Original Article

The Study of Retinal Structure in Frugivorous Bat (Rousettus Aegyptiacus) by Light and Electron Microscope

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ABSTRACT

Introduction: Vision is fundamentally important sense in animals for perceiving their environment, but in bats less well known.

Aim of the Work: Due to the importance of vision in bats as the only mammals that can fly, the retinal tissue of the frugivore bat (Rousettus aegyptiacus) was examined by light and electron microscope in present study.

Materials and Methods: 5 male bats weighing of 123±0.8g were anesthetized, dissected, and their retina was removed. The sections (1×1mm) were isolated, fixed in 4% glutaradehyde and 2% paraformaldehyde (1hour) and rinsed with sodium cacodylate buffer (0.1). They were post fixed in osmium tetroxid 1%. The semithin sections (0.5µm) were stained with Toluidine blue, and the ultrathin sections (70 nm) were prepared. The retinal cells and layer thickness were measured by Grids-sterolite software. Obtained data were analyzed by ANOVA and t test (p<0.05).

Results: Findings showed that the ratio of the eye's diameter to the body length was 1:18.83. Retina layers conform to the general mammalian blueprint, but in wavy pattern with 106.61±16.19µm thickness. Inner nuclear layer was the thickest layer (18.52±1.55). Retina is duplex with dominant rod cells especially in centralis [≈25cell/(100µ)2], Cone/ Rod ratios: 1/ 7.95, and four other cell types with different density and distribution were seen.

Conclusion: According to obtained electro-micrographs, retinal epithelial layer was thin with poor pigmentation and its surface was covered by microvilli. The arrangement of the photoreceptor parallel to the numerous choroidal papillae, and density of rod cells is higher than cone cells according to obtained results, this species of bat can be active in both dim light and daylight.

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Key Words: Dim light, duplex retinal, megabat, photoreceptor, stereological study.

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BACKGROUND

Eye is the most important sensory receptor in animals, and this organ is different in diurnal and nocturnal animals. The retina as a major area of image formation and object recognition is a neural layer with variety of cells include photoreceptor cells and associated neurons[2]. There is a significant difference in photoreceptor population and their distribution in diurnal animals compared to nocturnal one[1].

Bats with more than almost 1400 known species comprise about a quarter of mammals and widespread throughout world[3]. They are benefit animals in ecosystem and divided to subspecies of Megachiroptera with large eye[4] and Microchiroptera with small eyes and poor eyesight[5]. They have diurnal and nocturnal spices and respond to moonlight intensity[6].

Although the anatomy and histology of bat's eye is similar to that of other mammals, but due to their adaptation to different habitats and habits, proportional differences in thickness of layer, density and distribution of retinal cells, is seen[7,8]. The documented research showed that bat's vision is dichromatic color and UV-sensitive cones that aid them in orientation, foraging and prey detection in night[9,10].

Rousettus aegyptiacus (Geoffroy 1810) is frugivorous bat that unlike the other megabat use echolocation for orientation. They produce ultrasounds by the tongue banging on the mouth wall[11] or with their wing[4]. They live in dark roosts, but able to detect small differences in brightness and hunt during the day in sunlight. The histological study of their eye showed that their retina with undulating pattern is a unique feature among mammals[12]. Although some research about structure of eye in the

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domestic animal have been done in Iran[13-15], but present study about the retinal histology of R. aegyptiacus including layers thickness, cells morphology, cell density and distribution was performed for the first time in Iran.

MATERIALS AND METHODS

The consuming materials were prepared as follow

4.28g Sodium cacodylate powder was solvated in 100cc dislated water and solution (0.2M) was obtained. Osmium tetroxid (1gm) was mixed to 98ml Phosphate Buffer, and embedded by Alminum sheet. Then 50ml TAAB was mixed with 5ml MNA. 25ml DDSA and 3ml DMP were added to them and mixed, and 50ml Asetat uranil + 25ml ethanol + 25 ml distilled water were mixed and centrifuged. 1.33g lead Citrate powder +1.76g Na Citrat + 30ml distilled water were mixed in flask. After 30 minutes, 8 ml NAOH +10 ml distilled water were added to them (total volume = 50ml).

Sampling

In the present study, 5 male frugivore bats (Rousettus aegyptiacus) weighing of 123 ± 0.8g (Figure 1) were captured by mist net in Tadvan cave (Fars Province). They were transferred to the lab in cloth bag, anesthetized and dissected their both eyes. All the experimental procedures, in compliance with regarding the National Institute of Health for using the laboratory animals.

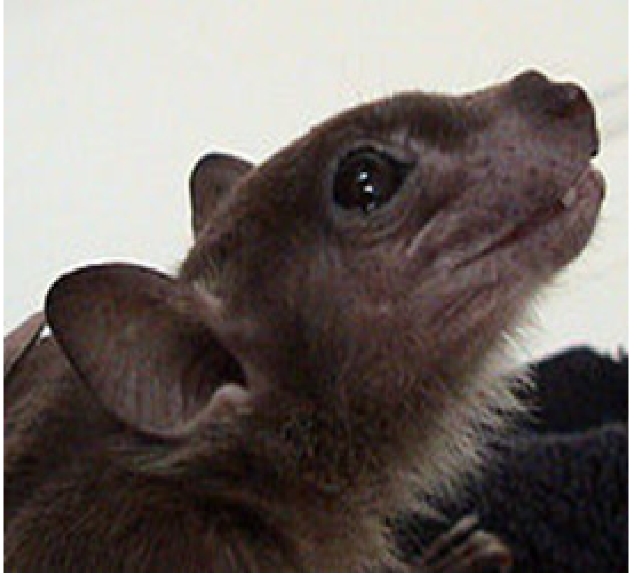


Fig. 1: Frugivore bat (Rousettus aegyptiacus)

The both eyes were removed from optic nerve local, and horizontal and vertical diameter of them using caliper were measured. Then they were immersed in 2% paraformaldehyde in 0.1 M phosphate buffer (pH=7.4) immediately. The specimens was removed by using steriomicrocop (zsm-1001). The sections (1×1mm) were isolated from peripheral (temporal and nasal corner) and central regions of the retina. They were fixed in 4% glutaradehyde (pH = 7.3) and 2% paraformaldehyde (1 hour) and rinsed with sodium cacodylate buffer (0.1) at 4°C for 2×20 minutes. The retinal tissue was separated near the optic nerve and fixed in buffer solution. They were post fixed in osmium tetroxid 1% and washed with distilled water for 2×10 minutes. After dehydration through graded ethanol series (50, 75, 95,100%), clearing in propylene oxide (100%), infiltrating with a mixture of propylene oxide and resin (TAAB) (1:1) (TAAB 812, DDSA, MNA, DMP30). Then they were incubated (65oC) overnight, and embedded in pure resin. The semithin sections (0.5µm) were prepared by ultramicrotum (C. reichert, Austria om U3) and stained with Toluidine blue. The prepared slides were examined with binocular light microscope[13].

The thickness of retinal layer and the size of photoreceptors were carried out by using Dino software and micrometer eyepiece in binocular microscope which was calibrated (using digital camera). Density and distribution of retinal cells in the central and peripheral parts of retina and their diameter in different directions were prepared by using Grids-stero lite software. So 10 slides from central and peripheral region of retina were selected, and 15 fields (70µm × 70µm) in every one were examined randomly at well-labeled positions across the retinas[16]. The number of cell types in the every field was counted (Figure 2). The mean number of cells was determined in each sample area and expressed as the number of cells (100µ)2. Since the total area of retina was not measured, This value, cells/ (100µ)2, for all cells were compared.

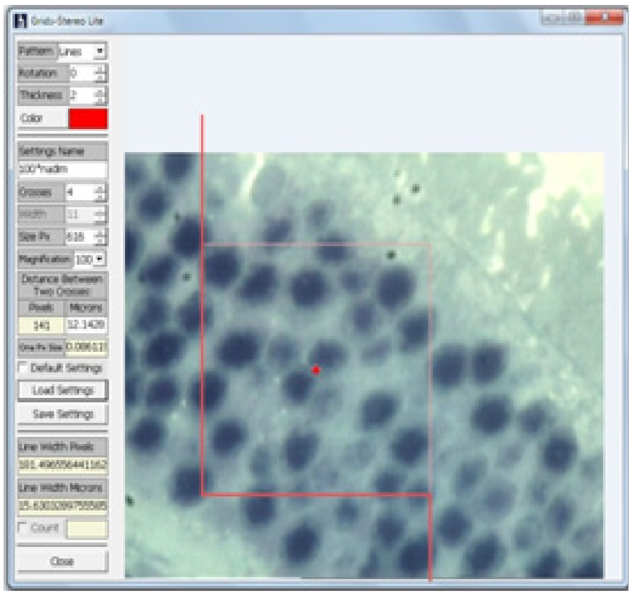


Fig 2: Retinal cell count by using Grids-stero lite.

The thin sections (70 nm) were prepared by ultramicrotum and placed on copper grids. They were stained with uranyl acetate and lead citrate. They were studied by transmission electron microscope (CM-10, Philips) in Tehran University. Also some of these sections (70nm) were dehydrated through aceton (50, 75, 100%), fixed in Hexamethyldisilazane solution, glued on the Aluminum steppes, coated gold, and studied by scanning electron microscope. The structure of retinal cells especially photoreceptor cells were studied by electron microscope. Obtained data were analyzed by using statistical Soft ware, ANOVA, and the parameters were compared by t test in significant level ($p<0.05$).

RESULTS

The macroscopic evaluation showed that R. aegyptiacus had large hemispherical eye (Figure 1), and ratio of the eye's horizontal diameter to the body length was 1:18.83 (Table 1). Although retinal tissue with ten layers follows the general mammalian scheme, but it was seen in undulating pattern (Figure 5A).

Total thickness of retina was ~106.61±16.19 µm, and retinal layers with different thickness in the centralis and peripheralis were observed (Table 2, Figures 3,5A). The pigment layer of retina was thin (9.29 ± 1.91µm) and composed of cuboidal cells which carry the melanocyte containing the melanin pigment (Table 2, Figure 5b).

Cone/rod ratio that is used as visual sensitivity index was different in central and peripheral parts of retina significantly (Table 1). Rod cell with the highest density spatially in the central region [~24.70 cell/(100 µm)2] and ganglion cell with the lowest density [~1.45 cell/(100 µm)2)] were seen, and they had no significant difference in the two regions except rod cells (Figure 4).

Inner nuclear layer with 3 to 4 rows of different cells were observed (Figures 5C,6E), and about four to five rows of nuclei with undulating arrangement were seen in outer nuclear layer (5C, 5D). Density of rod cells unlike other retinal cells were different in central and peripheral regions of retina ($p<0.05$).

According to obtained electro-micrographs (TEM & SEM), retina with undulating pattern was observed. The retinal epithelial layer was thin with poor pigmentation and its surface was covered by microvilli (Figure 6B). The inner and outer segments of photoreceptor are surrounded by outer limiting membrane (Figure 6A). The arrangement of the photoreceptor parallel to the numerous choroidal papillae (Figure 6A), and density of rod cells is higher than cone cells (Figures 6B,C)

The outer segment of the rod cells is longer than the cone cells, and include more discs which not connected to cell membrane whereas in cone cells attached to the outer segment membrane (Figure 6A). The inner segment both of photoreceptor containing mitochondria and other organelle which didn't observe clearly (Figure 6A). Cone cell with larger nuclei (Table. 1) and brighter cytoplasm are recognizable than rod cells nuclei (Figure 6D). The micrometric result of transmission electron microscopy showed that the cone cell nucleus is larger than rod nucleus (Table. 1).

The ganglion cells in different size and irregular arrangement are located in the most internal layer of retina (Figure 6F). A large nucleus which located in the corner of this cell. Also the bundles of nerve fiber that contacted with these cells were observed.

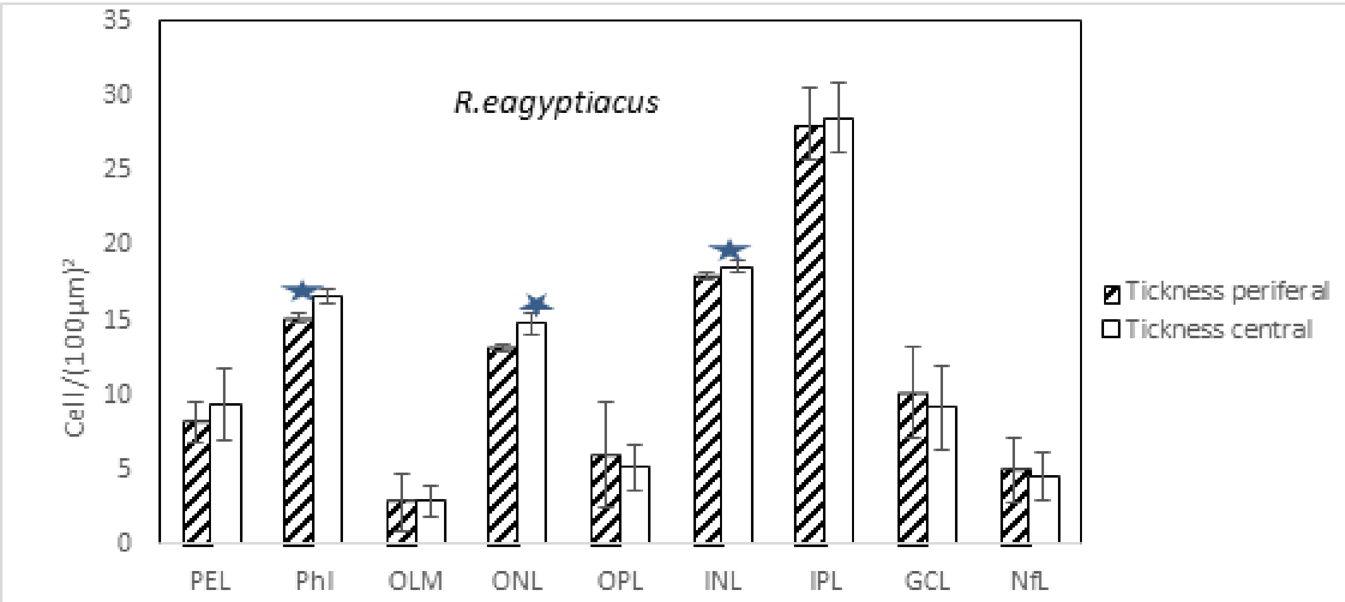


Fig. 3: Retinal layer thickness in central and peripheral region for R. aegyptiacus (Significant difference: $p<0.01$ Mean ± SD n=5)

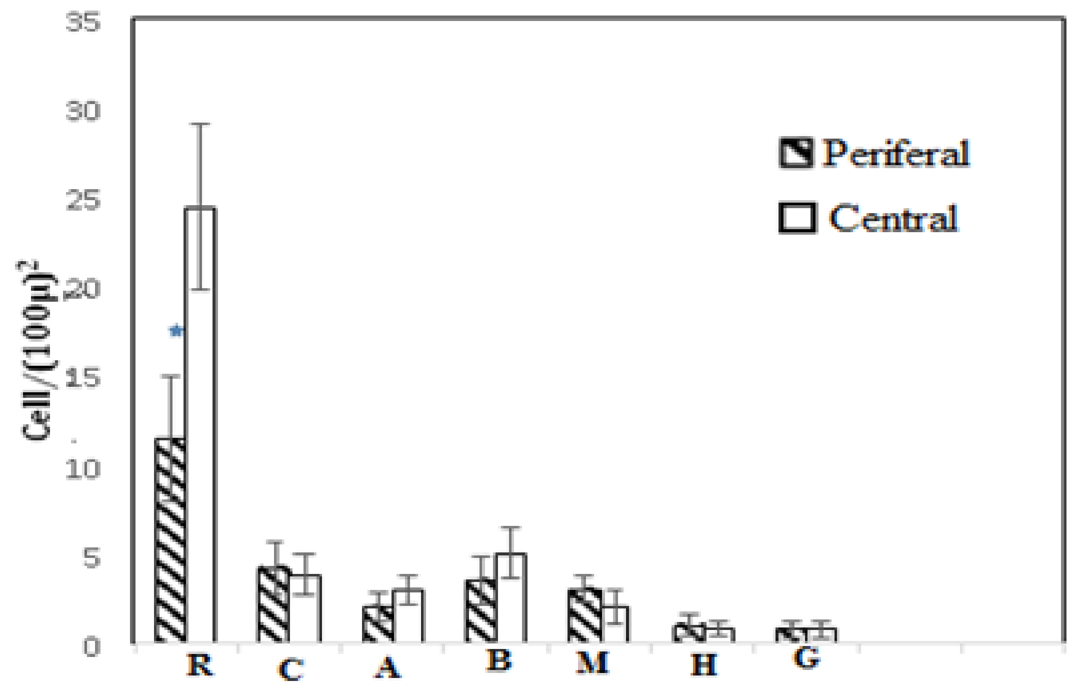
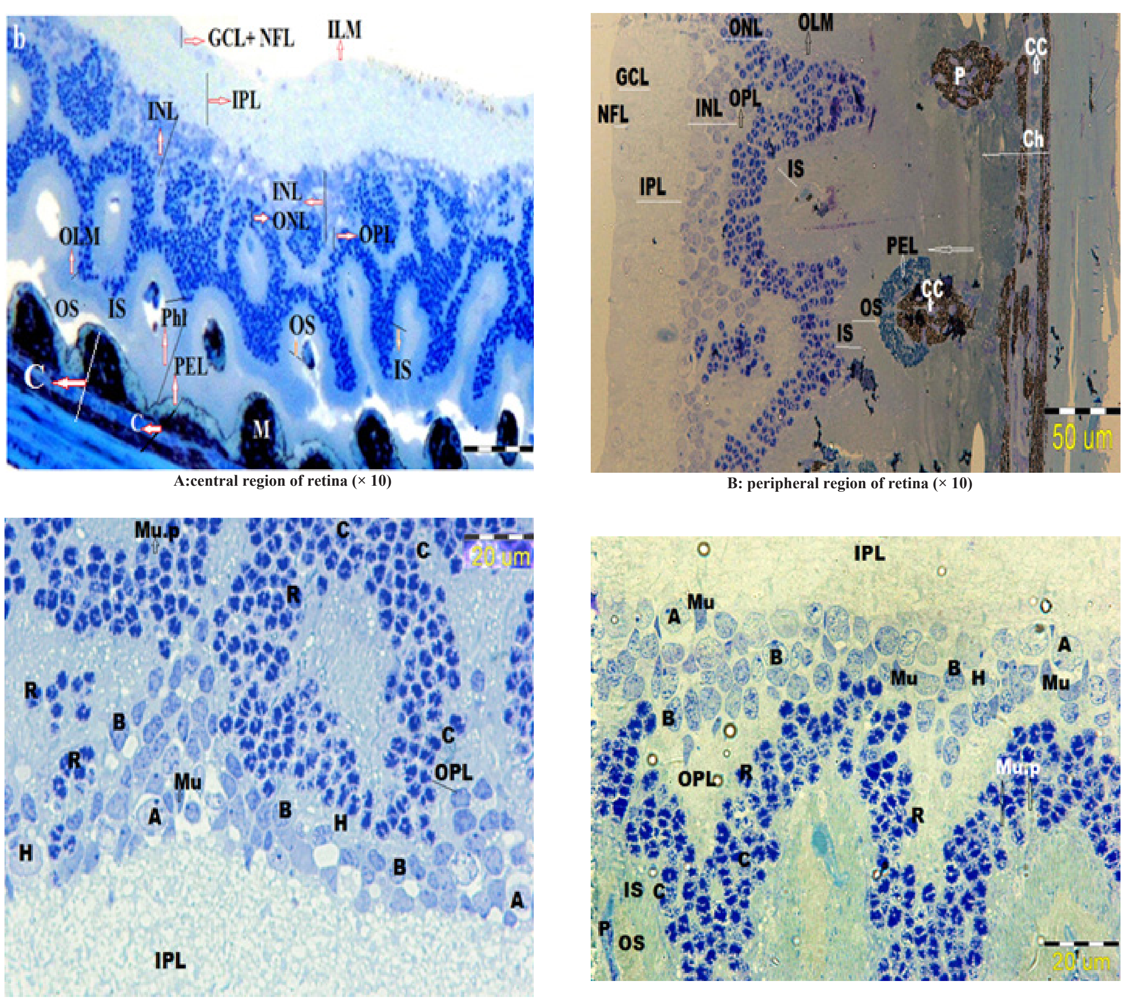


Fig. 4: The population of retinal cells in the central and peripheral of retina for R. aegyptiacus *: (Significant difference: p<0.01 Mean ± SD, n=5)



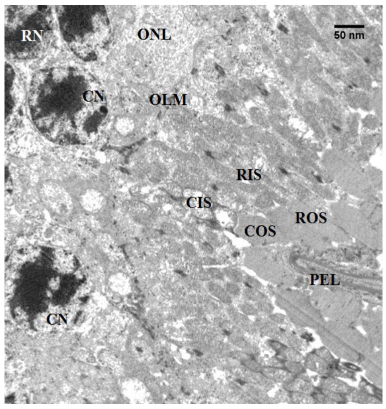
A:central region of retina (× 10)

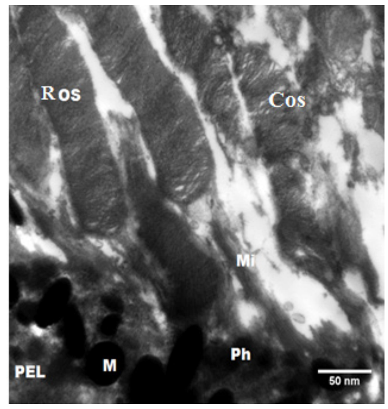
B: peripheral region of retina (× 10)

C: central region of retina (× 40)

D: peripheral region of retina (× 40)

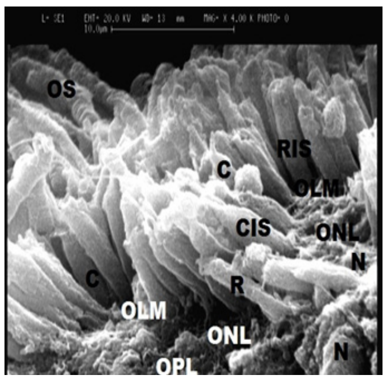
Fig. 5: Light micrographs of retinal layers and photoreceptors

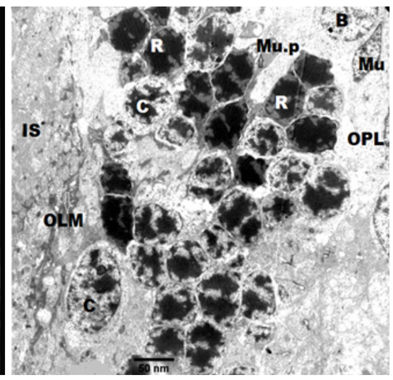




A: Retinal layers (TEM micrograph: x 2950)

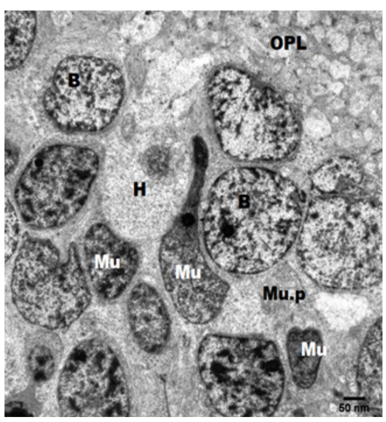
B: Retinal pigmented layer with photoreceptor outer segment (TEM micrograph: x 3950)

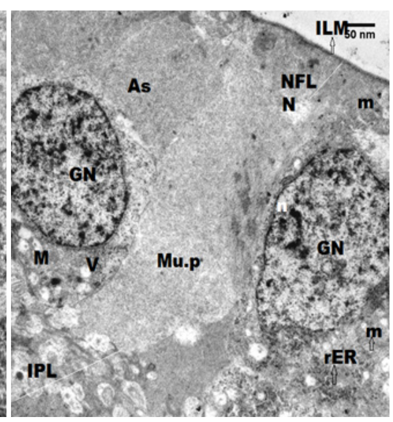




C: Retinal layers (SEM micrograph)

D: Outer nuclear layer (x 1650)





E: Inner nuclear layer with different cells (x 2200)

F: Ganglion cell layer and nerves (x 2200)

Fig. 6: Micrographs of retinal layers and photoreceptors (SEM &TEM)

Table 1: Eye parameters of R.aegyptiacus eye (Mean ± SD, n=5)

T.L(mm)	Eye HD (mm)	Eye VD (mm)	HD/TL	Cone/ Rod ratio	RND (µm)	CND(µm)
128. 03±0.12	6.8±0.14	6.08±0.28	1:18.83	P: 1/4.02 C: 1/ 7.95*	HD: 9.19 VD: 11.52	HD: 12.13 VD: 15.34

Horizontal Diameter (HD) - Vertical Diameter (VD) - Rod nuclear diameter RND - Cone nuclear diameter (CND) - Total body length (T.L) - central (C) - peripheral (P)* : Significant difference

Table 2: The thickness of retinal layers in for R. aegyptiacus (Mean ± SD, n=5)

	Retinal Layer	Thickness in Peripheral (µm)	Thickness in Central (µm)
1	Pigmened epitelium. L	8.14± 0.94	9.29 ± 1.91
2	Photoreceptor. L	14.05 ± 2.35	16.56 ± 3.60*
3	Outer limiting membrane	2.82 ±0.62	2.95 ± 0.45
4	Outer nuclear. L	12.08 ± 3.82	14.75 ±2.50*
5	Outer plexiform. L	5.98 ± 0.91	5.10± 1.94
6	Inner nuclear. L	16.89 ± 2.02	18.52 ± 1.55*
7	Inner plexiform. L	28.10 ± 1.84	28.44± 1.48
8	Ganglion cell. L	10.11 ± 1.17	9.10 ± 2.30
9	Nerve fiber. L	4.92 ± 0.86	4.52 ± 0.43
10	Inner limiting membrane	0.30 ± 0.59	0.38 ± 0.03
	Total retinal thickness	104.39 ± 15.12	109.61± 16.19

*: Significant difference The photoreceptor layer, outer and inner nuclear layer in centralis was significantly (P<0.05) thicker than peripheralis

DISCUSSION

In order to enhance our understanding of vision for this species of bat, R. aegyptiacus, quantitative analysis of some eye parameters were conducted. Its eye with a greater horizontal diameter than axial diameter is hemispherical. Accordance previous research, these features extend its visual field, vision acuity and sensitivity in dim light[17].

Hall *et al* (2012) Showed that large eye with large pupil[18], lens and more photoreceptors can gather more light and provide a long focal length and wide visual field, so the grater retinal image is formed[19]. These factors cause more visual acuity and sensitivity in nocturnal animals[20,21].

Almost of bats live in the cave with small entrance, so light can't be seen in all directions. These organisms have adapted to dim light by enduring changes in their eyes. Although the structure and function of the eye especially it's neural region, retina, in mammals follow a general pattern, but the difference is seen, due to adaptation to their different environments[20].

Our finding showed the ratio of eye diameter to body length in R. aegyptiacus (1:18.83) according to results were obtained in deep-sea fish (17, 21), in birds[22] and some species of microbat and megabat[23-25] indicated that its eye is large. This ratio related to visual acuity because if this ratio is low, retinal area is greater and number of photoreceptor cells increases, consequently ability to focus images is increased.

Corral-López *et al* (2017) showed the size of eye related to body size[26], and some research showed that in quick birds, eye size is directly related to brain size, not total size of the body[22,27]. This is probably due to the natural selection pressure. Small head of birds helps them to rotate head during flight, and large eye with high density of photoreceptors is fit in order to achieve high resolution especially in flight[28]. Among all mammals, bats similar to birds capable to flying, and sight are important in their activity[29]. The big eye in this bat species can be also similar to bird. Of course, other factors as size and shape of the lens and the cornea, size of the intracranial space, behavior and lifestyle of the organism effect on their eye size[30,31].

R.aegyptiacus is frugivorous and in order to feeding, it uses vision in daylight while the insectivore bats have small eyes, and they almost use echolocation to compensate for their visual impairment[32]. This species of bats uses both echolocation and visual ability to find objects[4].

The undulating pattern of retinal in this species not only isn't similar to most of bats, but also is unique in mammals. This obtained pattern is duo to choroidal indentation or choroidal papilla into the outer layers of retina that increased surface area of the retina, thus facilitate nocturnal vision. The difference between papilla's length causes a difference in the speed of light transmission which results in better light absorption and image formation. Schwab and Pettigrew (2005) believed this papilla deliver oxygen from choroid to retina. This pattern of retina would serve to increase the number of photoreceptors which aid in light gathering ability.

Retinal epithelial layer in this species of bat was thin with poor pigmentation that is in accordance with the finding of de Busserolles *et al* (2014) is related to[33] adaptation for vision in dim light. In addition the presence of numerous melaonocyts in the choroid increase the amount of light absorbed by the photoreceptors. Although taptum lucidum was reported in R. aegyptiacus by Bojarski and Bernard (1988), but it wasn't observed in present study. On the other hand, a lack of high blood vessels in their retina which improve light transmission was compensated by the numerous large choroidal papilla that represents suitable path for oxygen supply to inner retinal layers and capillaries below the pigmented retinal epithelium.

The retinal cells in different species of microbat and megabat with different distribution were demonstrated[8,9,34,35] which is related to its behavioral ecology[36]. According to present results, the dominance of rod cells retina implies that this species is nocturnal, but observation of cone cells, in contrary to a previous study[12] showed its retina is duplex. The presence of cone cell was confirmed in some microbat[7,9] and megabat[37].

Retina with wavy pattern increase the surface and has high density of photoreceptor. Due to the greater density of rod cells, it is suggested that this animal is nocturnal, but contrary to expectation and unlike all most nocturnal mammals, rod density in centralis was higher than periferalis[38]. The ability of this animal to sense daylight probably effect on the distribution of these cell. The significant decrease in the number of rod cells from centralis to periferalis of retina may be due to the lack of a macula. On the other hand, macula with more cone cells is in centralis which is unlike our findings. Therefore that is probably owing to the absence of macula. Bojarski and Bernard (1988) argues that the low retinal thickness, the presence of high choroidal pigments and numerous cells compensates the lack of macula.

The numerous photoreceptors especially rod cells comprise to bipolar and ganglion cells creates more light convergence. So sensitivity to small brightness contrasts and movement was increased, and the synapse some photoreceptors to one bipolar cell in peripheral of retina increases the sensitivity to dim lights[8,39]. Obviously the degree of convergence from photoreceptors to bipolar cell and then ganglion cell differs regionally within the retina[40].

Results of TEM and SEM analysis showed that the nucleus of cone cell significantly was larger than rod cell, and rod's outer segment was larger and narrower than cone cell. Light energy is transformed by the pigments in the outer segments of photoreceptors into electrochemical signals. The discs with more pigments in rod's outer segments absorb more light and increase visual acuity in dim- light.

CONCLUSION

According to obtained results, retina in R. aegyptiacus was duplex with high degree of development. Specific features as large and hemispherical eye, undulating pattern, more rod cells in central of retina and low density of pigment in the retinal epithelium layer showed that although this species of bat is nocturnal, but it is capable to see in day light, and it is consistent with its feeding strategy.

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CONFLICT OF INTERESTS

There are no cinflicts of interest.

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الملخص العربى

دراسة بنية الشبكية في الخفافيش المصرية الآكلة للفاكهة (Rousettus aegyptiacuss)

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مقدمة: الرؤية هي حاسة مهمة بشكل أساسي عند الحيوانات لإدراك بيئتها، ولكن في الخفافيش أقل شهرة.
هدف العمل: نظرًا لأهمية الرؤية في الخفافيش باعتبارها الثدييات الوحيدة التي يمكنها الطيران، تم فحص نسيج شبكية الخفاش فروجيفور (Rousettus aegyptiacus) بواسطة الضوء والمجهر الإلكتروني في هذه الدراسة.
المواد والطرق: تم تخدير ٥ ذكور من الخفافيش تزن ١٢٣ ± ٠,٨ جرام وتشريح وإزالة شبكية العين. تم عزل المقاطع (١ × ١ مم)، وتثبيتها في ٤٪ جلوتاراديهيد و ٢٪ بارافورمالدهيد (١ ساعة) وشطفها بمحلول كاكوديلات الصوديوم (٠,١). تم إصلاحها في رابع أكسيد الأوزميوم ١ ٪. تم تلوين المقاطع السيميثينية (٠,٥ ميكرومتر) بلون أزرق تولويدين، وتم تحضير المقاطع الرقيقة (٧٠ نانومتر). تم قياس خلايا الشبكية وسمك الطبقة بواسطة برنامج Grids-sterolite. تم تحليل البيانات التي تم الحصول عليها عن طريق اختبار ANOVA و t (P <0.05).
النتائج: أظهرت النتائج أن نسبة قطر العين إلى طول الجسم كانت ١: ١٨,٨٣. تتوافق طبقات شبكية العين مع المخطط العام للثدييات، ولكن بنمط متموج بسماكة ١٠٦,٦١ ± ١٦,١٩ ميكرون. كانت الطبقة النووية الداخلية هي الأكثر سماكة (١٨,٥٢ ± ١,٥٥). شبكية العين هي ازدواج مع خلايا قضيب مهيمنة خاصة في المركزية [٢٥ خلية / (١٠٠ µ) ٢]، ونسب مخروط / قضيب: ١ / ٧,٩٥، وشوهدت أربعة أنواع أخرى من الخلايا ذات الكثافة والتوزيع المختلفين.
الخلاصة: وفقًا للصور الكهربية التي تم الحصول عليها، كانت الطبقة الظهارية للشبكية رقيقة مع تصبغ ضعيف وكان سطحها مغطى بالميكروفيلي. ترتيب المستقبلات الضوئية الموازية للعديد من الحليمات المشيمية، وكثافة الخلايا العصوية أعلى من الخلايا المخروطية وفقًا للنتائج التي تم الحصول عليها، يمكن أن يكون هذا النوع من الخفافيش نشطًا في كل من الضوء الخافت وضوء النهار.