Biophysical and microstructural changes of swelling cornea caused by endothelial cells damage

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Summary

Biophysical properties and microstructural changes of swelling cornea which caused by endothelial cells damage will be evaluated. Swelling cornea models were established by endothelial cells damage in 114 Sprague Dawley rats. Relative gray value, swelling rate and light transmittance were measured to evaluated the biophysical properties and microstructure changes were observed by transmission electron microscopy. Relative gray value decreased while swelling rate rose along with time and both of them reached relative stability after 7d. Light transmittance showed a decline trend with time even after coreal thickness had reached stable stage. Observed by transmission electron microscopy, interfibrillar distance increased, fewer proteoglycans coating appeared and remnants proteoglycan branches became thinner and longer in 7d. Diameter of fibrils didn't change obviously with time. In cornea edema models caused by endothelial cells damage, the changes of biophysical property and the microstructure can help us evaluate corneal edema accurately and objectively.

Key words:

Swelling cornea; biophysical properties; microstructure

As the frontmost structure of the eyeball, cornea is the primary refractive

element when external light enters the eyeball. Under normal circumstances, cornea is in a state of relative dehydration. Maintaining corneal deturgescence is essential for keeping transparncy. When it is in pathological conditions and increases water content, cornea will lose its transparency. As a result, the vision will be severely affected. Comprising over 90% of corneal thickness, corneal stroma is made up mainly of collagen fibrils. They regularly pack with a highly ordered hierarchical organization and have a homogeneous diameter of 25-30 nm in human(Bancelin et al., 2014; Chen et al., 2015). It is known that in swelling corneas, the interfibrillar distance increased as well as overall lamellas disorganized(Muller et al., 2001). However, the biophysical properties and microstructural feasures were seldom analyzed simultaneously.

In this study, we established corneal edema model by endothelium damaged in rats. Using ultrasonic corneal thickness measurement technology, anterior segment photography, light transmission measurement and transmission electron microscope (TEM), we aim to display the biophysical properties and microstructural changes in swelling corneas.

Materials and methods

Sample Preparation

The study was conducted according to guidelines approved by the Ethical Committee in Animal Research at Zhejiang University and the tenets of the NIH Statement for the Use of Animals in Research. Five-week-old Sprague Dawley (SD)

rats with body weight ranging from 130 to 150g were pre-checked for corneal disease before obtained samples. They were housed in the animal labs at Zhejiang University for one week prior to use. After the rats sacrificed with intraperitoneal injection of overdosed anesthetic of chloral hydrate, eyeballs were obtained with epithelium intact immediately. A circle with a radius of 2 mm on the central corneal endothelium was stripped by a needle. Then, the eyeball was totally immersed in the Ringer's solution (sodium chloride: 0.85%; potassium chloride: 0.03%; calcium chloride: 0.033%) for 0h, 2h, 4h, 8h, 1d, 2d, 4d, 7d, 8d and 9d. The eyeballs were stored at 4℃ for incubation and the solution was replenished every 24 hours to maintain its compsition.

Anterior segment photography and relative gray value

Six eyeballs were performed anterior segment photography at each time point. To evaluate the opacity quantitatively and objectively, the relative gray value (RGV) was performed as described previously(Xu et al., 2005). Briefly, corneas were excised along cornea-scleral limbus and put onto a white paper with black crosses. Took photoes with same camera exposure settings and illumination conditions and then scaned them and obtained the data using IPP software (Image Pro Plus 6.0).

$$RGV = \frac{\text{grey value of black cross}}{\text{mean grey value of four adjacent white background}}$$

Central corneal thickness and swelling rate

Central corneal thickness (CCT) were measured at each time point by

ultrasound pachymeter (SP-3000, TOMEY, Japan). Each measurement consisted of 10 consecutive readings and the average was calculated after excluding the highest and lowest readings. To account for differences of corneal thickness between samples, swelling rate (SR) rather than CCT was evaluated.

$$SR = \frac{\text{change of CCT}}{\text{baseline CCT}} * 100\%$$

Light transmission measurement

Six corneas were cut with a 10-mm trephine at each time point. The cornea was washed 3 times using phosphate buffer solution (PBS) and placed on a 48-well microplate. Blank-corrected light absorbance was measured with microplate reader (Multiskan Spectrum, Thermo Scientific, USA) with wavelengths ranged from 400 nm to 800 nm. Light transmittance (LT) was calculated by the Beer–Lambert equation: transmittance (%) = $10^{2\text{-absorbance}}$.

Transmission electron microscopy

Corneas at 0d, 1d, 2d, 4d, 5d, 6d, 7d and 14d were fixed in 2.5% glutaraldehyde overnight. Each specimen from central corneas were cut with a 3-mm trephine and rinse with PBS. Stained the samples with 1% osmium tetroxide on ice for 1 hour and rinsed again. After dehydration with a graded series of ethanol solutions, the samples were embedded in Eponate 12 (Ted Pella, Redding,

CA) at 60°C for 2 days. Sections were cut by an ultramicrotome (Ultracut UCT; Leica, Austria) and stained with uranyl acetate. Then they were examined using a TEM (Philips TECNAL-10, Japan).

Statistical analysis

The data were analyzed by SPSS 20.0 statistical package. The normality of distribution of continuous variables was tested by one-sample Kolmogorov-Smirnov test. Continuous variables with normal distribution were presented as mean ± standard deviation (SD) and analyzed by Analysis of Variance (ANOVA). The Bonferroni test was used when the vriances were equal, and the Tamhane's T2 test was used when the variances were unequal. Non-normal variables were analyzed by Kruskal-Wallis test. A value of $P < 0.05$ was considered to be statistically significant.

Results

Anterior segment photography and relative gray value

As the immersing time prolonged, corneal oedema was aggravated (Figure 1A). At 0d, the corneas were transparent and the iris texture can be seen clearly. From 1d to 7d, the corneas gradually became opaque and edematous. At the time of 1w, corneas were severely edematous and iris was invisible.

Mean RGVs of swelling corneas at different time (0d, 2h, 4h, 8h, 1d, 2d, 4d, 7d, 8d and 9d) were (68.04 ± 3.94), (64.42 ± 3.31), (60.58 ± 0.39), (58.62 ± 1.14),

(54.54 ± 1.81), (52.86 ± 4.04), (49.68 ± 2.22), (39.52 ± 3.88), (36.35 ± 3.43) and (38.72 ± 3.69) respectively. It plunged from 0d to 1d, and reached relatively steady state after 7d. (Figure 1B).

Swelling rate

To reflect the changes of corneal thickness exactly, SRs were analyzed in this study. Mean SRs of swelling corneas at different time (2h, 4h, 8h, 24h, 48h, 4d, 7d, 8d and 9d) were (18.08 ± 4.04)%, (25.47 ± 4.31)%, (31.12 ± 4.24)%, (69.21 ± 11.38)%, (122.84 ± 15.62)%, (204.83 ± 24.51)%, (245.50 ± 21.22)%, (251.45 ± 26.00)% and (246.86 ± 26.50)%. Opposite to RGV, SR rose rapidly from the start, slowed down at 4d and reached steady state at 7d. (Figure 1C).

Light transmission

The LTs of swelling corneas declined throughout the visible light range as time went by, and rose slightly with the increase of wavelengths (Figure 1D). Since different lights had similar trends, LTs at 500nm wavelength (green light) were measured. The results showed LTs after 2d had significant difference compared with 1d ($P<0.05$). Besides, compared with 9d, the values before 4d were statistically different (Figure 1E).

Ultrastructure of the stroma

Cross sectioned and longitudinally sectioned collagen bundles of the central region of swelling corneas were observed by TEM. Regular arrangement of collagen fibrils within a lamellar was observed in fresh corneas. In early stage of corneal edema (within 1d), the basic structure of collagen fibrils was intact and the collagen fibers within a lamellar were in a regular arrangement. During the chronic phase or late stage (from 1d to 7d), the overall structure became irregular gradually. Not only interfibrillar distance increased, but the collagen fibrils themselves appeared different shapes, such as shorter, irregular and more disorganized, as well as necrosis cell debris appeared. In addition, the broken collagen fibrils within a lamellar were in extremely different orientations, which differed from the previous orderly arrangement situation (Figure 2A). On the other hand, collagen fibrils coated with proteoglycans arranged closely and tethered by short and thick branches of proteoglycans in early stage. However, fewer proteoglycans coating appeared and the remnants proteoglycan branches became thinner and longer with time (Figure 2B) . The density and diameter of

collagen fibrils of swelling corneas were calculated in TEM photoes. The result indicated that the density droped with time and stabilized after 6d (Figure 2C). However, the diameter of collagen fibrils didn't change apparently over time (Figure 2D).

The optical properties and microstructure of swelling cornea could be scaned from Figure 3.

Discussion

The corneal endothelium plays a key role in maintaining the hydration of cornea through 'pump-leak' mechanism. After we destroyed the endothelial cells, the pumps were not able to work properly. As a consequence, the balance between fluid absorption and exclusion was disturbed.

In this article, we obtained general structures of the cornea through anterior photography. The transparency of the cornea decreased significantly and the iris texture turned into invisible gradually over time. RGV plunged within 1d, then slowed down and reached a relatively stable stage after 7d. Changes in corneal thickness are very sensitive to corneal edema(Monti et al., 2002). In our study, SR increased rapidly within 4d, then slowed down and stabilized after 7d. These results showed that RGV had close relationship with SR. Interestingly, from beginning to 4d, trends of these two parameters were not completely reversed.

This indecated there were other factors participated in the process of corneal opacity except changes of corneal thickness.

LT is an important optic parameter for corneal tissue. In general conception, collagen fibrils were regularly packed and arranged as orthogonal layers or lamellae. This lattice-like structure produces minimal light scattering, or maximal LT, which is necessary for corneal transparency(Benedek, 1971; Farrell et al., 1973; Maurice, 1957). Once corneal edema occurs, swelling corneas will show remarkable periodic fluctuations in the refractive index over distances comparable to, or larger than one half the wavelength of light, which will be enough to increase light scattering. Change of refractive index was always accompanied by the changes in corneal ultrastructure. The existence of a large number of keratocytes in the stroma will make scattering problem even more perplexing. Keratocytes are the main cells in the corneal stroma. They are transparent except for their nuclei and contribute up to 15% of the total stromal volume(Huang and Meek, 1999). Without any doubt, when cornea swells to some extent, keratocytes may form 'lakes', which bring great light scattering(McCally et

al., 2007; Moller-Pedersen et al., 2000). This process was supposed to have relationship with the loss of protein expression or development of prominent actin filament bundles(Jester et al., 2005). Meek found that local fibrils disordering, refractive index mismatch and corneal thickness increasing together accounted for 20% increase in light scattering and additional scattering was probably caused by 'lakes'(Meek et al., 2003). Those may explain why we found LTs decreased with time even after SR had reached stable stage in this research.

Considering collagen density of corneas varies in different regions and depth of the cornea , we observed all specimen from central corneas(Boote et al., 2003; Freund et al., 1995). As TEM showed, the overall structure became irregular gradually with time increased. Not only the density of collagen fibrils and proteoglycans droped, but also the morphology of proteoglycans changed and fewer proteoglycan coating left. Fratz et al. suggested the fibrils start to be dehydrated after coating released all water in the two-stage drying model of cornea (Fratzl and Daxer, 1993). Cheng et al. pointed out that the proteoglycans attached to collagen fibrils play an important role in maintaining the order of the lattice, which is essential to keep corneal transparency(Cheng and Pinsky, 2013). Lewis et al. proposed mechanism on how the interfibrillar distances are maintained by proteoglycans(Lewis et al., 2010). The distances between adjacent

collagen fibrils are a consequence of the balancing of two opposing forces acting on the fibrils, which the repulsive forces between the fibrils provided by the pressure exerted by water molecules and the attractive forces provided by proteoglycans.

In our research, the destruction of endothelial cells led to corneal overhydration, which disrupted the equilibrium of adjacent fibrils. we observed the density and morphology of proteoglycans changed notably over time which may contribute to the change of corneal transparency. It is noteworthy that the electron microscopic images did not show significant differences in diameter of the collagen fibrils. Similar results were discerned in Müller's study(Muller et al., 2001). In this study, Müller made corneal stroma in extreme hydration by deionised water treatment. He found the diameter of the collagen fibrils did not be affected over time even after 6 months. Vitro experiments showed that the type V collagen molecule terminal domain was necessary to regulate the diameters of the formed fibrils(Birk et al., 1990). Moreover, additional mechanisms such as microfibrillar coiling and collagen-bound disaccharides also contribute to fibril diameter regulating(Meek and Knupp, 2015). It seems that fibril diameter would not affected by overhydration of stroma.

We found several properties and ultrastructure changes of the swelling corneas induced by ECs-damaged in rats. Rat corneas are different with the human corneas. Its endothelial cells can repair after mechanical injury involves

significant mitosis. However, we thought it had little influence to the outcomes because the enucleated eyeballs stored at relative low temparature. In further studies, expanding sample size and vivo studies will be considered.

Conclusion

In this study, we explored the changes among general structure, SR, optical properties and ultrastructure changes of the swelling corneas induced by ECs-damaged. The internal relationship between biophysical and microstructural changes may contribute to evaluating corneal microstructure and grade corneal edema accurately and objectively.

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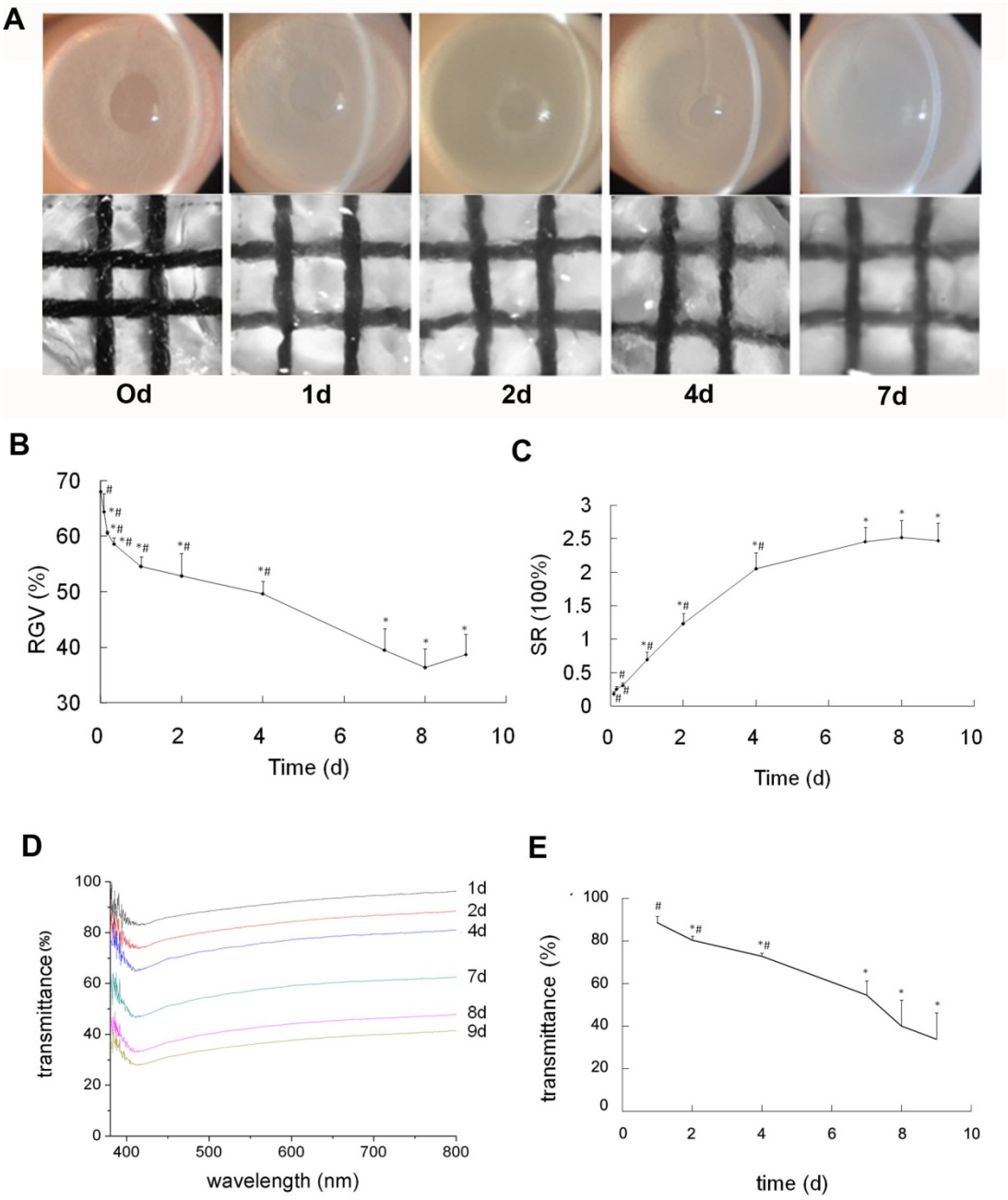

Figure 1. Biophysical changes of swelling cornea caused by endothelial cells damage at different times. **A**, anterior segment photography; **B**, RGV, * P < 0.05 compared with 0d , # P < 0.05 compared with 9d; **C**,SR, * P < 0.05 compared with 2h , # P < 0.05 compared with 9d; **D**, LTs of swelling corneas with different wavelengths; **E**, green light transmission of swelling corneas, * P < 0.05 compared with 2h , # P <

0.05 compared with 9d . Data are mean ± SD.

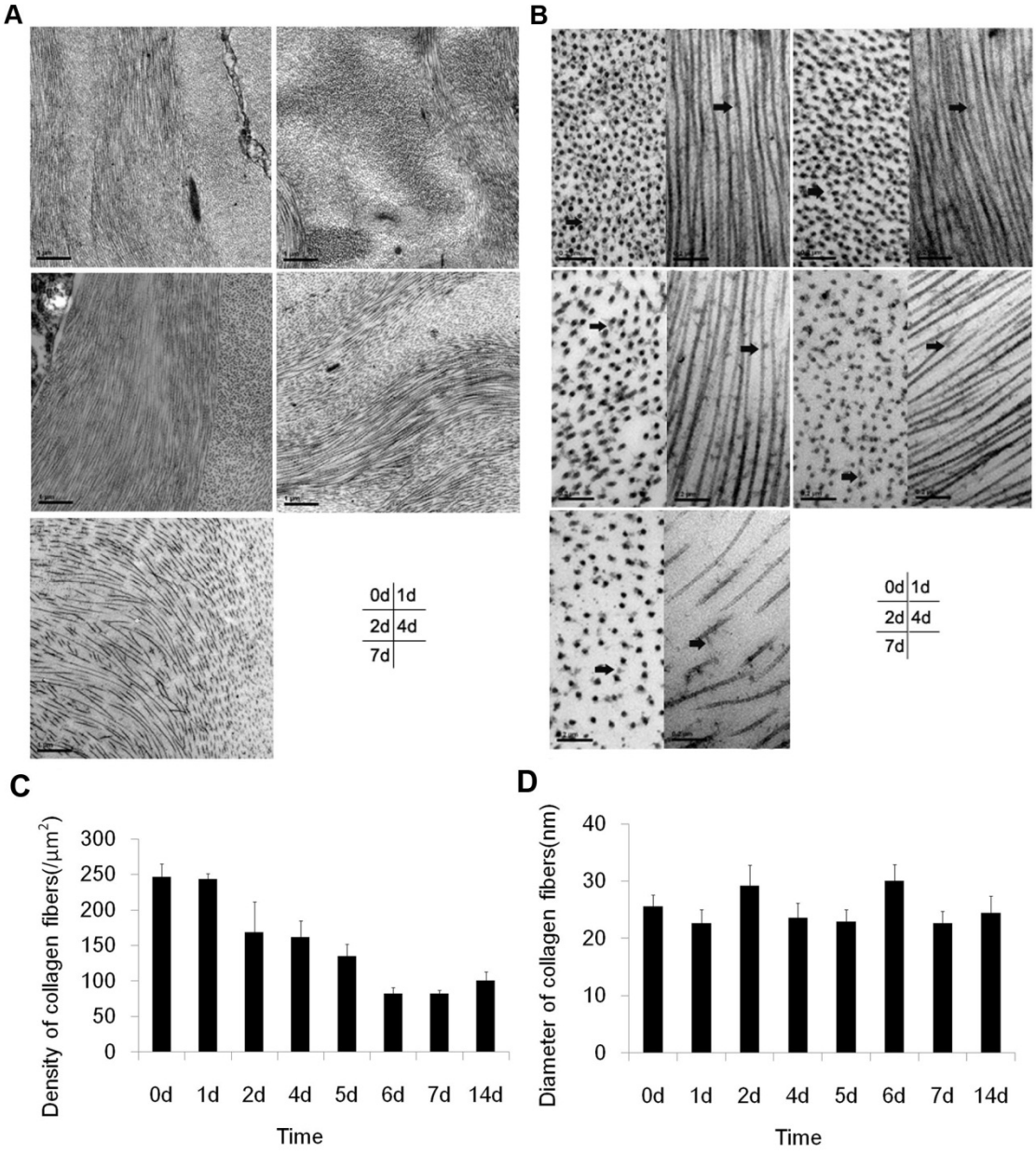

Figure 2. TEM of corneal stroma in swelling corneas. **A**, Ultrastructure became irregular and disorganized. The bars represent 1 μm (20,000×) ; **B**, Cross sectioned (left) and longitudinally sectioned (right) collagen fibrils were observed over time. Proteoglycans in interfibrillar space were showed by black arrows. The bars represent 0.2 μm (100,000×); **C**, Density of collagen fibrils in swelling corneas; **D**, Diameter of

collagen fibrils in swelling corneas.

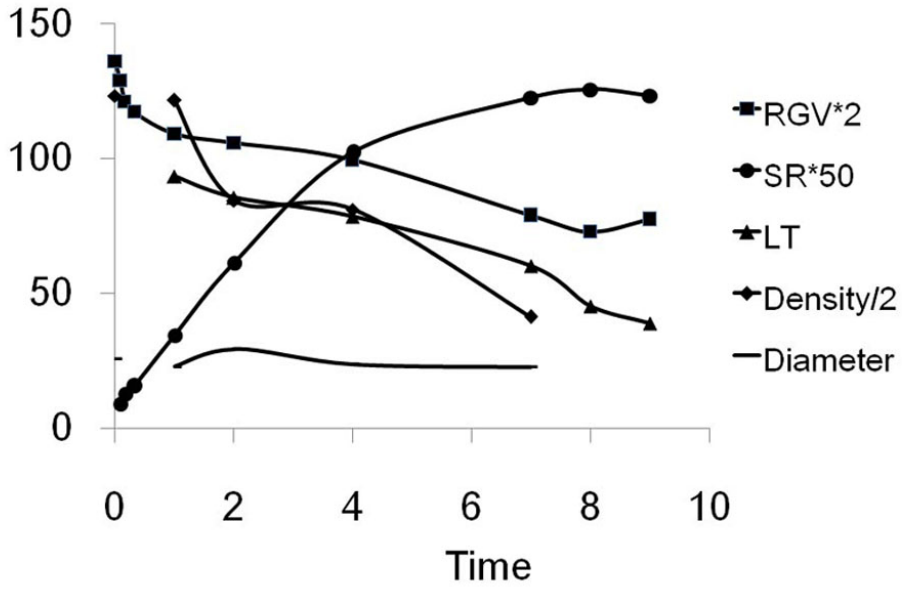

Figure 3. Changes of biophysical properties and microstructures of swelling cornea.