

Histomorphometrical and histochemical postnatal development of cornea in indigenous rabbits

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Abstract

The present study aims to clarify corneal development via analyzing of the histomorphologic and some histochemical parameters in local rabbits. Samples were collected from 25 Rabbits divided into five different age groups at 1, 10, 15, 30, and 40 postnatal day (PND), then the samples sectioned, processed, and stained with Hematoxylin and eosin stain and Masson's trichrome stain. Some sections were further stained with periodic acid Schiff (PAS) and toluidine blue (TB) stain for histochemical evaluation. Measurements of corneal layers performed for morphometric comparison among age groups. The finding revealed thickening of corneal epithelium, stroma, and corneal endothelium progressively with age and decrease of corneal layer's cellular density concurrently with alteration of corneal epithelium and corneal endothelium cell's type. The histochemical finding revealed late appearance of bowman's and Descemet's membranes after eyelid opening which responded strongly to PAS technique while stroma became metachromatic strongly responded to toluidine blue stain. In conclusion, cornea showed highly active histological development and cellular differentiation before and after eyelid opening.

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Introduction

Cornea is the transparent frontal portion of the eye that protects the pupil, iris, and the anterior chamber. Its main function is to concentrate and refract the majority of light as it passes into the eyes (1). The cornea composed of 5 layers: the corneal epithelium, corneal anterior membrane (Bowman's membrane) corneal stroma, corneal posterior membrane (Descemet's membranes) and the corneal endothelium (2), rabbit eyes commonly used for experiments and assessing of ophthalmic drugs so understanding of the histological development of eye may help to identify time-sensitive point and effect of tissue differentiation exerted by drug administration (3). In addition, rabbit eyes highly sensitive to irritants in comparison with those of humans (4) furthermore, rabbit's eyes sharing many histological and anatomical features with human and other domestic animal make their eyes

suitable for ophthalmic surgical evaluation and for inspection of new ophthalmic surgical strategies (5), Rabbit's eye development used to study several diseases related to age like dry eyes, age-related macular degeneration, glaucoma and cataract (6). In fact, the rabbit corneal model was used to understand and diagnose many diseases like fungal and bacterial keratitis (7) and to evaluate corneal wound healing, corneal epithelium regeneration (8), corneal refractive surgery (9), Moreover, corneal model development used in bioengineering grafting, corneal transplantation and also experimental histopathological researches of anterior chamber structures (10). The Histomorphometric study of the cornea highly considered to analyze the geometrical and biomechanical parameters that affect human and animals in relation to the age (11). Many other researchers targeting histological and histomorphometric development of the cornea in many species of animals, but rabbit corneal information still not

yet available was the reason to achieve the present study which was aimed to the demonstration of histomorphometric and histochemical development of the cornea in local rabbits (*Oryctolagus cuniculus*) after birth.

Materials and methods

Twenty-five healthy local rabbits were used in this study and were obtained from animal House, College of Veterinary Medicine, Mosul University, Iraq. Regardless of their sex, animals were divided into five different age groups at 1,10,15,30,40 postnatal days (PNDs). The animals euthanized before killing according to the ethical guidelines that were approved according to American veterinary medical association (12). Eyes and eyelids were excised using fine surgical tools and placed into physiological saline for the cleaning them from blood and tissue debris then were immersed into Davidson's fixative prepared according to Shariati (13) for 24 hours and to ensure rapid penetration. Caudal part of eye was injected with the same fixative above. The fixative was changed in the next day with 10% neutral buffer formalin and was kept for 48 hours.

The Samples were washed, cut, and were dehydrated in ascending series of graded alcohol concentrations 70, 80, 90, 100% for 2 hours each stage, later they were cleared with xylene for 30 minutes then were infiltrated with melted paraffin 58°C for 3 hours and casted into blocks, 5 micrometers thick sections were achieved using a rotary microtome and were mounted on glass slides, sections stained with Harries Hematoxylin and eosin stain according to the protocol of Bancroft (14) and Masson's trichrome stain according to (15), then they were examined under the light microscope (Olympus-Dm-CBAD, Japan) for routine histological assessments, histomorphometric evaluation, and for some histochemical analysis. Other sections were stained with Periodic Acid Schiff stain PAS to detect glycoprotein in Bowman and Descemet's membrane and with toluidine blue for detection of metachromasia through corneal stroma, and the reaction's intensity was measured according to different age periods (16).

Histomorphometric procedure

The Measurements performed to compare some parameters among groups using a 2.0 USB digital camera (scope image 9) provided with image processing software, parameters include the thickness of cornea as a whole, thickness of the corneal epithelium, thickness of corneal stroma, thickness of corneal endothelium and thickness of Descemet's membrane (17).

Statistical analysis

Data from each group was calculated to obtain the mean and the standard error followed by one-way analysis of variance (ANOVA) and Duncan post hoc test to understand

statistical differences among groups using IBM SPSS version 25.0 software at $P \leq 0.05$.

Results

Corneal epithelium

The corneal epithelium at PND 1 composed of two to three cell layers: basal cuboidal cell layer and flattened superficial layers (wing cells). The basement membrane (bowman's membrane) was indistinct at this period and the mean of total thickness of corneal epithelium was 7.2 ± 0.24 micrometers (Figure 1). The number of cell layers and the cell density increased at PND10 prior to eyelid opening and the cells changed from cuboidal-shaped cells to columnar in basilar layer. There was also no evidence of the basement membrane with a slight increase in epithelial thickness with the mean of 14.0 ± 0.32 micrometers (Figure 1). The corneal epithelium thickened noticeably at PND15 where mean was 24.0 ± 0.42 micrometers (after eyelid opening), epithelium changed into non-keratinized squamous cells of four to six layers. The superficial and basal cells became further packed than before (Figure 1). The corneal epithelium thickness continuously increased with mean of 32.9 ± 0.32 micrometers at PND30 as the basal cells began to extent upwards, and the superficial cells become even more wider. Using PAS, the positive content was found inside the cytoplasm of a basal region (Figure 1). The corneal epithelium cells reach maximum growth at PND 40 composed of five to six layers and the bowman membrane is very clear with a marked increase in total mean thickness 45.5 ± 1.93 (Figure 1). Morphometric results of corneal epithelial thickness show significant statistical differences among age groups (Table 1)

Corneal stroma

The corneal stroma at PND1 had two layers, the outer-sided (anterior) fibro areolar layer and the inner-sided (posterior) lamellar fibrous layer (Figures 1-3). The stromal connective tissue in the anterior fibro areolar layer was thin, and stromal keratocytes organized in a linear pattern. The posterior layer was composed primarily of collagen fibers bundles arranged in lamellar pattern each lamella was parallel to each other, but at different angles with neighboring lamella which was moderately thick in addition, to the high density of stromal keratocytes. Stromal thickness at PND 1 was thin and measured with mean of 92.1 ± 0.35 micrometers. Using Masson's trichrome stain, the Stromal keratocytes were flattened cells that appeared scattered between collagen lamellae with oval nuclei found in both layers (Figures 1). The collagenous extracellular matrix density increased in both the anterior and the posterior layers at PND 10 and the thickness of the whole stroma is slightly increased with mean of 111.7 ± 0.48 micrometers.

At PND15, the stroma is significantly thickened and was consisting of the lamellar fibrous layer only (after eyelid opening), with the disappearance of fibro areolar layer. There was a further rise in stromal thickness with mean of 139.7 ± 1.56 micrometers, with a decrease in the density of stromal keratocytes (Figure 1). The thickening is

continuous rapidly at PND 40 and reach maximum histological growth of 322.4 ± 0.86 micrometers. as well as the density of stromal keratocytes had decreased, Morphometric results of corneal epithelial thickness show significant statistical differences among age groups (Table 1).

Table 1: The micro-morphometric parameters of Cornea of rabbits at different age groups

Variables	Post Natal Day (mean±SE)				
	PND 1	PND 10	PND 15	PND 30	PND 40
Corneal epithelium thickness (µm)	7.2±0.24 ^A	14.6±0.32 ^B	24.0±0.42 ^C	32.9±0.32 ^D	45.5±0.39 ^E
Corneal stromal thickness (µm)	92.1±0.35 ^A	111.7±0.48 ^B	139.7±1.56 ^C	252.6±0.64 ^D	322.4±0.86 ^E
Corneal endothelium thickness(µm)	2.1±0.12 ^A	3.0±0.55 ^B	3.5±0.11 ^B	4.2±0.11 ^C	5.0±0.04 ^E
Total corneal thickness (µm)	101.7±0.89 ^A	133.5±0.86 ^B	172.1±0.62 ^C	293.2±1.24 ^D	397.2±1.93 ^E

Different letters among age in row indicate statistical significant differences at $P \leq 0.05$.

Corneal Endothelium and the corneal posterior membrane (Descemet’s membranes)

The Descemet’s membrane not noticeable, and the corneal endothelium was originally consisting of a one cuboidal cells layer at PND 1 (Figure 1) and the mean thickness was 2.1 ± 0.12 micrometers.

There was no distinguishable histological alteration in the morphology of endothelial cells at PND10 with a slight increase in thickness 3.0 ± 0.55 micrometers. The endothelium of the cornea was considerably flattened at PND15 (after eyelid opening). And Descemet’s membrane slightly thickened, at PND 40 the mean thickness progressively increased to reach 5.0 ± 0.04 micrometers, Descemet’s membrane thickened and became more obvious.

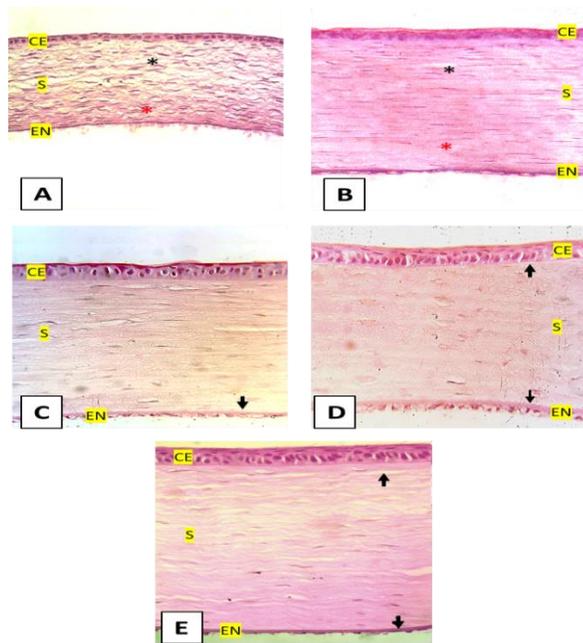


Figure 1: Microphotograph of corneal sections illustrated the histological development of cornea in different ages groups. Postnatal day 1 (A), fibro areolar layer (black asterisk), fibrolamellar layer (red asterisk). Postnatal day 10 (B) Postnatal day 15 (C), Postnatal day 30 (D), Postnatal day 40 (E). Bowman’s membrane (upper arrow), Descemet’s membrane (lower arrow), corneal epithelium (CE), corneal stroma (S), corneal endothelium (EN). (H&E, X400).

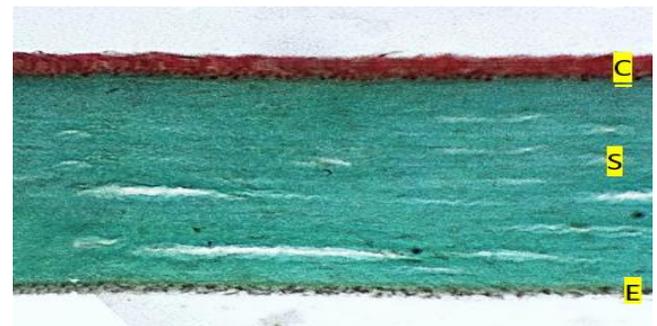


Figure 2: Microphotograph of cornea of a rabbit at PND1, keratocytes spread everywhere within corneal stroma (arrow), corneal epithelium (CE), corneal stroma (S), corneal endothelium (EN). (Masson’s trichrome stain, X100).

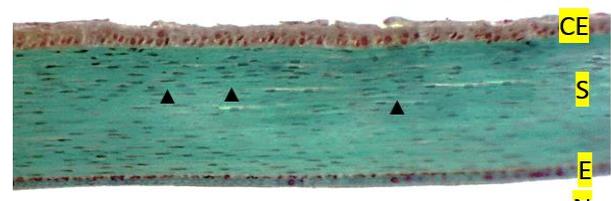


Figure 3: Microphotograph of cornea of a rabbit at PND 40, corneal epithelium (CE) corneal stroma (S), corneal endothelium (EN). (Masson’s trichrome stain, X100).

Histochemical findings

The Histochemical analysis of the cornea of rabbits at different age groups was revealed different levels of reaction to the carbohydrates. PAS staining technique shows positive reaction to basement membranes (Bowman’s membrane and Descemet’s membrane), which was strongly positive Responded in sections at 30 and 40 PND groups. Moderate reaction was observed at PND15 group, while there was no clear reaction was seen at PND1, and PND10 groups as the corneal stroma was poorly stained (Table 2, Figure 4). The metachromasia was demonstrated with the toluidine blue technique. The corneal stromal ground substance reacted positively to toluidine blue in a strong manner at PND 15, PND 30, PND 40 and moderately at PND1 and PND 10, while the corneal epithelium and corneal endothelium were negatively responded to this stain. (Figure 5).

Table 2: The reaction of Corneal structures to PAS and TB stains at different age groups

Variables	Post-natal day					
	1	10	15	30	40	
PAS	Bowman’s m.	-	-	++	++	+++
	Descemet’s m.	-	-	++	+++	+++
T. B	Corneal stromal	++	++	+++	+++	+++

m: membrane; +++: very strong; ++: strong; +: moderate; -: no reaction

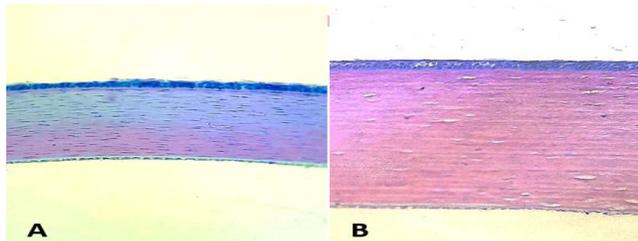


Figure 4: Microphotograph of rabbit's cornea, showing metachromasia through corneal stroma (purple color), PND 1(A),PND 40 (B). (Toluidine blue stain, X100).

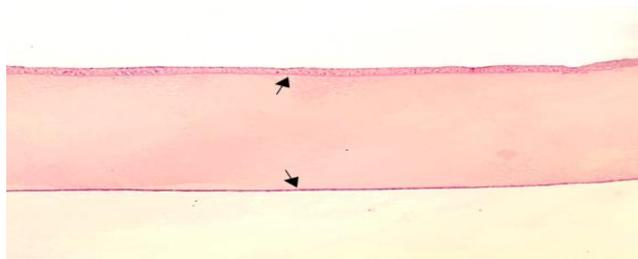


Figure 5: Microphotograph of cornea of rabbit at PND 40, Bowman’s membrane (upper arrow), Descemet’s membrane (lower arrow), (PAS stain, X100)

Discussion

The present study aims to clarify the corneal development of local rabbits using the histomorphometric and some histochemical parameters at different age periods from PND 1 to PND 40. The corneal histological development and differentiation highly active in the period from PND 1 to PND 15 as it were associated with eyes opening. Researchers were attributed the postnatal development of cornea and conjunctiva after eyes opening to light stimulation or exposure to light and atmosphere, which had a greater effect on mitosis in corneal surface (18).

Corneal epithelium results showed features of rapid histological growth starting from PND 1 which composed of 2-3 cells layer, which became nearly a double at PND10. In addition, there was a change of the basal layer cells shape from flattened or low cuboidal to columnar type cells and the space among cells decreased (tight junction). Corneal epithelium from PND15 to PND 40 was continuously thickened to reach 5-6 layers of stratified squamous type cells, furthermore, the bowman’s membrane thickened and became more evident and positively react to PAS staining technique, all these findings were similar to the researcher’s findings in Rabbit (19), and in mice (20).

The corneal stromal findings from PND1 to10 PND before eyes opening showed that stroma composed from 2 layers, thin fibro areolar and a thick fibro-lamellar layer with high cellular density. These findings are resembling to those of other works who have attributed these changes to the swelling of stroma before eyes opening and due to the presence of water molecules within stromal tissue (20,21).

They explained the fibro areolar layer’s disappearance after eyes opening to sulfation of proteoglycans and the synthesis of keratin sulfate and absorption of water. These findings also explained the strong metachromasia after eyes opening and the strong reaction to toluidine blue technique in the present study. The stromal thickness was markedly increased and the cellular density was drastically decreased and the bundles of collagen lamellae became denser and thicker in the period from PND15 to PND40. These results confirmed the results of in rabbits (22) in chickens, and in mice (23).

The corneal endothelium observations showed the fewer changes than those of corneal epithelium and corneal stroma, a slight rise of thickness starts from PND10 to PND 40. The cell density was increased after eyes opening and the cells became noticeably flattened, smaller and packed. These findings were similar to (24) findings.

The Descemet’s membrane was thickened gradually from PND 15 and became more obvious at PND 40 and it was highly responded to PAS staining technique which indicate the elevation of the carbohydrate content. These results agreed with (25).

Conclusion

The findings of the corneal development in local rabbits were showed highly active histological growth, significant morphological differentiation, different levels of histochemical responses to some stains with significant measurements differences particularly in the periods before and after eyes opening. These results provided precise data about developmental milestone with the postnatal course of time, which has a similar approach in domestic animals

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Conflict of interest

Authors declared that there is no conflict of interests.

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دراسة شكلية قياسية وكيميائية نسيجية لتطور القرنية في الأرانب المحلية بعد الولادة

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الخلاصة

أجريت الدراسة الحالية لتوضيح على التطورات النسيجية القياسية والكيميائية النسيجية في قرنية الأرانب المحلية. حيث جمعت العينات من ٢٥ أرنب سليم وقسمت إلى ٥ مجاميع بعمر ١، ١٠، ١٥، ٣٠ و ٤٥ يوم بعد الولادة، بعد ذلك تم إجراء التقطيع النسيجي للعينات وصيغت بملون الهيماتوكسلين والأبوسين واملون الماسون الثلاثي ولغرض إجراء الفحص الكيميائي النسيجي صيغت بعض الشرائح تقنية صبغة حامض البيريودك-شيف واملون التلودين الأزرق ثم أجريت القياسات المجهرية لطبقات القرنية المختلفة للمقارنة بين المجاميع

وديسيميت بعد انفتاح الجفون إلى العمر ٣٠ و ٤٠ يوم بعد الولادة وظهور التغيرات اللونية في سدى القرنية وتباين شدة الاستجابة لكلا الملونين في الفئات العمرية المختلفة، ونستنتج من الدراسة ازدياد النشاط التطوري والتمايز الخلوي خلال الفترة قبل وبعد انفتاح الجفون.

العمرية. أظهرت الدراسة القياسية زيادة في سمك ظهارة القرنية والسدى وبطانية القرنية بالتزامن مع تقدم العمر بالإضافة إلى انخفاض الكثافة الخلوية وتغيرات شكلية نسيجية في ظهارة وبطانة القرنية وأظهرت الدراسة الكيميائية النسيجية تأخر ظهور غشائي بومان