Morphologic and biochemical changes in the retina and sclera induced by form deprivation high myopia in guinea pigs

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Abstract

Background Our aim was to study the morphologic and biochemical changes in the retina and sclera induced by form deprivation high myopia (FDHM) in guinea pigs and explore the possible mechanisms FDHM formation. Methods Forty 3-weeks-old guinea pigs were randomized into the blank control (Group I, 20 cases) and model groups (20 cases). In the model group, the right eyes of the guinea pigs were sutured 8 weeks to induce FDHM (Group II) and the left eyes were considered a self-control group (Group III). The refractive errors were measured were measured with retinoscopy. The anterior chamber depth, lens thickness, vitreous depth and axial length were measured using ultrasonometry A. Retinal and scleral morphology and ultrastructural features were observed with light and electron microscopy. The content of malondialdehyde and the activity of superoxide dismutase in the retina and sclera were detected with a chemical colorimetric assay. Results After remaining stitched for eight weeks, the refractive errors of Group II changed from (+3.59±0.33) D to (-7.96±0.55) D, and these values were significantly higher than those of Group I (+0.89±0.32) D and Group III (-0.55±0.49) D. The vitreous chamber depth (4.12±0.13) mm and axial length (8.93±0.22) mm of Group II were significantly longer than that of Group I [(3.71±0.23) mm and (7.95±0.37) mm, respectively] and Group III [(3.93±0.04) mm and (8.01±0.15) mm, respectively] ($P < 0.05$). With the prolongation of form deprivation, the retina and scleral tissue became thinner, the ganglion cell and inner and outer nuclear layers of the retina became decreased, and the arrangement was disordered. In Group II, the activity of SOD was significantly lower than that in Group I and Group III, and the content of MDA was significantly higher in Group II than in Group I and Group III. The differences were statistically significant ($P < 0.05$). Conclusions These findings suggested that in the FDHM guinea pigs model, the refractive errors, the vitreous chamber depth, and axial length increased significantly with prolongation of monocular FD time, and morphological structural changes in the retina and sclera were observed. Oxygen free radicals might participate in the formation of FDHM.

Background

Myopia is a global epidemic ametropia caused by a combination of genetic and environmental factors [1]. Worldwide, the number of people with high myopia (HM) is about 163 million, accounting for 2.7% of the total population. It is estimated that by 2050, the number of people with HM will increase to 938 million, accounting for 9.8% of the total population. The number of people with HM in China is 87 million, accounting for 6.3% of the total population. It is estimated that by 2050, the number of people with HM will exceed 175 million, accounting for 13% of the total population [2]. Surveys have shown that the prevalence of HM in Chinese adolescents is 6.69-38.4%; that is, China is a typical country with a high HM incidence [3,4]. With myopic refractive errors increasing, especially up to HM, the incidence of fundus lesions, risk of blindness and medical costs also increase [5]. It has been reported that pathological myopia (PM) is an important cause of global vision loss, and the prevalence of PM is 0.9 to 3.1%. The prevalence of PM associated with visual impairment in European countries is 0.1% to 0.5%, and the value in Asian countries is high, ranging from 0.2% to 1.4%. Studies in China have shown that PM is the most common cause of blindness, accounting for 26.1% of the cases [6]. At present, the occurrence of myopia

tends to be higher in younger people and is in a more advanced stage. HM fundus lesions, especially macular degeneration, are the main causes of blindness in East Asian countries [7].

The form deprivation myopia (FDM) theory was founded in 1977. After Wiesel et al. [8] sutured the eyelids of newborn rhesus monkeys, the sutured eyes caused obvious axial myopia. According to the research needs, some scholars have carried out myopia research on the eyes of animals such as tree shrews [9, 10], chickens [11], mice [12], rats [13], and guinea pigs [14]. Studies have confirmed that form deprivation (FD) in young animals leads to abnormal growth of the eye axis and formation of obvious myopia [15, 16]. Guinea pigs are widely used in the current research on myopia because the eyeball structure and emmetropization mechanism is similar to that of human myopia. The mechanism of HM formation is very complicated, and the local retinal regulation mechanism is a focus of research. It has been shown that in myopic eyes, the axial length increased, vascular changes would be observed such as retina vascularization diminishing and narrowing [17]. Shih's research reveals that along with the increase of myopia refractive errors, ocular pulse amplitude, which is generated by choroidal blood flow, becomes lower [18]. Not only is oxidative stress (OS) involved in the development of myopia, but also accompanied by the complications of HM. The elevated level of malondialdehyde (MDA) in the vitreous of myopic subjects strongly suggests a retinal involvement in the genesis of the human myopic cataract [19]. Compared with the control group, oxidative stress levels in the aqueous humor were lower in patients with high myopia [20]. Simonelli et al. [21] have found that the level of MDA was higher in cataractous lenses, diabetes and high myopia than in clear lenses, idiopathic forms. MDA would induce the accumulation of soluble proteins and fragmentation of the membrane structure. Compared with age-related cataracts, the content of superoxide dismutase (SOD) was lower in myopic patients [22]. Compared with healthy controls, the MDA level in plasma was higher. Shkrebets have reported that the antioxidant capacity in tears is weakened in HM patients [23]. To some extent, the results of OS can represent the degree of circulatory disturbance in the development of myopia. SOD is the first line of defense against OS and an important component of the enzymatic antioxidant defense system in the retina. SOD can increase activity of endogenous peroxide detoxifying activity. Xu Huibi et al. [24] reported that the activities of SOD decreased in the retina of the myopic eyes in form-sense-deprived chicks. But fewer studies about the activities of total SOD in form deprivation high myopia in guinea pigs could be checked. This study intended to establish a guinea pig FDM model to simulate HM, observe changes in retinal and scleral morphology, determine the content of MDA in the retina and sclera, and assess the activity of SOD. The relationship between oxygen free radicals and the formation of form deprivation high myopia (FDHM) provides new ideas and a basis for further research on myopia, especially HM.

Methods

Animal Model and Grouping

Forty pigmented guinea pigs at two weeks of age weighing 100 g to 140 g were obtained from Beijing Keyu Animal Breeding Center. {SCXK (Jing) 2017-0002}. After acclimation for one week, the guinea pigs were randomly assigned to two groups: the blank control group (Group I) with 20 guinea pigs and the

model group with 20 guinea pigs. The model group was divided again into the FDHM group (Group II, eyelids on their right eyes were stitched for eight weeks) and the self-controlled group (Group III, left eyes received no intervention). All animals had access to abundant food and water at the Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences Central Animal Laboratory {SYXK (Jing) 2016-0013}, and vitamin-rich feed and fresh vegetables were given to supplement vitamin C. This study was approved by the Ethics Committee of the China-Japan Friendship Hospital (ethics review number: 180103) and was conducted in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmology and Vision Research and the Declaration of Helsinki. Before the experiment, the anterior segment and fundus of all guinea pig eyes were examined with a slit lamp microscope and an ophthalmoscope, and those with eye diseases were excluded.

Main equipment and reagents

A strip light inspection mirror was purchased from Suzhou Liuliu Vision Technology Co. Ltd. A lens box was purchased from Danyang Medical Instrument Factory. A type A ophthalmic ultrasound instrument (ODM-1000) was purchased from Tianjin Maida Medical Technology Co. Ltd. Total SOD (KGT001100) and an MDA (KGT004) test kit were purchased from Beijing Benuowei Biotechnology Co. Ltd.

Biological measurements

All guinea pigs were treated with mydriatic optometry before and after FD for eight weeks. Before FD, tropicamide eye drops were used to fully enlarge the pupil and the ciliary muscle was paralyzed. The optometry was performed in a dark room with a band-shaped photoreceptor. Each eye was measured three times to obtain an average value. The astigmatism degree was transformed into half of the count and this was included in the spherical mirror. Surface anesthesia was performed with 0.4% oxybuprocaine hydrochloride eye drops. The length of each component of the eyeball was measured with ultrasonometry A. The probe of ultrasonometry A was perpendicular to the cornea and located in the center of the pupil. Meanwhile, the cornea was not pressed. When the pattern was stable and clear, the posterior capsule of the lens and the double peak of the retina were higher than the baseline, and the image and results were recorded. Each eye was repeatedly measured 8 times to obtain an average value. Recorded data included the anterior chamber depth (AC), lens thickness (L), vitreous chamber depth (V) and axial length (AL).

Light microscopy Specimen Preparation

After FD for eight weeks, guinea pigs were anesthetized with an overdose intraperitoneal injection of 1% pentobarbital sodium (100 mg/kg). Five eyeballs of each group were removed with the optic nerve 1 to 2 mm long and fixed in 4% paraformaldehyde (Solarbio, Beijing, China). The eyecups were made by cutting the eyeball along the limbus and removing the anterior segment, and then the eyecups were fixed again in 4% paraformaldehyde at 4 °C for 24 hours. After gradient alcohol dehydration and xylene transparent and paraffin machine embedding, the sclera and retina were continuously sectioned along the

longitudinal axis. Sections had a thickness of 4 μm and were stained with haematoxylin-eosin (HE). A microphotographic system (OLYMPUS BH-2, Japan) was used to observe the histology and morphology of the sclera and retina.

Electron microscopy Specimen Preparation

Fives eyeballs in each group were fixed in 2.5% glutaraldehyde solution (Solarbio, Beijing) at 4 °C for 72 hours. First, the sclera and retina were sliced to a 2mm × 2 mm size and washed three times with phosphate buffer (PBS) for 10 minutes each time. Second, the eyeball samples were fixed in 1% citric acid for two hours and rinsed twice with double distilled water for 10 minutes each time. Third, ethanol acetone was used to dehydrate stepwise. Fourth, the samples were infiltrated and embedded with Epon812 epoxy resin. Then, the slice was cut into a 10 nm sample and double stained with uranyl acetate and lead citrate. Finally, a Hitachi H-600 transmission electron microscope was used to observe the sclera and retina ultrastructure and take photos.

Colorimetric detection of the MDA content and SOD activity

After FD for eight weeks, ten guinea pigs in each group were selected and then the eyeballs were removed after the same administration of anesthesia as described above. After removing the anterior segment of the eye on an ice cube, the sclera and retinal/choroidal unity were peeled off with a microscopic tweezer and put into a cryotube. The wet weight was weighed with a precision analytical balance, and double-distilled water was added to make a homogenate. The homogenate was centrifuged at 3000 r/min for 5 minutes, and the supernatant was taken for testing. According to the SOD vitality test kit instructions, we used a 96-well plate to set the sample well and the blank control well. We then added the sample to be tested and other various solutions in turn, and we added the reaction start working solution, mixed it well, and incubated it at 37 °C for 30 minutes. We measured the absorbance at 450 nm, and calculated the SOD vitality according to the formula in the instruction manual. In reference to the MDA kit standard tube absorbance, we took 0.1 ml as the standard. The standard tube absorbance minus the standard blank tube absorbance was 0.103-0.112. We calculated the MDA content according to the formula in the manual. After the experiment, the guinea pigs were euthanized by increasing an anesthetic dose pentobarbital sodium (50mg/kg ip). We have concerned for the welfare of animals and our method of euthanasia followed the AVMA Guidelines for the Euthanasia of Animals.

Statistical Analysis

SPSS statistics 22.0 software was used to analyze the results. Data were recorded as the mean ± standard deviation (`x±s). Comparisons between blank control and model group were performed using independent t test. Comparisons between Group II and Group III were performed using paired sample t test. A value of $P < 0.05$ was considered statistically significant between the groups.

Results

Changes in the refractive status of guinea pig eyeballs

The guinea pigs were born with hyperopia. The refractive errors of the 3-week-old guinea pigs were about +3.50D. Table 1 shows that there was no significant difference in the refractive errors among the groups before FD ($P > 0.05$). After receiving FD for eight weeks in the right eyes, the refractive errors of Group II were significantly increased compared with Group I ($P < 0.05$). The refractive errors of Group II were higher than that of Group III and the differences were statistically significant ($P < 0.05$).

Table 1 Changes of refractive errors at three and eleven weeks of age in each group of guinea pigs

	Group I (n=20)	Group II (n=20)	Group III (n=20)	P Values among 3 groups	P values of post hoc Comparison		
					I vs. II	II vs. III	I vs. III
Refractive errors, D (**3-week-old guinea pigs**)	+3.65±0.27	+3.59±0.33	+3.61±0.29	0.8352			
Refractive errors, D (**11-week-old guinea pigs**)	+0.89±0.32	-7.96±0.55	-5.55±0.49	<.0001	<.0001	<.0001	<.0001

Ultrasonometry A measurement results and statistics

After FD, the vitreous cavity of group II was deepened and the axial length of the eye was longer. Compared with group I, the difference was statistically significant ($P < 0.01$). The depth of the vitreous cavity and the axis of the right eye in the guinea pigs in group III were compared. The length was less than in group II, and the difference was also statistically significant ($P = 0.000$). There was no significant difference in the depth of the anterior chamber and the thickness of the lens among the groups before and after FD ($P > 0.05$; Table 2).

Table 2 Changes in measurement parameters of ultrasonometry A at three and eleven weeks of age in each group of guinea pigs

	Group I (n=20)	Group II (n=20)	Group III (n=20)	P Values among 3 groups	P values of post hoc Comparison		
					I vs. II	II vs. III	I vs. III
AC, mm (3-week-old guinea pigs)	1.22±0.15	1.24±0.06	1.23±0.34	0.3216	NS	NS	NS
AC, mm (11-week-old guinea pigs)	1.31±0.14	1.39±0.24	1.31±0.41	0.2130	NS	NS	NS
L, mm (3-week-old guinea pigs)	3.05±0.15	3.05±0.31	3.03±0.28	0.1356	NS	NS	NS
L, mm (11-week-old guinea pigs)	3.11±0.21	3.14±0.16	3.12±0.17	0.2031	NS	NS	NS
VC, mm (3-week-old guinea pigs)	3.42±0.13	3.43±0.24	3.44±0.16	0.0645	NS	NS	NS
VC, mm (11-week-old guinea pigs)	3.71±0.23	4.12±0.13	3.97±0.03	0.0046	0.0029	0.0036	0.0014
AL, mm (3-week-old guinea pigs)	7.26±0.42	7.27±0.16	7.27±0.62	0.1124	NS	NS	NS
AL, mm (11-week-old guinea pigs)	7.95±0.37	8.93±0.22	8.26±0.15	0.0102	0.0013	0.0002	0.0023

NS no significance

Observation of guinea pig retinas under light microscope

Figure 1 showed that the retinal structure of group I was clear, the thickness of the ganglion cell layer was normal, the monolayer was distributed, the nucleus was round, the nucleolus was clear and neatly

arranged, there was no cell loss or deformation, and the retinal pigment epithelium (RPE) included the visual cells. The outer segment protruded as small protrusions, arranged in a brush-like shape and it was neatly dense (Fig. 1A). After FD for eight weeks, the retinas of group II became thinner, the ganglion cell and inner and outer nuclear layers were all reduced, the nucleus was small and uneven, and the cells were arranged in a disorderly manner. The microvilli protruding from the RPE layer were shorter and even fused to break (Fig. 1B). The retinas of group III showed only a decrease in ganglion cells, a small nucleus, and clear structures in the remaining layers (Fig. 1C).

Observation of guinea pig retinas under an electron microscope

Figure 2 showed that under an electron microscope, the retinal disks of group I were arranged neatly and tightly (Fig. 2A). Compared with group I, the membrane disk of group II showed swelling and deformation (as shown by the red arrow in Figure 2B). Figure 3 showed that the nuclear layer of cell membranes was smooth and completed with uniform chromatin (Fig. 3A). Compared with group I, irregular contraction of the inner and outer nuclear layers and RPE cell membrane, irregular accumulation of the nuclear chromatin, mitochondrial swelling, deformation and vacuolization were observed in group II (as shown by the red arrow in Figure 3B). The pathological damages of retinal membrane disk and inner nuclear layers in group III are weaker than that in group II (Fig. 2C, Fig. 3C).

Observation of guinea pig sclera under a light microscope

Figure 4 showed that the scleral thickness of group I was normal. The fibroblasts were stained blue-purple and were fusiform or oblong, the extracellular matrix was pink, the collagen fibers were neatly arranged, and the diameters were uniform (Fig. 4A). The sclera of the guinea pigs in the group II had obviously become thin, the fibroblast cell nuclei were distorted, the collagen fibers were sparsely distributed, the diameter was obviously reduced, the arrangement was disordered, twisted, broken, and separated, the interfiber space was increased, and the extracellular matrix was increased (Fig. 4B). The scleral thickness of the group III was slightly thinner, but the morphology was not significantly abnormal (Fig. 4C).

Observation of guinea pig sclera under an electron microscope

Figure 5 showed that the scleral tissue was composed of fibroblasts and collagen bundles parallel to the wall of the eyeball. The collagen fibers constitute the framework of the scleral tissue. The cross-sectional diameter of the collagen fibers was observed. There was no significant difference between the sclera of group I and group III. Compared with the two groups, the diameter of the FDHM was significantly reduced and the fiber density was decreased.

MDA content and SOD activity in the retinal/choroidal unity and sclera of guinea pigs

The statistical results showed that the activity of SOD and MDA, the main products of lipid peroxide, had an opposite trend. That is to say, under normal circumstances, the SOD of the retinal/choroidal unity and sclera remained high in the normal group, while MDA was at a low level. However, after FD, compared

with Group I, the content of SOD in the retinal/choroidal unity and sclera of guinea pigs in Group II decreased, while the content of MDA increased ($P < 0.01$). There was no significant difference compared with Group III ($P > 0.05$; Table 3, 4).

Table 3 Content of SOD and MDA in retinal/choroidal unity of guinea pigs (n=5,`x ± s）

Group	SOD（µg/g）	MDA（µg/l）
I	230.01±14.97	4.33±0.43
II	173.35±29.69[ab]	9.23±0.85[ab]
III	187.73±18.31	8.70±1.59

compared with Group I, [a] $P < 0.01$, compared with Group III, [b] $P > 0.05$

Table 4 Content of SOD and MDA content in sclera of guinea pigs (n=5,`x± s）

Group	SOD（µg/g）	MDA（µg/l）
I	261.60±2.30	0.44±2.37
II	197.60±2.40[ab]	1.99±1.73[ab]
III	227.99±4.54	1.01±2.40

compared with Group I, [a] $P < 0.01$, compared with Group III, [b] $P > 0.05$

Discussion

Since the refractive status and orthotopic mechanism of guinea pigs are similar to that of humans, guinea pigs are born with hyperopia. In three weeks, the axial length of the guinea pigs grows rapidly and the refractive errors decrease. The refractive state of 11-week-old guinea pigs is stabilized in the mild hyperopic eye. The FDM model has been widely used in experimental research on myopia [25, 26]. Studies have shown that, after FD, the cornea is flattened, the lens is thickened, the anterior chamber is deepened, the axial length is increased, and the refractive errors are decreased [27]. In this study, 3-week-old guinea pigs were selected as a myopic animal model. At this time, guinea pigs were in a critical period of visual development and were sensitive to the model manipulations. By prolonging the time of FD, the aim was to cause high myopia [28]. The retina is a tissue with a high oxygen demand, strong metabolism and active redox reaction. Under physiological conditions, the intraocular tissues, such as the retina, are continuously stimulated by external light to cause photooxidation, which promotes the formation of oxygen free radicals. At the same time, effective antioxidants, such as SOD, in the cells can promptly scavenge oxygen free radicals. The formation and degradation are in a dynamic equilibrium, which provides protection for tissues such as the retina and sclera. Under pathological conditions, excessive generation of oxygen free radicals or insufficient antioxidant capacity of the body can trigger lipid peroxidation, and its main metabolite, MDA, causes damage to cells and tissues. Oxidative stress can

lead to retinal damage under hypoxic circumstance [29, 30]. This could be used to explain the relationship between OS and FDHM since hypoxic situations would exist chronically. The content of MDA reflects the degree of attack on oxygen by oxygen cells. SOD is an important antioxidant enzyme in the body, and its activity reflects the body's ability to scavenge oxygen free radicals [31, 32].

The results of this study showed that, after eight weeks of FD due to guinea pig eyelid suture, the myopic refractive errors decreased, with the highest at -8.00D refraction, and the biometric data of Ultrasonometry A increased, in which the vitreous cavity was elongated and the axial length became longer. Compared with Group I, the difference was statistically significant ($P < 0.01$), which indicated that the high myopia model was successful. The results are trustworthy since refractive errors were examined by a skilled optometrist and repeated three times to obtain the average value. The guinea pig model was first presented by McFadden and Wallman at ARVO in 1995. They showed that guinea pigs develop deprivation myopia in a reasonably short period of time (a few days) [33]. The guinea pig model is the most common animal model for myopia in Asian laboratories [34]. As mentioned in a myopia animal model, there exists 3,00 D difference approximately between model group and self-controlled group after occlusion for fourteen days [35]. There may be species differences between guinea pigs and tree shrews. The specific mechanism for these differences needs further investigation. There was no significant difference in biochemical parameters between Group II and the Group III (both $P > 0.05$). Deprivation in Group II did not interfere with the natural development of the contralateral eye, which is consistent with the findings of other researchers such as Zhao [36]. Yang J et al [37] have detected retinal metabolic changes in guinea pigs induced by form deprivation myopia. Although they have not directly measured the SOD and MDA level, results they reported represented retinal metabolite levels. That is, no statistically significant differences have been revealed between FD and self-controlled eyes. In our study, the SOD and MDA content has a decreasing and increasing trend respectively when compared with Group I. There are several potential possibilities to account for the mismatch with the refraction and biometric parameters. It is well known that the myopia development is a time-dependent process. It is necessary to measure biochemical parameters at more time-points in further study. We expect that these biometric parameter differences would reach significance if the sample size was larger.

Conclusions

In summary, this experiment produced a HM status in the guinea pigs FD for eight weeks. We observed the retinal, scleral morphology, refractive errors, vitreous cavity depth, axial length change and SOD, MDA expression. Disorders in the oxygen free radical level may be involved in the development of FDHM. The results of this study provided new ideas for the pathogenesis and prevention of HM. However, the specific mechanism of action is not fully understood and further research is needed.

Abbreviations

HM: High myopia; PM: Pathological myopia; FDM: Form deprivation myopia; FD: Form deprivation; MDA: Malondialdehyde; SOD: Superoxide dismutase; FDHM: Form deprivation high myopia; AC: Anterior

chamber depth; L: Lens thickness; V: Vitreous depth; AL: Axial length; HE: Haematoxylin-eosin; PBS: phosphate buffer; RPE: Retinal pigment epithelium

Declarations

Ethics approval

This study was approved by the Ethics Committee of the China-Japan Friendship Hospital (ethics review number: 180103) and was conducted in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmology and Vision Research and the Declaration of Helsinki.

Consent for publication

Not applicable.

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request at any time.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

YX Z and MJ designed the study, analyzed the data and wrote the manuscript. YX Z, YD, JR Z, and MQ J performed the animal study. YL Q and TT D revised and made suggestions to the manuscript. All authors read and approved the final manuscript.

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Figures
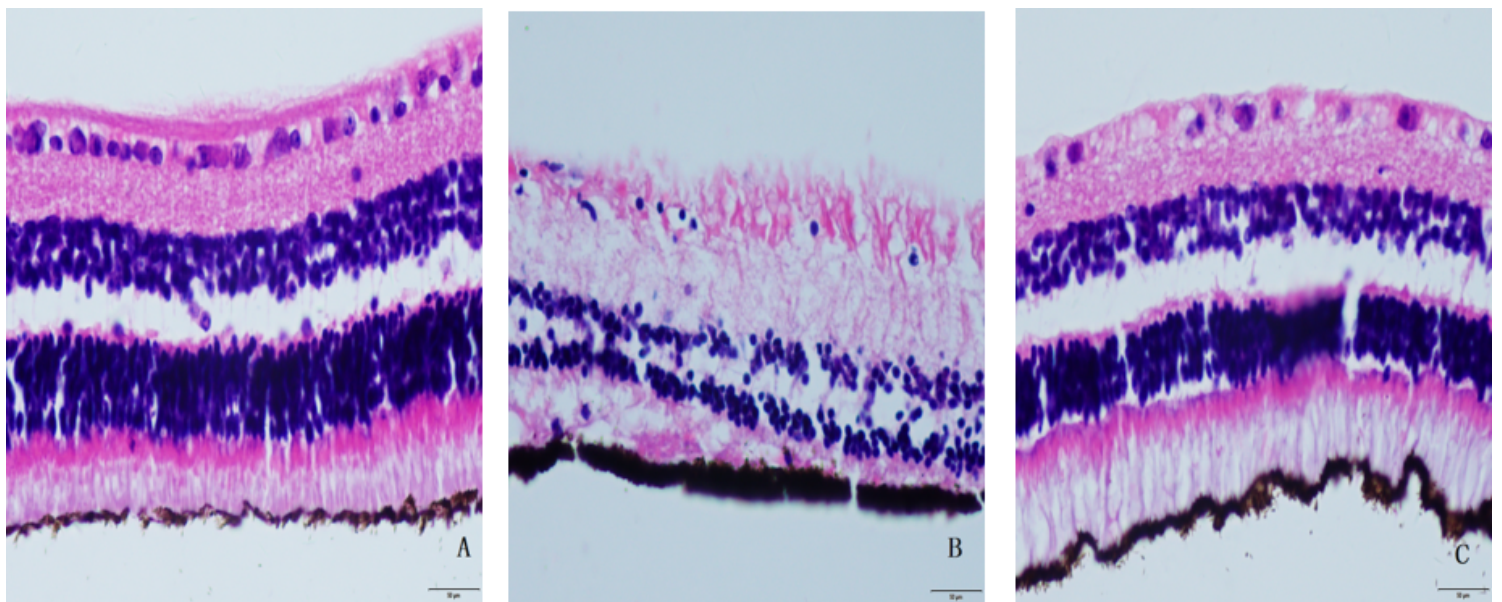
Figure 1

Observation of light microscopic observation of guinea pig retina (HE×400)
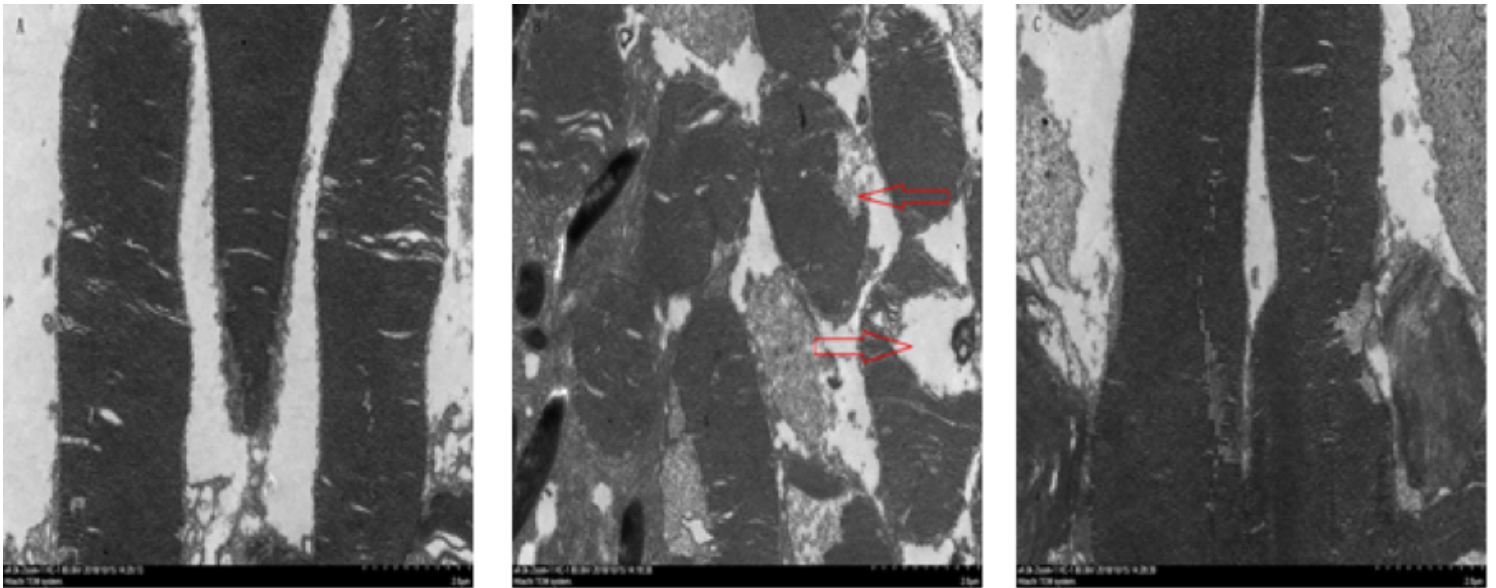
Figure 2

Observation of electron microscopic observation of guinea pig retina membrane (×4000)
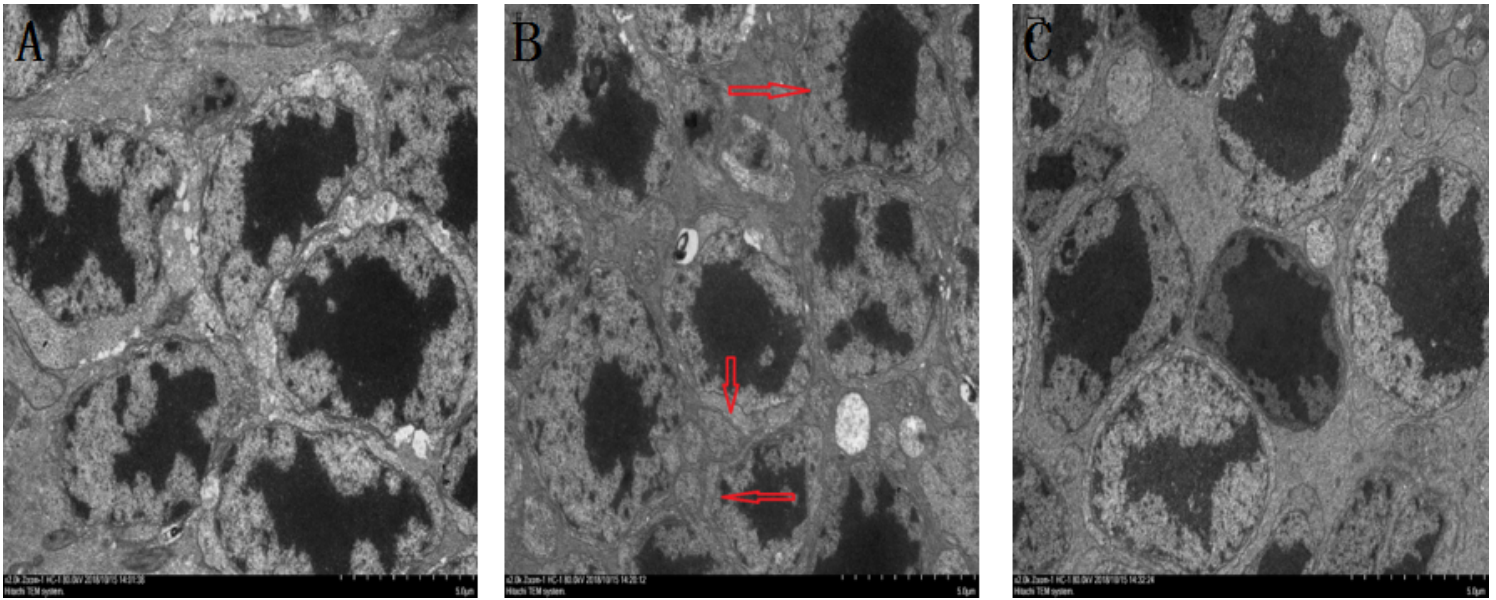
Figure 3

Observation of guinea pig retinal inner nuclear layers under electron microscopic (×4000)
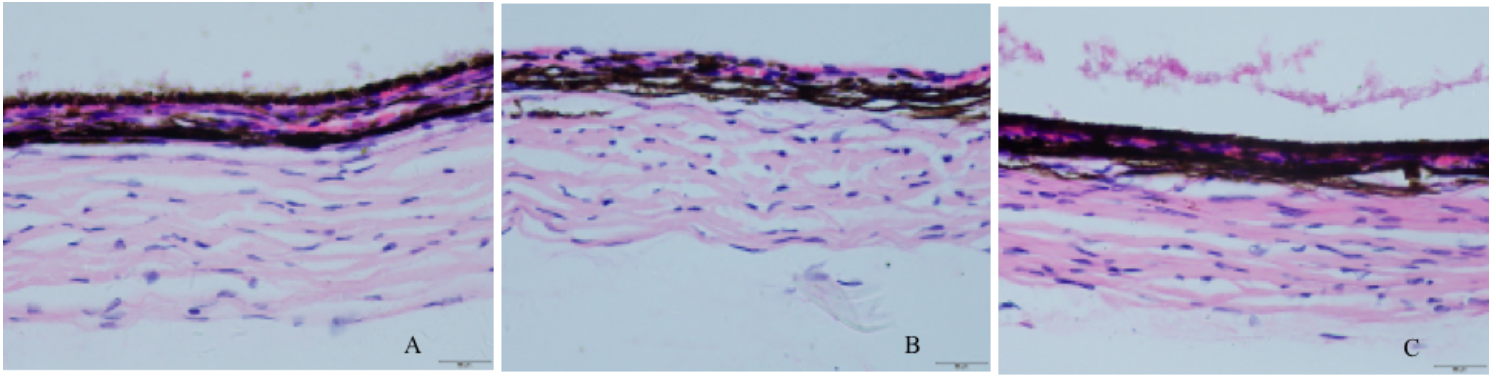
Figure 4

Sclera morphological structure changes in guinea pigs of each group.
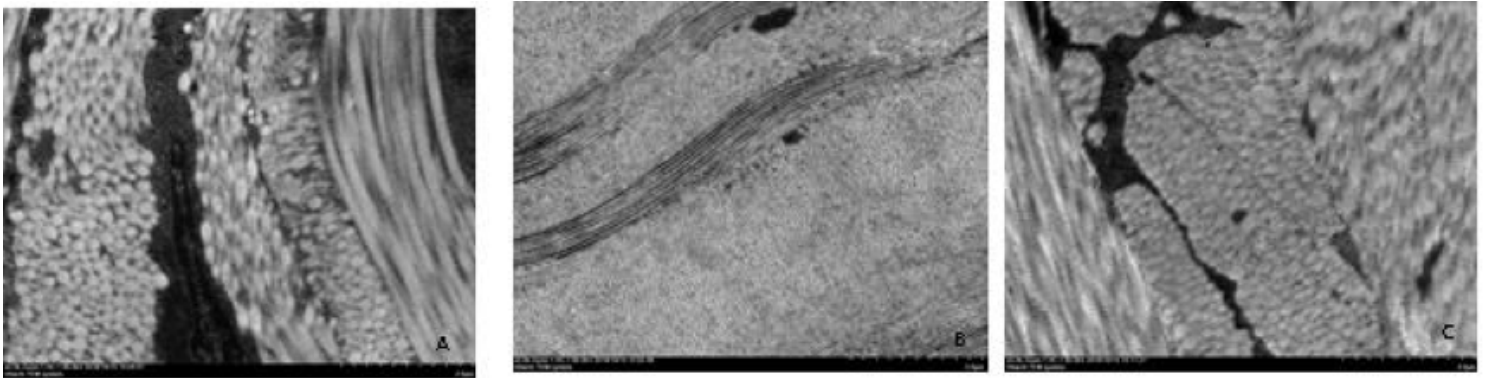
Figure 5

Sclera ultrastructure changes in guinea pigs of each group.