
	Anterior stroma	Posterior stroma	Anterior stroma	Posterior stroma
1	64.78±10.49	34.56±4.07	41.33±6.75	34.44±3.94
2	51.67±10.39	36.78±5.83	52.22±7.45	32.22±4.69
3	57.89±8.78	34.67±9.82	51.56±6.21	31.00±3.64
4	51.77±5.65	41.22±4.79	50.44±6.75	39.33±6.75
5	49.23±7.72	32.64±6.77	39.74±5.12	34.07±3.56
6	50.27±7.18	35.38±4.42	37.64±6.12	33.53±5.83
	P<0.01	p<0.01		

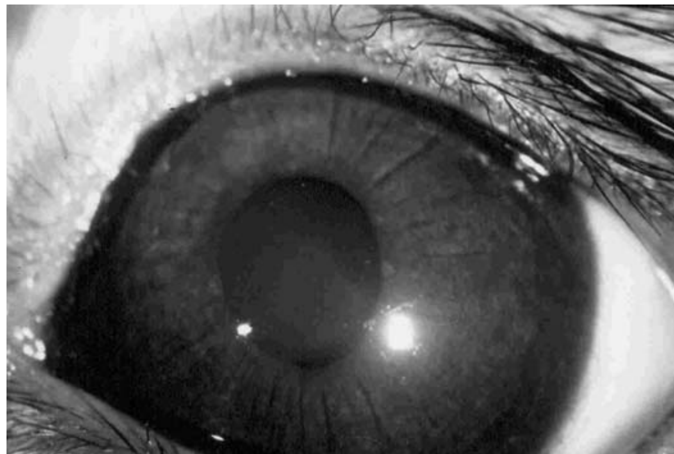

Figure 1. Rabbit cornea after deepithalization

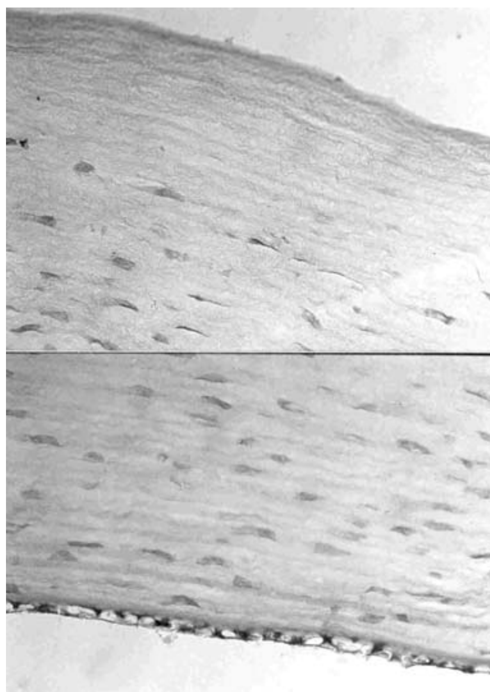

Figure 2. Hypocellular anterior storma of rabbit cornea after deepithelization

DISCUSSION

Studies of corneal wound healing requires an understanding of the interaction of corneal epithelium and stroma (Kim *et al*., 1999, Gopinathan *et al*., 2001). Hirst *et al*. (1981) have shown that an early decrease in the density of keratocytes after superficial injury is followed by an increased number of these cells in the underlying stroma, as well as by increased production of collagen. This stromal regrowth is clinically related to subepithelial haze (Ivarsen *et al*., 2004). The fact that the keratocytes disappear in anterior stroma beneath the area of deepithelization within a very short time and the lack of detritus may indicate a mechanism of self-destructive apoptosis (Gerschenson and Rotello, 1992, Wilson, 1997, Li *et al*., 2000).

Interactions between the epithelial cells and the extracellular matrix are mediated by integrins, heterodimers with transmembrane components which transduce biochemical signals (Hynes, 1992, Stramer *et al*., 2003). Growth factors are also elements of complex biological signalling. They are potent regulatory factors that coordinate the proliferation, migration and differentiation of cells and synthesis of extracellular matrix. Epidermal growth factor (EGF), transforming growth factor (TGF), and fibroblast growth factors, acidic and basic (aFGF, bFGF), stimulate proliferation of epithelial cells and stromal fibroblasts, as well as increase collagen synthesis (Grant *et al*.,1992, Kruse and Tseng, 1993).

Fibroblast growth factors comprise a family of at least five proteins involved in the control of cell growth and differentiation (Hecpuet *et al*., 1990). Basic FGF is present mainly in the epithelial cells and ocular basement membrane and requires heparin or heparan sulfate (HS) for receptor binding and activation (Knorr *et al*., 1996). An injury of the epithelium and basement membrane enables deposition of bFGF in the anterior stroma and promotes stromal fibroblast activity. Heparin has been identified as an important participant in FGF signalling (Krufka *et al*., 1996). Binding of FGF to heparin or HS was proposed to stabilise and protect the FGF molecule against inactivation or to facilitate FGF receptor binding and oligomerization resulting in transmembrane signalling and biological response. It has also been reported that heparin binds to the FGF signalling receptor itself (Spivak-Kroizman *et al*., 1994, Miao *et al*.,1997).

Our intention was to prevent keratocyte loss after deepithelization by local application of heparin, assuming that the interaction with the heparin-binding growth factors and their receptors will ensue.

Topical application of heparin drops, 24 hours after deepihelization have decreased keratocyte loss, thus it could change the cellular response of the corneal stroma after the epithelium has been removed.

Treatment of ocular surface damage with bandage contact lenses is not new. Contact lenses used as mechanical shields, have been shown to improve epithelial healing, reduce stromal edema and decrease inflammatory cell infiltration after stromal incisions in rabbits (Aquavella *et al*., 1987, Shaker *et al*., 1989, Simsek *et al*., 1996). They could also be beneficial for the treatment of corneal surface conditions associated with epithelial defects, and enhance stromal cells activity and wound healing.

Address for corespondence :
Dr Katarina Misailović
Clinical Centre Zvezdara,
The Eye Clinic "Prof. Dr Ivan Stanković"
Dimitrija Tucovića 161
11000 Beograd, Serbia&Montenegro
e mail: kate@ eunet.yu

REFERENCES

1. *Aquavella JV, del Cerro M, Musco PS, Ueda S, De Paolis MD,* 1987, The effect of collagen bandage lens on corneal wound healing, *Ophthalmic Surg*, 18, 570-3.
2. *Campos M, Hertzog L, Wand XW, Fasano AP, McDonnell PJ,* 1992, Corneal surface after deepithelization using a sharp and a dull instrument, *Ophthalmic Surg*, 23, 618-22.
3. *Campos M, Raman S, Lee M, McDonnell PJ,* 1994, Keratocyte loss after different methods of deepithelization, *Ophthalmol*, 101, 890-4.
4. *Dua HS, Gomes JAP, Singh A,* 1994, Corneal epithelial wound healing, *Br J Ophthalmol*, 78, 401-8.
5. *Gerschenson LE, Rotello RJ,* 1992, Apoptosis: a different type of cell death. *FASEB J*, 6, 2450-5.
6. *Gopinathan U, Ramakrishna T, Willcox M, Rao CM, Balasubramanian D et al.,* 2001, Enzymatic, clinical and histologic evaluation of corneal tissues in experimental fungal keratitis in rabbits, *Exp Eye Res*, 72,4, 433-42.
7. *Grant MB, Khaw PT, Schultz GS, Adams JL, Shimizu RW,* 1992, Effects of epidermal growth factor, fibroblast growth factor and transforming growth factor-beta on corneal cell chemotaxis*, Invest Ophthalmol Vis Sci*, 33, 3292-301.
8. *Hecpuet C, Morisset S, Lorans G, Plouet J, Adolphe M,* 1990, Effects of acidic and basic fibroblast growth factors on the proliferation of rabbit corneal cells, *Curr Eye Res*, 9, 429-33.
9. *Hersh PS, Rice BA, Baer JC, Wells PA, Lynch SE et al.,* Topical nonsteroidal agents and corneal wound healing, *Arch Ophthalmol*, 108, 577-83.
10. *Hirst LW, Kenyon KR, Fogle JA, Hanninen L, Stark WJ,* 1981, Comparative studies of corneal surface injury in the monkey and rabbit, *Arch Ophthalmol*, 99, 1066-73.
11. *Hynes RO*, 1992, Integrins: Versatility, modulation and signalling in cell adhesion, *Cell*, 69, 11-25.
12. *Ivarsen A, Laurberg T, Moller-Pedersen T*, 2004, Role of keratocyte loss on corneal wound repair after LASIK, *Invest Ophthalmol Vis Sci*, 45,10,3499-506.
13. *Kim WJ, Helena MC, Mohan RR, Wilson SE,* 1999, Changes in corneal morphology associated with chronic epithelial injury, *Invest Ophthalmol Vis Sci,* 40,35–42.
14. *Knorr M, Wunderlich K, Steuhl KP,* 1996, Effect of heparin and ascorbic acid on growth behaviour of cultivated corneal epithelial cells of the rabbit, Ophthalmologe, 93, 3, 275-8.
15. *Krufka A, Guimond S, Rapraeger AC,* 1996, Two hierarchies of FGF-2 signalling in heparin: mitogenic stimulation and high-affinity binding/receptor transphosphorylation, *Biochemistry,* 35, 11131-41.
16. *Kruse FE, Tseng SC,* 1993, Growth factors modulate clonal growth and differentiation of cultured rabbit limbal and corneal epithelium, *Invest Ophthalmol Vis Sci*, 34, 1963-76.
17. *Kulkarni PS, Srinivasan BD,* 1985, Comparative in vivo inhibitory effects of nonsteroidal anti-inflammatory agents on prostaglandin synthesis in rabbit ocular tissues, *Arch Ophthalmol*, 103, 103-6.
18. *Lai YH, Wang HZ, Lin CP, Chang SJ,* 2004, Mitomycin C alters corneal stromal wound healing and corneal haze in rabbits after argon-fluoride excimer laser photorefractive keratectomy, *J Ocul Pharmacol Ther*, 20, 2,129-38.
19. *Li Q, Ashraf MF, Bekoe NA, Stark WJ, Chan CC et al.,* 2000, The role of apoptosis in the early corneal wound healing after excimer laser keratectomy in the rat, *Graefes Arch Clin Exp Ophthalmol,* 238, 853–60.

20. *Miao HQ, Ornitz MD, Aingorn E, Ben-Sasson AS, Vlodavsky I,* 1997, Modulation of fibroblast growth factor-2 receptor binding, dimerization, signalling, and angiogenic activity by a synthetic heparin-mimicking polyanionic compound, *J Clin Invest*, 99, 7, 1565-75.
21. *Miller D, Gruenberg P, Miller R, Bergamini MVW,* 1981, Topical flurbiprofen or prednisolone: effect on corneal wound healing in rabbits, *Arch Ophthalmol,* 99, 681-82.
22. *Nassaralla BA, Szerenyi K, Pinheiro MN, Wee WR, Nigam A, et al,* 1995, Prevention of keratocyte loss after corneal deepithelization in rabbits, *Arch Ophthalmol,* 113, 506-11.
23. *Nishida T,* 1993, Extracellular matrix and growth factors in corneal wound healing, *Curr Opin Ophthalmol*, 4, 4-13.
24. *Podskochy A, Fagerholm P,* 2001, Repeated UVR exposures cause keratocyte resistance to apoptosis and hyaluronan accumulation in the rabbit cornea*, Acta Ophthalmol Scand*, 79,6, 603 -12.
25. *Shaker GJ, Ueda S, LoCascio JA, Aquavella JV,* 1989, Effect of a collagen shield on cat corneal epithelial wound healing, *Invest Ophthalmol Vis Sci*, 30, 7, 1565-8.
26. *Simsek NA, Ay GM, Tugal-Tutkun I, Basar D, Bilgin LK,* 1996, An experimental study on the effect of collagen shields and therapeutic contact lenses on corneal wound healing, *Cornea*, 15, 6, 612-6.
27. *Song IK, Joo CK,* 2004, Morphological and functional changes in the rat cornea with an ethanol-mediated epithelial flap*, Invest Ophthalmol Vis Sci*, 45, 2 ,423-8.
28. *Spivak-Kroizman T, Lemmon MA, Dikic I, Ladbury JE, Pinchasi D et al.,* 1994, Heparin-induced oligomerization of FGF molecules is responsible for FGF receptor dimerization, activation, and cell proliferation, *Cell*, 79, 1015-24.
29. *Stramer BM, Zieske JD, Jung JC, Austin JS, Fini ME,* 2003, Molecular mechanisms controlling the fibrotic repair phenotype in cornea: implications for surgical outcomes, *Invest Ophthalmol Vis Sci*, 44, 10, 4237-46.
30. *Yanagishita M*, 1993, Function of proteoglycan in the extracellular matrix, *Acta Pathol Jpn*, 43, 283-93.

PREVENCIJA GUBITKA ĆELIJA STROME POSLE POVRŠINSKE RANE ROŽNJAČE KUNIĆA

MISAILOVIĆ KATARINA, RISOVIĆ DUŠICA i DUJIĆ MIRJANA

SADRŽAJ

Oko kunića je najčešće korišćen eksperimentalni model za istraživanje toka zarastanja rana posle traume rožnjače. Cilj ove eksperimentalne studije bio je da ispita da li se primenom heparina i kontaktnog sočiva može uticati na broj ćelija u stromi posle uklanjanja epitela rožnjače kunića.

Ispitivanje je urađeno na dvadeset jednom Činčila kuniću, podeljenim u 5 grupa. Površinska rana na rožnjači pravljena je kod 18 kunića, raspoređenih u 4 grupe. Posle deepitelizacije rožnjače u prvoj grupi primenjen je lokalno heparin u toku 24 sata, u drugoj grupi subkonjunktivalno heparin u toku sedam dana i trećoj grupi je postavljeno kontaktno sočivo u toku pet dana. Četvrta grupa posle traume rožnjače nije dobijala terapiju, i tri životinje su žrtvovane 24h , a ostale tri 7 dana posle povrede.U petoj, kontrolnoj grupi rožnjača je ostala intaktna.

Posle 24 sata od uklanjanja epitela, došlo je do značajnog pada broja keratocita u prednjoj polovini strome. Sedam dana posle povrede, u prisustvu regenerisanog epitela u prednjoj stromi rožnjače uspostavljen je normalan broj keratocita.

Oči koje su bile lokalno tretirane heparinom u toku 24 sata pokazuju značajno manji pad broja keratocita prednje strome ($p<0.05$). Subkonjunktivalno primenjen heparin nije značajno uticao na broj keratocita ($p>0.05$). Broj keratocita posle primene kontaktnog sočiva značajno je veći u odnosu na grupu tretiranu heparinom i kontrolnu grupu ($p<0.05$). Posle deepitelizacije rožnjače kunića lokalno primenjen heparin u toku prva 24 sata i kontaktno sočivo u toku prve sedmice mogu da smanje gubitak keratocita i ovaj protektivni uticaj bi se mogao iskoristiti u terapiji rana na rožnjači.