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Experimental repair of deep corneal defects using a bio-construct comprising a collagen type I matrix loaded with buccal epithelial cells

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The research objective *was to study the reparative effects of the collagen type I bio-construct loaded with buccal epithelial cells, on the rabbit cornea after experimental keratectomy at various stages of treatment (on the 3rd, 7th, 14th, 30th days).*

Material and methods*. The experiments were conducted on 20 rabbits of the Chinchilla breed that were operated on cornea of both eyes aiming to inflict epithelial and stromal cornea defects. The collagen-based bio-construct bearing buccal epithelial cells was placed over the cornea of the experimental eyes. The cornea of the control eyes was covered with smooth contact lens. After the surgery, a temporal blepharorrhaphy was performed and kept for 3 days. We studied macro- and microscopic pattern of corneal regeneration at 3, 7, 14, and 30 days of experiment.*

Results. *When using the collaged-based bio-construct containing buccal epithelial cells, the complete epithelialization of the corneal defect occurred at mean 7 days earlier compared to that in the control eyes. Thus,*

the offered bio-construct stimulated the cell migration and proliferation at early stages of treatment (3–7 days) reducing the inflammation activity.

Conclusion. *The bio-construct comprising a collagen type I matrix loaded with buccal epithelial cells can provide an effective treatment option for corneal defects.*

Keywords: corneal defect, experimental study, buccal epithelium, collagen, bio-construct

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The urgency of treatment optimization for one of the most common anterior eye pathologies, namely, the corneal erosions and ulcers of various origin, is primarily dictated by frequently developing complications, such as recurrent erosions, descemetocele, corneal perforations, persistent opacification, etc. that often result in disability [1, 2]. Transplantation of progenitor cells seems to be the most promising trend in the corneal pathology treatment [3]. In 2003, Nakamura and Kinoshita in their experimental research demonstrated a high regenerative potential of cultured buccal epithelial cells upon transplantation onto a damaged cornea [4-7]. The cells of the buccal epithelium were noted to maintain the proliferative activity of the cornea native tissue without the necessity to stimulate keratinization. The corneal tissue regeneration processes were significantly accelerated even with a short exposure time of the cultivated buccal mucosa allograft [8, 9]. However, the method to deliver the buccal epithelial cells and keep them on the defect site remains a challenge. The most optimal solution would be the development of a bio-construct containing a matrix-carrier and cells.

The optimal carrier for cells can be a collagen-containing matrix [10, 11]. The advantages of collagen matrices include elasticity, durability, biodegradable nature, a high ability to attract and retain adhesive cells, as well as their microvesicles [12]. Type I collagen-based dressings are widely known among collagen grafts; along with a simple use and low manufacturing cost, they possess marked reparative properties, particularly in combination with cellular material. The reparative effect of cell-containing collagen dressings has been demonstrated in the treatment of burn and bite wounds, and some trophic ulcers [12-14]. There are reasons to believe that similar bio-engineered grafts will also be effective for stimulating the regeneration of the damaged cornea, but this treatment technique requires a considerable optimization.

The purpose of the study was to investigate the effect of collagen type I bio-construct loaded with buccal epithelial cells on reparative process in the cornea of rabbit eyes at various stages of treatment after experimental keratectomy.

Material and methods

The laboratory animals used in the experiment were rabbits of Chinchilla breed with a body weight of 2.0-2.5 kg. The experiment was made in conformity with the *European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes* No. 123 of 18.03.1986 and the USSR Healthcare Ministry Order No. 755 of 12.08.1975 "On Measures to Further Improve the Organizational Forms of Work with the Use of Experimental Animals".

The clinical efficacy and regenerative effect of the bio-construct was investigated using the rabbit model of keratectomy. After the removal of the nictitant membrane in 40 eyes of 20 rabbits, a dosed keratectomy of 8 mm in

diameter was performed. The thickness of the removed corneal flap was about 160 microns. Further, the originally developed bio-construct containing buccal epithelial cells on a collagen type I matrix was placed on the damaged right eye cornea of each rabbit. The cornea of the contralateral left eye was covered with a soft contact lens. After the surgery was completed, a temporary blepharorrhaphy was performed on all the eyes for the period of 3 days.

The eyes after the bio-construct transplantation made up an experimental study group (20 eyes); the eyes coated with a soft contact lens (20 eyes) were used as a comparison group. For 10 postoperative days, each animal received an antibacterial medication (Oftaquix drops) in instillations of 1 drop 3 times a day both into the experimental, and comparison group eye.

After the suture removal, the size and depth of the corneal defect as well as the rate of the defect reduction were comparatively assessed between the groups. The observation period was 30 days. Macro- and microscopic assessments of the therapy effect were made on days 3, 7, 14, and 30 of the experiment. The size and depth of the corneal defect were assessed macroscopically, and the rate of the defect reduction was compared between the groups. For the corneal microscopic evaluation, the animal eye samples were fixed with 10% formalin, and the standard histological preparations were made, stained with hematoxylin and eosin, and then studied using light microscopy. The collagen fiber pattern, inflammatory cell infiltration, and epithelialization were evaluated. The structural integrity of the corneal collagen fibers was also studied considering their autofluorescence in histological preparations at fluorescence microscopy (λ excitation = 510-560 nm, λ emission: from 575 nm, the exposure: 1 second) [15].

In the treatment of rabbits, we used an originally developed bio-construct based on collagen type I with buccal epithelium cells. The bio-construct consisted of three components:

- the underlayer: a soft contact lens iWear dr Comfort;

- the matrix-carrier of rabbit collagen type I. The collagen was obtained from rabbit tendons by acid extraction, then the biomaterial was diluted with a standard aqueous 0.05% chlorhexidine solution, lyophilized, and sterilized by ultraviolet irradiation to reduce the acetic acid concentration. As a result, a 1-2 mm thick sponge was obtained, which was applied on the contact lens;

- cultured cells of the rabbit buccal epithelium. Concentrated cell suspension (1 million/mL in isotonic 0.9% sodium chloride solution) in an amount of 1-2 ml was applied to contact lenses containing collagen matrix; and then, the bio-construct was immediately dispatched for use 16].

Results of the study

When analyzing the data of an experimental study on a rabbit keratectomy model to investigate the effect of the original bio-construct on the regeneration processes in a damaged cornea, the following results were obtained.

On day 3 after keratectomy, the inflammation signs such as conjunctival hyperemia, scanty mucous discharge, grade 1-2 corneal edema were seen in the experimental eyes, and in the comparison group eyes. The erosion area was significantly smaller in the experimental group vs. the comparison group eyes, making 24.48 ± 2.18 mm^2 vs. 32.92 ± 2.64 mm^2 ($t_{emp}= 2.3$ at $p < 0.05$). In one of the experimental eyes, there was no fluorescein staining of the defect.

On observation day 7, the inflammation was controlled in all the eyes. In the experimental group, a residual defect of mean 3.73 ± 1.46 mm^2 by area persisted in 5 of 15 eyes. The remaining 10 eyes displayed a complete epithelialization, including 2 eyes where the cornea returned its initial transparency and smoothness characteristics. In the comparison group, the defect persisted in 11 of 15 eyes, its mean area being 6.22 ± 2.21 mm^2. One of the eyes of that group showed the epithelium roughness in the defect area. We noted that the epithelialization was significantly more likely in the experimental group than in the comparison group ($\chi^2 = 4.821$ at $p < 0.05$).

On day 14, the mean area of the residual corneal defect in the experimental group was 1.334 ± 0.035 mm^2, with complete epithelialization observed in 6 of 10 eyes. A slight cloud-like opacification (grade 2-3 by Voino-Yasenetsky Scale) was revealed in 2 eyes. In the comparison group, only 4 of 10 eyes were completely epithelialized, the mean area of the defect in the others was 4.75 ± 0.1 mm^2. Meanwhile, a recurrent corneal erosion was found in 2 eyes. Cloud-like opacities (grade 3-4 by Voino-Yasenetsky Scale) were seen in 6 of 10 eyes.

On observation day 30, a recurrent erosion with the defect area of 0.78 mm^2 was noted in one case and a slight cloud-like opacification (of grade 2-3 by Voino-Yasenetsky Scale) was identified in 3 cases of the experimental group (5 eyes). In the comparison group (5 eyes), a corneal defect of 3.14 mm^2 retained in one eye. The corneal opacification of grade 3 by Voino-Yasenetsky Scale was revealed in 3 eyes.

Histological specimens of the comparison group after 3 days (n = 5) demonstrated the absence of continuous epithelium in all the animals; the epithelium contained only 1 layer of cells at the wound edges in 4 of 5 cases (Fig. 1); a basal membrane was seen formed and extending long in the

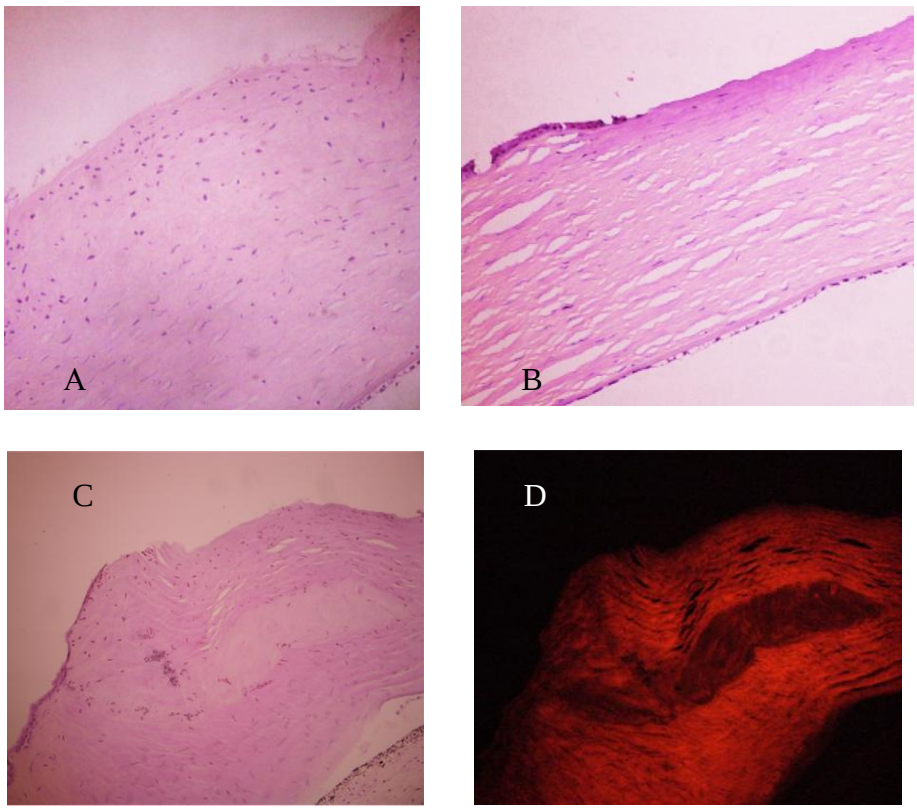

Fig.1. Histological view of the corneal defect after 3 days of the experiment in the comparison group: A, the bottom of the defect, inflammatory cell infiltration; B, the edge of the defect, one layer of the epithelium, eosinophilia of fibers; C, stromal edema; D, autofluorescence of stroma collagen fibers, edema. Stained with hematoxylin and eosin. Magnification 200x

cornea of 2 eyes. Edema and eosinophilia of collagen fibers of the intercellular matrix were found deep in the wound, the intensive infiltration with inflammatory cells, mainly by neutrophils and eosinophils, was noted with a lowered migration activity of keratocytes. In the experimental group 1 eye was found to have the epithelial layer over the entire corneal length; in 2 eyes, the epithelium at the edges of the wound had 2-3 layers of cells (Fig.

2). In 3 of 5 cases, an intensive migration of keratocytes with weakly condensed chromatin (synthetically active cells) was observed in the cornea; as a rule, the migration occurred along the deep lying fibers and Descemet's layer (Fig. 2B). The swelling of the corneal fibers in the experimental eyes

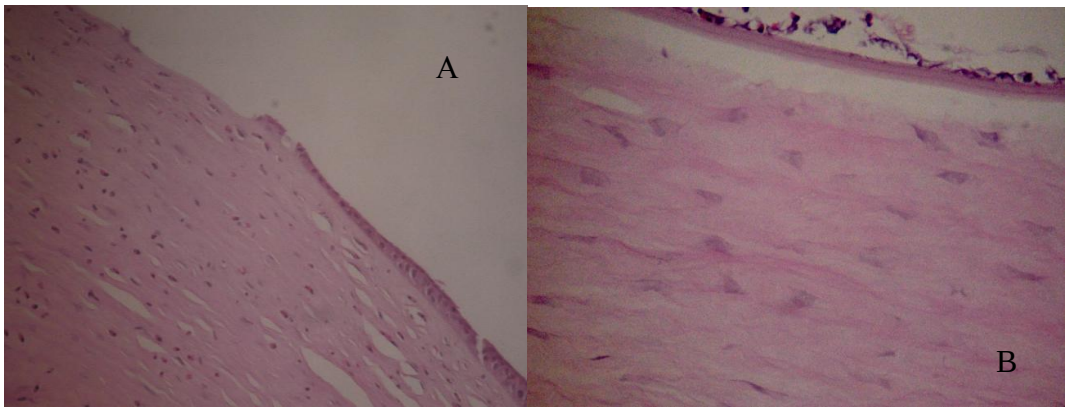

Fig. 2. Histological view of the corneal defect after 3 days of experiment in the experimental group: A, the edge of the defect, 2-3 layers of epithelium; B, the migration of undifferentiated keratocytes in the stroma. Stained with hematoxylin and eosin. Magnification 200x

was less pronounced than in the comparison group, meanwhile, the fibers with pronounced eosinophilia were observed both in the experimental and in the comparison group. Increased eosinophilia of corneal collagen was not associated with its decondensation; in both groups, the eosinophilic fibers had the same level of autofluorescence as the normally stained fibers, whereas in the fibers with pronounced edema, this parameter was significantly decreased. In general, the overall pattern of intercellular stromal fibers in the experimental keratectomy area was seen less distinctly than in the non-damaged areas.

After 7 days, the continuous epithelium was formed on the cornea of 1 eye in the comparison group and 3 eyes in the experiment group; and only in 1 case in the experimental group, the epithelium had 3-4 layers and the normal cell orientation in them. In all the animals with complete epithelialization, we identified the zones where the number of epithelial cell layers was higher than normal, and superficial epithelial proliferating tissues could be observed (Fig. 3). In the experimental group, the epithelial proliferating tissues were observed also in the stroma (submerged proliferating tissues), which indicates a higher intensity of epithelial growth than that in the comparison group (see Fig. 3B). The infiltration with inflammatory cells (mainly eosinophils) persisted, but its level in the experimental group was lower than in the comparison group. In both groups, the keratocyte migration was observed, mainly in the outer layers of the stroma (Fig. 4). Meanwhile, edema and a disrupted orientation of the collagen fibers were seen in experimental, and comparison group eyes.

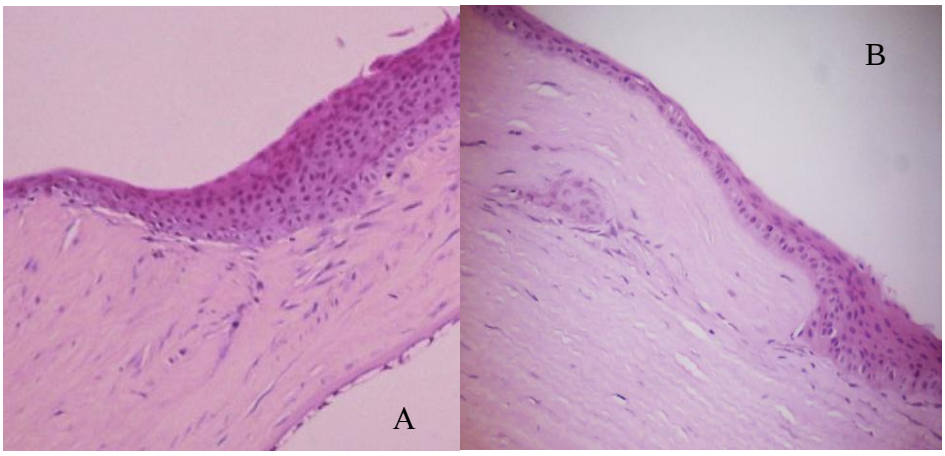

Fig. 3. A multilayered epithelium formation in the cornea after 7 days of the experiment in a comparison group eye (A), and in an experimental group eye (B). Stained with hematoxylin and eosin. Magnification 200x

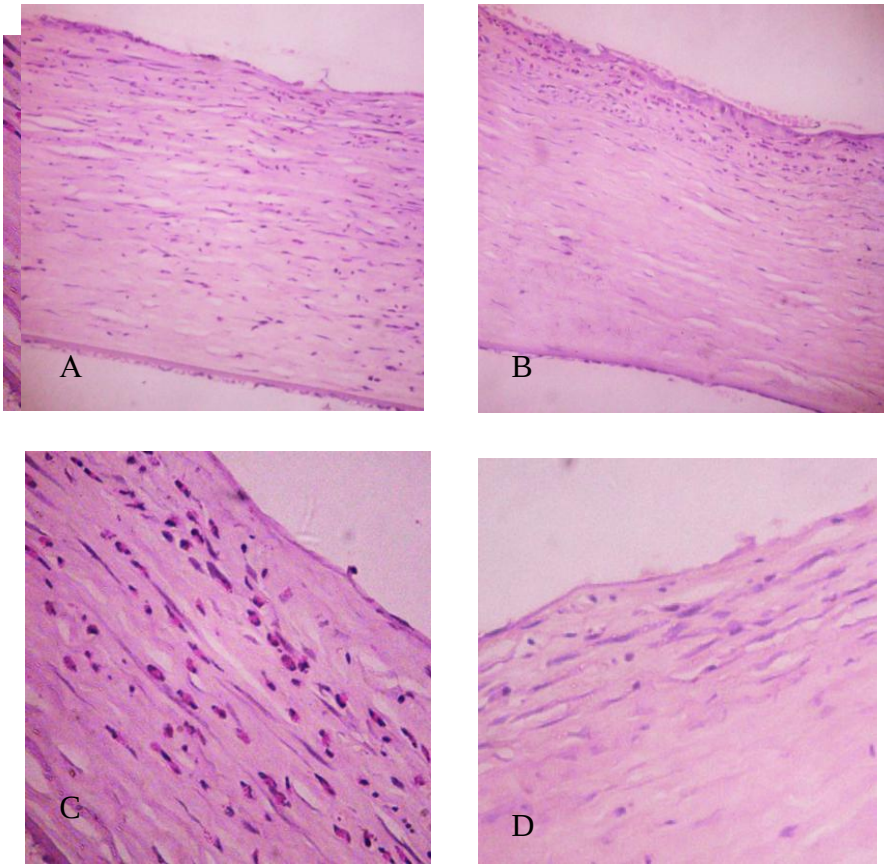

Fig. 4. The inflammatory cell infiltration and the migration of keratocytes on day 7 of the experiment: A, a comparison group eye; B, an experimental group eye (magnification 200x); C, a comparison group eye; D, an experimental eye (magnification 400x). Stained with hematoxylin and eosin

After 14 days, the surface proliferating tissues with an increased number of cells were clearly identified in the epithelial layer, that resembled the pattern observed on day 7; the submerged proliferates were absent (Fig. 5) both in the experimental eyes, and the comparison group eyes. The cornea in the keratectomy area was covered with continuous epithelium, while in most cases, the stromal fibers adjacent directly to the epithelium were clearly thinner than normal ones, which was a predisposing factor for epithelial

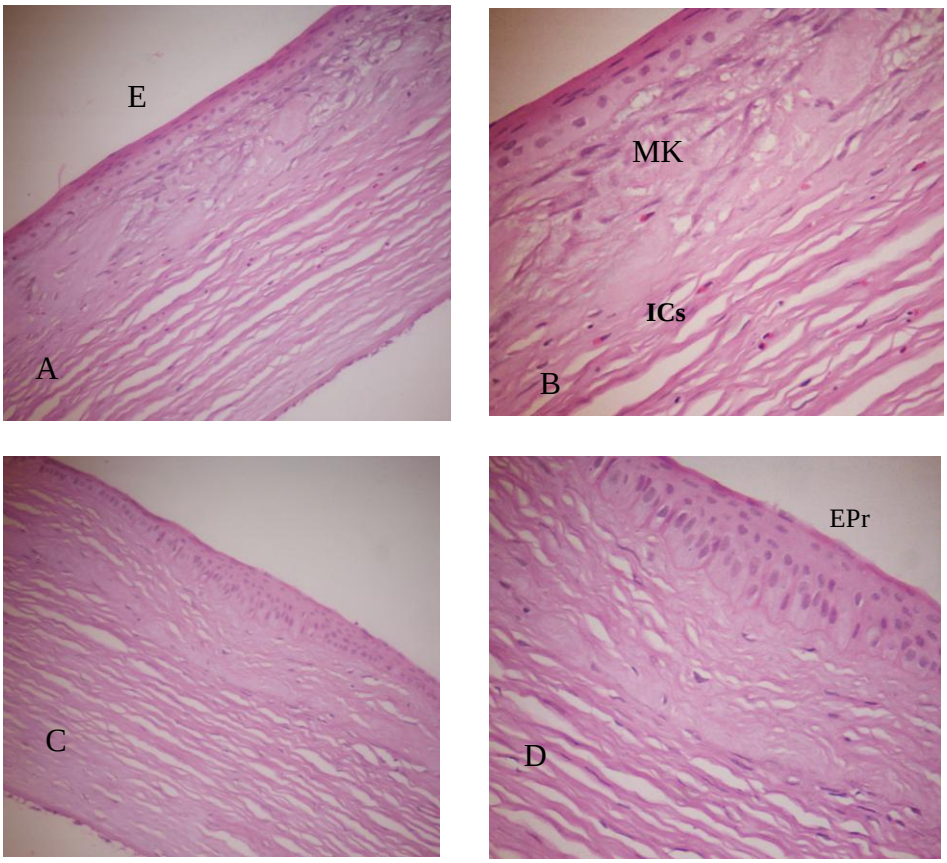

Fig. 5. Histological view of the corneal defect after 14 days of the experiment: A, the comparison group (magnification 200x); B, the comparison group (magnification 400x); C, the experimental group (magnification 200x); D, the experimental group (magnification 400x). Stained with hematoxylin and eosin

Note: E: epithelium; EPr: epithelial proliferating tissue; ICs: inflammatory cells; MK; migration of keratocytes.

detachment. Similar thinness of fibers was noted in all comparison group eyes, and in 3 of 5 experimental eyes. In addition, all specimen still showed edematous fibers. A quite normal architecture of the stromal intercellular matrix and the normal arrangement of keratocytes were noted in 2 experimental eyes. The migration activity of cells (including those with

weakly condensed chromatin) in the experimental group had decreased by that time of observation, which could have indicated the completion of the stromal repair process. In the comparison group, on the contrary, there was an increased cell migration, mainly in the outer layers of the stroma.

After 30 days, the cornea was covered with a continuous epithelium (Fig. 6) in 4 animals both in the comparison group, and in the experimental group; In one eye from each of the groups there were areas where the corneal epithelium was either absent (comparison group) or detached (experimental group). We should note that the cornea in most animals with

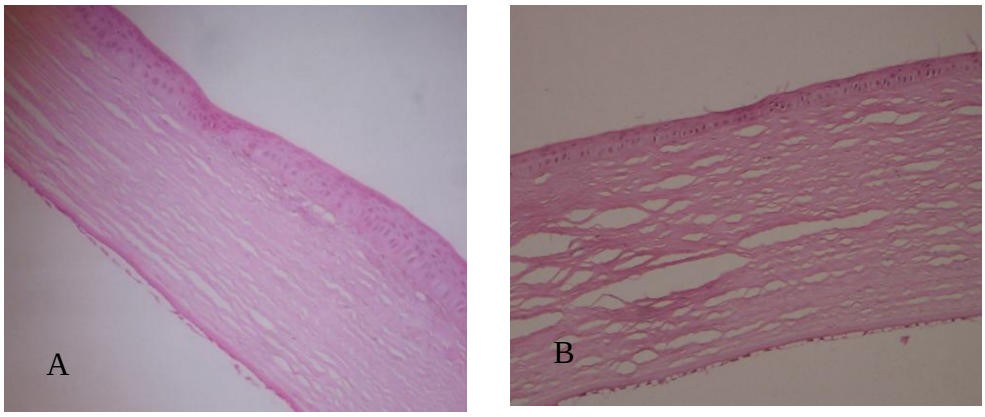

Fig. 6. Histological view of the cornea at 30 days after the treatment initiation: A, the comparison group; B, the experimental group. Stained with hematoxylin and eosin (left). Magnification 200x

complete epithelialization had a rather uneven surface (see Fig. 6A); that "tuberosity" was formed due to the protrusion of the epithelium and stroma or by the presence of superficial epithelial proliferating tissues. Marked epithelial proliferating tissues were seen in 3 comparison group eyes and in 1 experimental group eye (where an epithelial detachment also occurred). The underlying stroma fibers were thinned, the same as at earlier stages (on days

7-14); the stromal edema persisted in all the comparison group eyes and in 3 experimental eyes. In general, the normal corneal stroma arrangement was noted only in 2 eyes of the experimental group; in others, the architecture of intercellular fibers and the cell orientation were not fully consistent with normal. The infiltration with inflammatory cells was observed in the area of the cornea contact with conjunctiva.

The study has shown that the offered bio-construct accelerates the migration and proliferation of cells at early stages of repair (at 3-7 days after the treatment initiation), and also reduces the severity of the inflammatory reaction. This may be explained by the action of cytokines released from the buccal epithelium cells. The most active corneal repair processes occurred on day 7 in the experimental eyes, and on day 14 in comparison group eyes. We should note that the bio-construct contacts an eye for a short time only, and the buccal epithelium cells give a pronounced but short-lived effect, which diminishes over time. We had also found that the continuous epithelium formation was evidently faster than the underlying stroma recovery. As a result, in addition to swelling, the immature collagen fibers were detected in the stroma of most animals. Apparently, it is the presence of such defective fibers in the epithelium-adjacent layers that creates the risk of recurrence. The literature reports demonstrated that the grafts saturated with hormones or cultured cell components accelerated two-fold the corneal defect epithelialization; on the other hand, those studies did not describe the underlying stroma condition. Apparently, it is the restoration of normal topography and architecture of the stroma that is necessary for the complete recovery of the corneal defect. Epithelium, due to its high proliferative cellular potential, is able to actively increase its volume and fill in the open zones of the stroma; therefore, the process of corneal repair primarily

demands corneal subepithelial layers to be restored. The surface epithelial proliferating tissues (with an increased number of cells) were seen in many animals on day 30 of the study. Probably, their presence at a macro level can be manifested in the form of a varying severity opacification. One should not rule out the temporary presence of epithelial proliferating tissues in the cornea, and bear in mind that they will disappear at later stages after injury.

Conclusion

The originally developed bio-construct based on collagen and buccal epithelium stimulates reparative processes in the cornea at early stages of treatment. The question remains, how long this effect would last. Probably, a single application of bio-construct is not quite enough to increase the treatment effect, and repeated applications would be required. The development of the technique to treat deep corneal defects using cell-tissue grafts is a challenge for future research.

Summary

1. The proposed bio-construct based on collagen type I containing buccal epithelium cells significantly accelerates the healing of corneal defects at early stages of treatment (days 3-14). In the experimental eye group, the area of the residual corneal defect after 3-14 days of treatment was 1.5-3.5 times smaller than in the comparison group, with less pronounced edema of stromal fibers and inflammatory cell infiltration.

2. The use of the bio-construct reduces the cornea opacification intensity at day 14-30 of treatment.

3. A concurrent restoration of the epithelial lining and the underlying stroma fibers is necessary for the efficient corneal regeneration. A recurrent corneal erosion may occur due to a low rate of stroma regeneration.

4. The reparative and regenerative effect of the offered bio-construct should be investigated with its repeated applications.

The authors state there is no conflict of interests to declare

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