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Morphological Characteristics of Rabbit Cornea in Norm and Wound Healing Cytoarchitecture

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Cornea is an avascular structure with an important role in vision, which could be impaired by different conditions. The most suitable animal model in ophthalmology research, comparable to human, is the rabbit model. Data on the morphology of rabbit cornea are capable to predict or to be used for comparison of the fundamental processes in corneal wound healing. In this study the morphological aspects of corneal postoperative wound healing processes were assessed in comparison with the normal histology of corneal layers in rabbits. Our findings demonstrated that the corneal wound healing was between the phases of proliferation and maturation-tissue regeneration restored the integrity for three months but lamellar organization and remodeling still were not completed. Strong postoperative keratitis was observed in one case and structure similar to the human pre-Descemet's layer was noticed.

Key words: morphology of rabbit cornea, wound healing, tissue regeneration

Introduction

Cornea is an avascular part of the fibrous tunic (corneoscleral layer) – the outermost layer of the eye with an important role in focusing vision. It provides also transparency, refractivity and light transition into the retina - the innermost layer of the eye and gives mechanical strength of the eye. These functions could be deteriorated by corneal infections and injuries resulting into inflammation and ulcers, keratoconus, Fuchs endothelial corneal dystrophy, pterygium, etc. Such problems are found in animals, as well as in humans and have a high social and economic impact for the last, especially if the disease ends in blindness. Some superficial injuries can be treated by PRK (photorefractive keratectomy) or LASIK (laser assisted in situ keratomileusis) but often a corneal transplantation is recommended – penetrating or endothelial keratoplasty, or deep anterior lamellar keratoplasty. In such cases the usage of donor tissues from a human or xenotransplantation from a pig, because of similarities in biomechanical properties of human and pig corneas [13], is needed. The cornea is an immunologically privileged site and incidences for tissue rejection of corneal allografts or xenografts are less common than in other tissues requiring passage

through immunological barriers. However, there are many incidents of unsuccessful operations and along with the factor of cadaveric donation the demand for substitutes and new materials for medical implants in ophthalmology is determined. These facts require strict analysis in real situation – testing on living organisms, as this is the most suitable form of prediction of medicine or medical devices' behavior in humans. Usually, the ophthalmic surgery encounters difficulties due to micro-volumes of the work and challenges connected with the integration of different biomaterials with host tissues and preserving the corneal transparency, biomaterial degradation or aggressive wound healing response, which can even worsen the vision. Rapid restoration of corneal integrity after defects is a crucial factor for intraocular inflammation prevention and avoidance of permanent blindness. To study and evaluate the safety and biocompatibility of materials, the researcher has to be familiar with the histoarchitecture and morphological specificity of vision organs in norm and in pathology. Particularly, the corneal wound healing consists of complex events, involving different cell types and biochemical conditions demanding an understanding of the anatomical and physiological characteristics of the cornea and its multilayered structure. The establishment of the morphological characteristics of these specific cellular and subcellular structures in norm and during healing could be a helpful paraclinical tool in ophthalmology. The corneal histological structure is well-known [21], but as a surface structure it is in a constant state of morphological reconstruction due to epithelial regeneration or dynamic healing processes after physical or chemical injuries. In 1989 studies on cell kinetics indicated the presence on the ocular surface of proliferative cell compartments of stem cells and transitional amplifying cells [12]. Since then, the scientific research in the direction of transplantology and tissue-repair by stem cells usage in regenerative ophthalmology has developed rapidly. The most appropriate model, in research concerning ophthalmology comparable to human, is the rabbit model – *(Oryctolagus cuniculus)* and particularly the New Zealand White [26]. Rabbits are suitable specifically with their relatively large eyes and similarities with some anatomical features in humans – eyeball size, its internal structure and optical system, biomechanical and biochemical features, as well as conjunctiva cavity volume [27] and for age-related changes studies. Along with the comparable with human eye structures exist differences in some segment, for example: larger anterior segment, resulting in iris bulging and curvature of the anterior chamber [26], non-uniform changes in human and rabbit lens dimensions after excision, despite their similar size in eye [25], capacity to resist blinking due to lipids in their tears, produced by Hardarian gland, absent in primates [15], differences in the lamina cribrosa and its vascular supply, prelaminar optic nerve head and a retinal ganglion cell layer [2], partially myelinated by oligodendrocytes retina [19]. In the study of Ojeda et al. [18] was found that all epithelial cells exhibited microplicae regardless of their location and the stromal lamellae organisation and keratocytes in both species are not functionally homogeneous. Differences were also found: in the collagen fiber patterns of the epithelial basement membranes and the surface of keratocytes located near Descemet's membrane – in humans exhibited small fenestrations, which were absent in rabbits. In brief, histological investigations of rabbit cornea can be used for comparison of the fundamental processes in corneal wound healing on the basis of the known similarities and differences in both species.

This study aimed to investigate and highlight some morphological aspects of corneal postoperative wound healing process and cell interplay and to compare them with the normal histology of corneal layers in rabbits.

Materials and Methods

In vivo study: White New Zealand male rabbits (n=6), 6-7 months of age and mean body weight of 3,5 kg were divided into 2 groups as follow: 1st group – the control intact animals (n=2, 4 intact corneas); 2nd group (n=4, 4 intact corneas of the left eyes and 4 corneas of the right eyes with surgically made incisions – modified micropocket assay) and screened for corneal regeneration. After a postoperative period of three months, rabbits were sacrificed with an intravenous injection of 5-6 ml Euthanasin "N", Vetprom and Magnesium sulphate 5 mg/kg body mass and the corneas were excised. Several criteria for corneal healing or inflammation, visible with naked eye or after histological procedure were evaluated: signs for corneal layers regeneration and lack of ulceration or inflammation of the cornea (keratitis) and the adjacent sclera (signaling for uveitis). Macroscopically were evaluated: signs of inflammation (eye redness, pain, light sensitivity, signs for decreased vision and visible neovascularization, optical transparency; histologically – endothelial decompensation (dystrophy), fibrosis, stromal vascularization, keratoconus, interstitial keratitis, etc. *Surgery techniques*: According to Chan et al. [6] the rabbit cornea has uniform thickness of 407 +/- 20 μ, with an average thickness variation of only 7μ. In other study, it was found that the corneal thickness increased gradually from the center to the periphery of the 6 mm measured and the center corneal thickness of the right eyes was 387 ± 19.8 μ, while of the left – 384 ± 20.2 μ [24]. Based on these data, in our study a small perforation in the central cornea (debt 200μ) was made on the right eye through the anterior corneal stromal layer. The procedure was done under standard general ketamine/xylazine anesthesia with starting points of 10 mg/3 mg/kg. After the operation, both eyes of each rabbit from 2nd group were treated 21 days (three weeks × 4-3-2 times daily) with TobraDexEye Drops (tobramycin and dexamethasone, 3 mg/1 mg/ml), Alconand Crystal Vision Eye Drops (INN hydroxypropylmethylcellulose), Antibiotic-Razgrad AD. The left corneas were left intact but treated with both types of eye drops for comparison. *Histopathology: hematoxylin-eosin (H&E) staining*. After surgical excision tissue samples from the central corneas were routinely fixed in 10% buffered formalin, rinsed in water, dehydrated in graded ethanol, cleared in xylene, embedded in paraffin and subjected to histopathological H&E processing and evaluation by light microscope Leica DM 5000B, Wetzlar, Germany. *DAPI nuclear staining.*The materials fixed and embedded in paraffin were cut to 3-5 μm thick tissue sections, dewaxed in xylene and were DAPI [4,6-Diamidino-2-phenylindole, dihydrochloride] (10 mM solution in water, Abcam) stained and examined under UV light (359 nm excitation and 461 nm emission) by fluorescent microscope Leica DM 5000B, Wetzlar, Germany.

All the experiments with animals were carried out in the Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences (Permit number: 11 30 127) in accordance with the national Regulation № 20/01.11.2012 regarding laboratory animals and animal welfare and European legislation. The medical procedures were strictly guided by the Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals used for scientific purposes and approved by the Ethics Committee in IEMPAM-BAS.

Results

During the experiment daily checks of the health status of all rabbits and observations of both eyes were made. Visible changes during the period of daily treatment with eye drops were not noticed. One week after the end of the eye drop course one of the rabbits from the second group showed clinical manifestation of eye irritation and inflammation, perhaps connected with external contamination of the cornea. In the end of the experiment in the control group histological observations of rabbit corneas revealed standard histoarchitecture composed of a stratified non-keratinized squamous epithelial layer, Bowman acellular collagenous layer beneath the epithelial basement membrane; a stromal layer with regular collagen organization, evenly interspersed with keratocytes, Descemet's basement membrane and endothelial cell monolayer of polygonal cells (**Fig. 1D**). On day 90 after the operation, in 3 of the rabbits from the second group, the histological aspects of corneal healing revealed wound regeneration processes at the site of the operative area on the right corneas with some individual differences observed (**Fig. 1A, B, C**). All layers of the cornea comprised of: epithelium layers, Bowman's layer, anterior and posterior collagen stroma, Descemet's membrane and the endothelium were present as in cornea of the rabbits from the control group described above. In these rabbits epithelial events involving the superficial squamous cells, central suprabasal cells and especially the single layer of inner columnar basal cells generating the other two types and populating the defect were observed microscopically. In the central corneal region, corresponding to the operative defect, were noticed focal proliferations, reattachment and differentiation of the epithelium forming more layers, compared to control left eye, perhaps due to initiated migration of epithelial cells as well from the periphery, but also from the columnar basal cell layer, leading to complete regeneration and covering of the corneal defect. Thus were formed prominent islets of epithelium, alternating with focal thinnings, similar to mid-stage keratoconus, but lack of the other main keratoconus characteristics was found. The stroma consisted of well-arranged collagenous fibers forming lamellae with parallel distributions, but in some regions were perpendicular to each other, following the curves of the anterior stroma and epithelium. Slight focal noncellular edema was present in some collagenous lamellae (**Fig. 1C**). Stromal resynthesys and reorganization phase was still present. Endothelial or Descemet's membrane damage was not observed. Inflammatory cell response, intrastromal neovasculature or other signs of chronic ocular inflammation were not seen in three of the rabbits from the second group at the end of the third month. Also lack of ulcerations, hyalinization, keratocyte loss, necrosis, stromal calcification after topical steroid therapy (as the described calcareous degeneration of the corneal stroma by Schlötzer-Schrehardt et al. [20]) was found. In one of the rabbits from the second group clinical signs for eye irritation were evident and the microscopic description confirmed acute inflammation and keratitis (**Fig. 2**). Distinct hyperplasia of the stratified squamous corneal epithelium lining was observed, along with histopathological signs of corneal fibrosis, strong neovascularization in the stroma, abundant inflammatory cell infiltrates in the upper lamellar layers. Keratocytes' homogeneity and intensity presented significant differences of populating the stromal lamellae, compared to the controls. Obvious stratification and drainage of the lamellae were found, different from fixation artifacts. In the same individual irregular Bowman's layer with taperings or tearings was noticed and moreover – a clearly visible layer – thin, acellular composed of collagen fibers was observed, resembling the pre-Descemet's layer – Dua's layer found in humans (**Fig. 3**), but

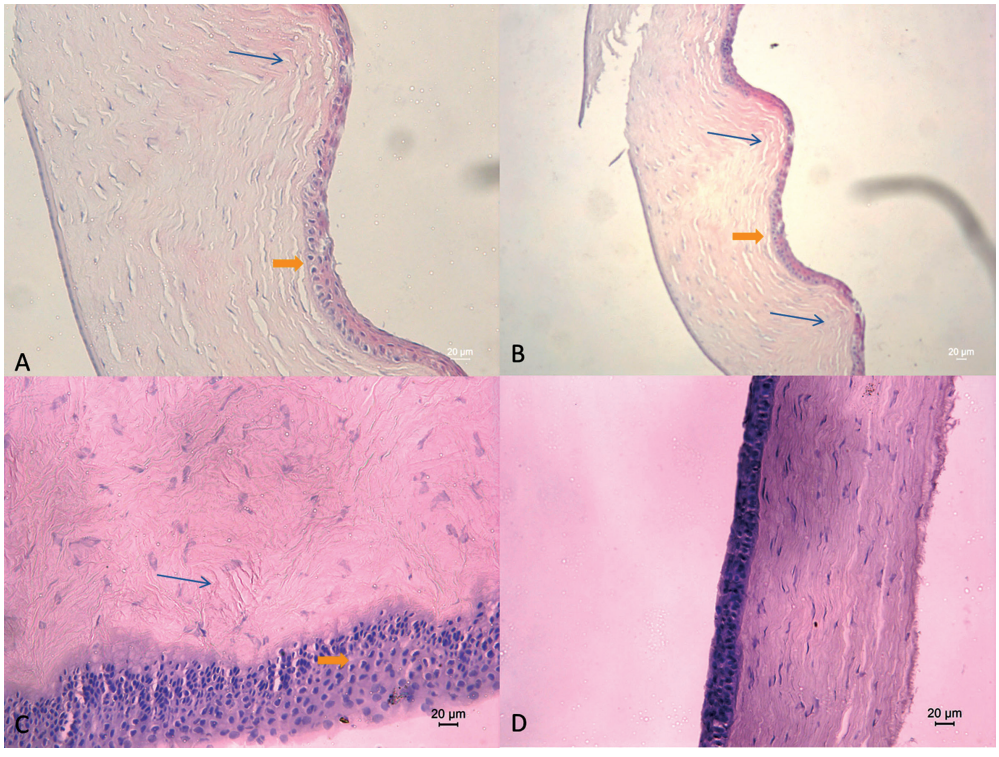

Fig. 1. Central region of rabbit cornea at the site of the operation: two individuals from the second group (A, B-one specimen; C – second specimen): obvious epithelial layer thickening (short orange arrow) and changed partial corneal thickness, irregular Bowman's layer and anterior stroma curves with changed lamellae organization towards perpendicular (long narrow blue arrow), A, B – enlargement of basal epithelium, C – changed inner basal cell layer of columnar cells connected with regeneration and slight lamellar edema; D – control cornea from an animal from the first group. H&E. Scale bar=20µm.

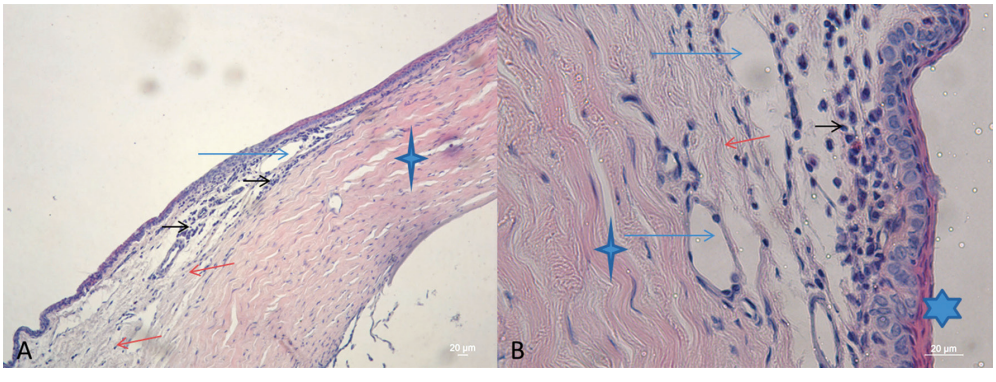

Fig. 2. Keratitis in rabbit: Inflammatory cells in the basal epithelium and Bowman's layer (small black arrow, pointing to the right), fibrosis (longer orange arrow, pointing to the left) and neovasculature (the longest blue arrow, pointing to the right) in stroma (four – point star), enlarged basal cells and epithelial squamous hyperplasia (six – point star). H&E. Scale bar=20µm.

thinner, which has to be studied in detail in separate study. DAPI staining revealed a similar picture reaffirming the multiplying of epithelial layers in operated eyes with a focus on the inner basal cell that changed their placement and nuclear morphology from elongated nuclei, but preserved uniform distribution of chromatin without signs of apoptosis and pyknosis (**Fig. 4**). Also, lack of inflammatory stromal response was confirmed in the three healthy animals with even distribution of keratocytes on the collagen lamellae.

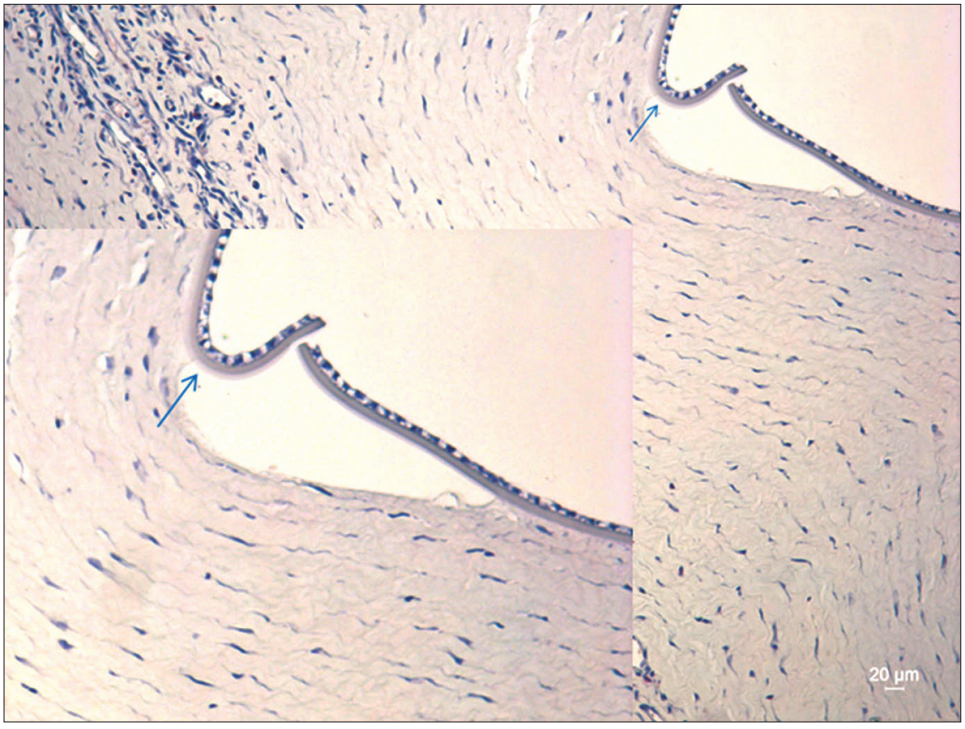

Fig. 3. Rabbit cornea-acellular layer (blue arrow) resembling the pre-Descemet's layer – Dua's layer in humans, <10µm. H&E. Scale bar=20µm.

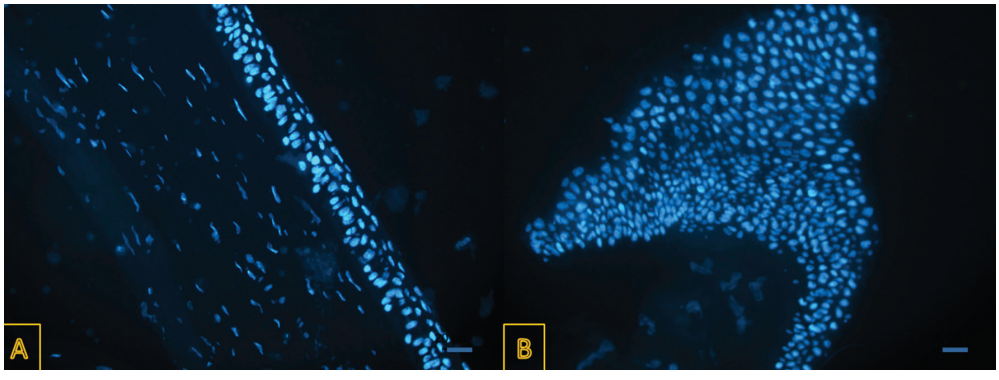

Fig. 4. Central region of rabbit cornea at the site of the operation: A-control, B-obvious epithelial layer thickening and changed nuclear morphology of inner columnar epithelium. DAPI. Scale bar=20µm.

Discussion

Wound healing of cornea is a similar process to skin wound healing, where the inflammatory processes are fundamental responses [16]. Our histopathological findings on 90 day confirmed postinflammatory phases and corneal repair in 3 of the operated animals and demonstrated that the corneal tissue regeneration restored the integrity but was still under maintenance and maturation. This complex process, orchestrated by growth factors that largely overlaps skin repair, was described in other studies [1, 4, 14]. The corneal immune system is an important factor in wound healing and regeneration along with corneal angiogenic privileges essential for homeostasis and functiontionality. Cornea is perceived as a privileged place in this regard, but when the stimulus is strong enough, a network of signals is triggered and an immune response and blood supply is released. Our observations provided insight into the cellular events in corneal wound healing and also revealed strong corneal inflammatory reactions in one case. The incidentally made observation in our study – notable inflammatory process and well developed new vascularization in rabbit stroma, indicated the principal opportunity and capacity of the cornea to react as a consequence of immune cell function against antigens and to rule the outcome of the healing response. This confirms the possibilities for easy rejection of graft transplants or frustration of other surgical invasions, essential in ophthalmology. The epithelium normally is constantly renewing itself at every 7–10 days [4]. In our study we visualized in healing corneas adhesion and migration of superficial epithelial cells and observe the advanced formation of multilayered cell stratum covering the defects. In this population cell-related and protein-related events are involved. As it was elucidated, stem cells migrate from the limbal palisades towards the corneal center and differentiate to transient amplifying cells and basal cells to replenish the epithelium [8, 17], but except this vertical movement from deep layers to superficial layers, a centripetal migration takes place [17]. Protein-related events include the extracellular matrix and basement membrane proteins promoting the chemotactic and haptotactic migration of cells, especially fibronectin, laminin, and type IV collagen with major role as reported by Cameron et al. [5].The stroma represents tightly-packed collagen fibers organized in lamellae and arranged in right angles relative to the fibers in neighboring layers [10] and sparsely-spread resting keratocytes, secreting the collagen and proteoglycans components of the matrix. Keratocytes are the cells activated and involved in corneal repair after injury and are quickly replenished. In our case at the site of injury they restored to some extend the histoarchitecture after 3 months with the presence of occasional focuses of noncellular edema, connected with extacellular matrix transformation and not fully completed lamellar organization. This result is comparable to the reported reestablishment for at least one year of the lamellar organization of the stromal collagen across the site of the incision [9], but this remodeling process and restoration to perfect transparency can take also years [7]. In this study, the endothelium had not been directly surgically manipulated or ruptured. The endothelial capacity to preserve the barrier function and to resume the draining activity towards excessive fluids during wound healing was observed. The corneal layer of endothelium consists of a single layer of hexagonal-shaped cells which ensure corneal dehydration at about 78% and preserve from swelling in norm. These cells are situated on a basal Descemet's membrane, composed of collagen [3, 10]. Here we found focal microedema in the anterior stroma, connected with extracellular matrix events, while in the posterior near

the endothelium it was insignificant, testifying preserved postoperative desiccating functionality. In contrast, in one of the cases, we observed interlamellar edema and a lot of new intrastromal blood and lymph vessels as clinicopathological correlation during inflammation. In this particular case was found keratitis supposed to be a residual effect of external contamination. Normally, the corneal vessels are found in the limbus region, but are nonspecific for the central cornea. Only severe inflammatory conditions can result in a massive upregulation of proangiogenic growth factors overwhelming the antiangiogenic mechanisms, resulting in stromal vessels ingrowth to the center [4]. Neovasculature was accompanied by an abundance of incoming inflammatory elements, anterior and posterior corneal stromal differences in keratocyte populations and the development of fibrous tissue and disorganized lamellae in the process of restoration of the stromal defect. In this case could be seen injury responses in eye, which determine an exacerbation of the normal physiological processes, influenced by extracellular matrix proteins and growth factors, as was previously described [22]. Moreover, we observed visible layer, resembling the pre-Descemet's layer - Dua's layer discoved in 2013 by the team of Harminder S. Dua [11]. It was announced that the new layer in the human cornea supports the endothelium thus having many surgical and clinical implications. In literature such layer was not indicated in rabbit cornea, which provoked our interest in the direction of tracking more deeply the existence and histological specifics, if this layer is proved in future studies.

Conclusion

The involvement mainly of corneal epithelium, stroma and endothelium formed a complex and dynamic cascade of microevents restoring the entirety of eye cornea, but also depending on the type and strength of the injury process. Histological diversity from the intact cornea prolongs months after the invasion and could be aggravated in certain postoperative situations.

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