Morphological and vascular characteristics of the optic nerve head in normal guinea pigs

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Abstract

The morphological and vascular characteristics of the optic nerve head (ONH) in normal guinea pigs remain unknown. Therefore, we aimed to investigate these parameters using optical coherence tomography (OCT) and optical coherence tomography angiography (OCTA). We measured the refractive error, axial length, and intraocular pressure (IOP), and performed OCT and OCTA of the ONH in 3- and 4-week-old tricolour guinea pigs. A total of 208 right eyes from 208 normal guinea pigs were examined. The refractive error in the 3-week group was significantly larger than that in the 4-week group ($p < 0.001$), the IOP in the 3-week group was significantly lower than that in the 4-week group ($p = 0.014$), and the circumpapillary retinal nerve fibre layer (cpRNFL) thickness in the 3-week group was significantly larger than that in 4-week group ($p = 0.048$). There were no significant differences in the average vessel area, vascular density, total number of junctions, total vessel length, total number of endpoints, and vascular diameter between the two groups. However, an age-adjusted linear regression analysis revealed that total vessel length was positively associated with the cpRNFL thickness ($p = 0.024$) and negatively associated with IOP ($p = 0.016$). This is the first report on morphological and vascular characteristics of the ONH in normal guinea pigs based on in vivo OCT and OCTA imaging and quantification of ONH parameters. These results may contribute to further research on myopia and glaucoma using guinea pig models.

Introduction

Myopia has been reported to be associated with an increased risk of developing primary open-angle glaucoma (POAG).¹ The optic nerve head (ONH) is an important structure involved in the pathogenesis of both myopia and glaucoma. Guinea pigs have been increasingly used in experimental models of myopia and, recently, in a novel experimental model of glaucoma.² In an in vitro study, Ostrin et al. had reported the structural anatomy of retinal and ONH in guinea pigs.³ Haematoxylin and eosin-stained sections had shown retinal ganglion cell axons organised into fascicles in the prelaminar and laminar regions of the ONH, and scanning electron microscopy had revealed a well-defined lamina cribrosa with radially oriented collagen beams.³ Therefore, guinea pigs are considered better models than rats and mice for elucidating similar glaucomatous changes in the ONH in humans.

Optical coherence tomography (OCT) and OCT angiography (OCTA) are recent technological advancements in the diagnosis, treatment, and follow-up of many retinal diseases due to their ability to capture high-resolution characteristics of the retinal state within seconds.⁴ In our previous study, we had found that in OCT images, a U-shaped neural canal and a lower ratio of Bruch membrane opening (BMO) to minimum rim width were early indicators of retinal nerve fibre layer (RNFL) defects in preperimetric glaucomatous eyes with normal circumpapillary RNFL (cpRNFL) thickness.⁵ Jnawali et al. had observed that histological sections of all the retinal layers in guinea pigs were consistent with their OCT images and that, specifically, hyper-reflective regions of the OCT images had corresponded to the retinal pigment epithelium, inner/outer segment junction, outer plexiform layer, inner plexiform layer, and RNFL.² However, the anterior lamina cribrosa surface cannot be visualised in OCT images because the thick RNFL and

vascular sheath obscure the ONH, which decreases the penetrance of the scanning laser. On the contrary, vascular density (VD) reduction observed on OCTA may potentially indicate the progression of glaucoma in eyes with advanced glaucomatous damage and may provide information on the risk of its rapid deterioration.⁶ In addition, OCTA-related parameters — including VD, number of vessel junctions, total vessel length, and number of vessel endpoints — have been introduced to assess choroidal neovascularisation (CNV) in rat models; these parameters had shown significantly higher values in the intermediate and deep vascular plexi of CNV when obtained by OCTA than when obtained by fluorescein angiography (FA).⁷ Vascular dysfunction in the ONH and the peripapillary retina is considered important in the pathogenesis of POAG.⁸

Silva et al. had reported that POAG eyes show significantly lower VD and flow in deep ONH and peripapillary vessels compared with control eyes.⁹ Similar to the ONH in humans, the ONH of guinea pigs also contains vascular tissue. Protruding vascular tufts in the ONH have been reported in guinea pigs;² however, little is known about the normal imaging characteristics of their blood perfusion and OCTA parameters. Quantification of such parameters in normal guinea pig models is critical for characterising vascular structural changes that are expected to occur in conditions like myopia and glaucoma.

In the present study, we aimed to investigate and set the normal ranges for both morphological and blood perfusion characteristics of the ONH in normal guinea pigs using OCT and OCTA, and to investigate the factors influencing blood perfusion in the ONH. We conducted this study to assess whether guinea pigs could be considered as potentially valuable models for investigating the association between myopia and glaucoma in humans.

Methods

Animals

Our study was conducted on 3-, and 4-week-old tricolour guinea pigs that were stratified by their age. Because there are no statistically significant differences between the refractive and biometric measurements (intraocular pressure [IOP] and axial length [AL]) in the right and left eyes,^{2,10} only the right eye was selected as the study eye in all groups. Guinea pigs with congenital cataracts, corneal abnormalities, or other ocular diseases that may affect vision were excluded at the baseline. All animal care and examinations in this study conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and all experimental protocols were approved by the Animal Care and Use Committee of China Medical University. The study is reported in accordance with ARRIVE guidelines (<https://arriveguidelines.org>).

Parameter measurements

Initially, cycloplegic retinoscopy was performed in all eyes to record the following measurements: refractive error using astreak retinoscope (YZ-24, Suzhou Liuliu, China), IOP using a tonometer (Icare

Finland Oy, Vantaa, Finland), and ocular AL using an A-scan ultrasound (Aviso, Quantel Medical Inc., Cournon-d'Auvergne, France; probe diameter, 5 mm; ultrasonic resolution, 0.01 mm; ultrasonic transmission frequency, 11 Hz) under topical anaesthesia. The IOPs of guinea pigs exhibit diurnal variations, peaking in the early morning and decreasing across the day;³ therefore, measurements were always taken at the same time of day (afternoon) to avoid any confounding effect of diurnal rhythms. Three sets of six measurements were recorded, and the average IOPs were calculated. Guinea pigs do not require anaesthesia for IOP measurements; therefore, anaesthesia-induced alterations in IOP can be avoided.²

The ONH parameters were measured using spectral-domain OCT (SD-OCT, Cirrus HD-OCT, Zeiss, Germany) in the ONH mode. The analysed parameters were average cup-to-disc ratio (CDR), cpRNFL thickness (μm , the RNFL of the circle with a diameter of 3.4 mm centered at the disc), rim area (mm^2), disc area (mm^2), and cup volume (mm^3). Blood flow signals of the ONH were also recorded on OCTA (Cirrus HD-OCT, Zeiss, Germany) in the $6 \times 6\text{-mm}$ range. SD-OCT and OCTA measurements were performed under general anaesthesia with 1% pentobarbital sodium (0.33 ml/100 g). The guinea pigs were subjected to intraperitoneal anaesthesia, and 0.5% proparacaine hydrochloride (Alcaine, Belgium) eye drops were dropped into the conjunctival sac before injection.

Analysis of OCTA images

The blood flow signal maps of ONH were obtained using the OCTA device, and retinal avascular layers mode were segmented and selected; subsequently, these target images were imported to the AngioTool software,¹¹ which automatically measured the vessel area, VD, total number of junctions, total vessel length, and total number of endpoints of vessels of the ONH (Fig. 1).¹² The vascular diameter was defined as the vessel area divided by the total vessel length.

Statistical analyses

All data were analysed using the SPSS 24.0 statistical software (IBM, SPSS statistics, Version 24, SPSS Inc., Chicago, USA). The data were expressed as mean $\pm$ standard deviation. Independent-samples t-test was used for pair-wise comparisons of AL, IOP, refraction, and OCT- and OCTA-related parameters between the 3- and 4-week old groups. Pearson's test was used to determine the correlations between VD, total vessel length in the ONH, and all other parameters. Moreover, the influencing factors for VD and total vessel length of the ONH were determined by linear regression analysis. A p-value < 0.05 was considered statistically significant.

Results

General information

A total of 208 right eyes from 208 normal guinea pigs were enrolled, which comprised 83 3-week-old (3-week group) and 125 4-week-old (4-week group) guinea pigs' eyes. The average AL, refractive error, and IOP were 7.68 ± 0.205 mm, 2.38 ± 2.095 D, and 17.4 ± 3.04 mmHg, respectively. No significant difference was found in AL between the 3-week and 4-week groups (7.67 ± 0.190 mm vs. 7.69 ± 0.215 mm, $t = 0.432$, $p = 0.666$). The refractive error in the 3-week group was significantly larger than that in the 4-week group (3.02 ± 1.266 D vs. 1.96 ± 2.373 D, $t = 3.710$, $p < 0.001$). The IOP in the 3-week group was significantly lower than that in the 4-week group (16.7 ± 3.19 mmHg vs. 17.8 ± 2.88 mmHg, $t = 2.483$, $p = 0.014$).

Morphological parameters of the optic disc based on OCT

The CDR, cpRNFL thickness, area of the rim, area of the disc, and cup volume of the 208 eyes were 0.38 ± 0.127 , 53.0 ± 14.74 μ m, 0.77 ± 0.206 mm², 0.92 ± 0.216 mm², and 0.030 ± 0.0179 mm³, respectively. The cpRNFL thickness in the 3-week group was significantly larger than that in 4-week group (55.4 ± 16.27 μ m vs. 51.3 ± 13.45 μ m, $t = 1.993$, $p = 0.048$). However, no significant differences were found in the CDR, area of the rim, area of the disc, and cup volume between the 3-week and 4-week groups (Fig. 2).

Blood perfusion in the ONH based on OCTA

The vessel area, VD, total number of junctions, total vessel length, total number of endpoints, and vascular diameter were 924.64 ± 226.329 pixels, $48.86 \pm 7.116\%$, 6.84 ± 3.540 , 180.70 ± 49.435 pixels, 7.93 ± 3.198 , and 5.25 ± 1.027 pixels, respectively. There were no significant differences in any of these parameters between the 3-week and 4-week groups (Table 1).

Table 1
Blood flow parameters of the optic disc head based on OCTA

	N	Vessel area (pixels)	Vascular density (%)	Total number of junctions (N)	Total vessel length (pixels)	Total number of endpoints (N)	Vascular diameter (pixels)
3-week group	83 eyes	920.63 ± 227.994	48.44 ± 7.493	6.6 ± 3.39	180.81 ± 49.427	7.6 ± 2.88	5.24 ± 1.167
4-week group	125 eyes	927.31 ± 226.097	49.15 ± 6.871	7.0 ± 3.64	180.63 ± 49.640	8.2 ± 3.38	5.26 ± 0.927
		$t = 0.208$, $p = 0.835$	$t = 0.700$, $p = 0.485$	$t = 0.897$, $p = 0.371$	$t = 0.026$, $p = 0.979$	$t = 1.394$, $p = 0.165$	$t = 0.118$, $p = 0.906$

Factors influencing VD and the total vessel length of the ONH

VD of ONH was positively correlated with vessel area ($r = 0.508$, $p < 0.001$), total number of junctions ($r = 0.348$, $p < 0.001$), total vessel length ($r = 0.265$, $p < 0.001$), and vascular diameter ($r = 0.241$, $p < 0.001$) and was negatively correlated with the total number of endpoints ($r = -0.460$, $p < 0.001$). However, an age-adjusted linear regression analysis revealed that VD was positively associated with vessel area ($B = 0.036$; 95% confidence interval [CI] = 0.029 to 0.043; $p < 0.001$) and total number of junctions ($B = 1.363$; 95%CI = 1.096 to 1.629; $p < 0.001$) and was negatively associated with the total vessel length ($B = -0.172$; 95%CI = -0.211 to -0.132; $p < 0.001$), total number of endpoints ($B = -1.418$; 95%CI = -1.576 to -1.260; $p < 0.001$), and vascular diameter ($B = -0.647$; 95%CI = -1.490 to 0.197; $p < 0.001$).

Total vessel length of ONH was positively correlated with vessel area ($r = 0.870$, $p < 0.001$), VD ($r = 0.265$, $p < 0.001$), total number of junctions ($r = 0.853$, $p < 0.001$), and total number of endpoints ($r = 0.257$, $p < 0.001$). However, it was negatively associated with the vascular diameter of ONH ($r = -0.469$, $p < 0.001$). Both cpRNFL thickness ($r = 0.110$, $p = 0.113$) and IOP ($r = 0.076$, $p = 0.273$) failed to show any relationship with total vessels. However, an age-adjusted linear regression analysis revealed that total vessel length was positively associated with vessel area ($B = 0.166$; 95%CI = 0.155 to 0.177; $p < 0.001$), total number of junctions ($B = 4.413$; 95%CI = 3.632 to 5.194; $p < 0.001$), and cpRNFL thickness ($B = 0.110$; 95% CI = 0.015 to 0.205; $p = 0.024$) and was negatively associated with VD ($B = -1.622$; 95%CI = -1.973 to -1.271; $p < 0.001$), total number of endpoints ($B = -2.319$; 95%CI = -3.002 to -1.636; $p < 0.001$), vascular diameter ($B = -13.209$; 95%CI = -14.920 to -11.497; $p < 0.001$), and IOP ($B = -0.611$; 95%CI = -1.108 to -0.114; $p = 0.016$).

Discussion

Guinea pigs have become increasingly popular models for studying human myopia, which carries an increased risk of glaucoma.³ Therefore, it is important to have data on the normal development of the eyes of guinea pigs. The main goals of this study were to set the normal ranges for both morphological and vascular parameters of the ONH in normal guinea pigs and to analyse the factors influencing blood perfusion in the ONH.

Our results showed that no significant differences were found in the CDR, area of the rim, area of the disc, and cup volume between the 3-week and 4-week groups. However, as no related analyses have been reported, we were unable to compare them to any corresponding reference values. Jnawali et al. had reported that the BMO area of 2.5-year-old pigmented guinea pigs is $0.192 \pm 0.023 \text{ mm}^2$ and that the diameter is $493.79 \pm 31.89 \text{ }\mu\text{m}$.² RNFL thickness is an important clinical diagnostic measure in glaucoma.¹³ In our study, the cpRNFL thickness in the 3-week group ($55.4 \pm 16.27 \text{ }\mu\text{m}$) was significantly larger than that in 4-week group ($51.3 \pm 13.45 \text{ }\mu\text{m}$). Jnawali et al. had reported that the RNFL thickness in 2.5-year-old pigmented guinea pigs is $59.2 \pm 4.5 \text{ }\mu\text{m}$,² which is larger than our result.

Patel et al. had reported that VD is greater within the BMO of rhesus monkeys and significantly decreases with increasing eccentricity.¹⁴ Our study also focused on the vasculature of the ONH. The vessel area, VD, total number of junctions, total vessel length, total number of endpoints, and vascular diameter were 924.64 ± 226.329 pixels, $48.86 \pm 7.116\%$, 6.84 ± 3.540 , 180.70 ± 49.435 pixels, 7.93 ± 3.198 , and $5.25 \pm$

1.027 pixels, respectively. Previously, it had been reported that the VD within BMO of rhesus monkeys was 42.7% (10 mmHg), 43.5% (20 mmHg), and 43.3% (30 mmHg).¹⁴ However, there were no significant differences in the above parameters between the 3-week and 4-week groups.

Patel et al. had reported that the peripapillary inner retinal capillary density in rhesus monkeys gradually decreases with increasing IOP, reaching statistical significance when pressure exceeds 50 mmHg but returns to normal when IOP is reduced.¹⁴ Similarly, age-adjusted linear regression analysis revealed that cpRNFL thickness is positively associated with total vessel length, and that IOP is negatively associated with total vessel length. Manalastas et al. had reported that the thicknesses of cpRNFL and the macular ganglion cell complex are more strongly associated with VD of the ONH ($r = 39.2\%$ and 26.7% , respectively) of glaucoma patients, glaucoma suspects, and healthy individuals.¹⁵ Moreover, age-adjusted linear regression analysis revealed that VD is positively associated with vessel area and total number of junctions and is negatively associated with total vessel length, total number of endpoints, and vascular diameter.

Our results revealed that the AL, refractive error, and IOP were 7.68 ± 0.205 mm, 2.38 ± 2.095 D, and 17.4 ± 3.04 mmHg, respectively. Jnawali et al. had reported that the mean AL was 9.997 ± 0.12 mm in guinea pigs aged 2.5 years.² In addition, two previous studies on normal guinea pigs (age unknown) reported that the IOP was 16.5 ± 3.2 mmHg among 100 normal guinea pigs¹⁶ and 18.27 ± 4.55 mmHg among 31 normal guinea pigs.¹⁷ In our study, the IOP in the 3-week group was significantly lower than that in the 4-week group (16.7 ± 3.19 mmHg vs. 17.8 ± 2.88 mmHg). Similarly, Jnawali et al. had reported that IOPs showed an early small increase and thereafter appeared to stabilise with age.²

Our study showed that the refractive error in the 3-week group was significantly larger than that in the 4-week group (3.02 ± 1.266 D vs. 1.96 ± 2.373 D). Jnawali et al. had also reported that young guinea pigs were hyperopic, with a mean spherical equivalent refraction of $+3.50 \pm 0.77$ D.² These results may support the suggestion that IOP is a driving force for the process of emmetropisation.

No difference was found in AL between the 3-week and 4-week groups (7.67 ± 0.190 mm vs. 7.69 ± 0.215 mm). Similarly, Zhou et al. had reported that the increase in AL is relatively rapid during the first week, with little change during the next three weeks, and then, rapidly increases after five weeks.¹⁰ The emmetropisation process in guinea pigs is mainly related to the increase in the vitreous chamber length, and the axial development of the vitreous chamber in guinea pigs appears to be associated with posterior scleral tissue growth.¹⁰

This study has some limitations. First, due to an increase in retinal pigmentation with age, the accuracy of judgment during retinoscopy for older guinea pigs would have been affected, which might have resulted in a fluctuating variance of the refractive data. Second, we did not measure the refractive power of guinea pigs at birth. These limitations should be addressed in further research.

Nevertheless, this is the first report on morphological and vascular characteristics of the ONH in normal guinea based on in vivo OCT/OCTA imaging and quantification of ONH parameters. Hence, our results may contribute to further research on myopia and glaucoma using a guinea pig model.

Declarations

Conflicts of Interest: The authors have no conflicts of interest related to this article to declare.

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Authors contribution statement

R.H., L.G., and J.T. made substantial contributions to conception and design, analysis and interpretation of data; R.H., and L.G. took part in drafting the article or revising it critically for important intellectual content; L.G., Y.T., S.C.C., and X.Z. performed the experiment and acquired data; all authors agreed to submit to the current journal; all authors gave final approval of the version to be published; all authors agree to be accountable for all aspects of the work.

References

1. Xu L, Wang Y, Wang S, Wang Y, Jonas JB. High myopia and glaucoma susceptibility the Beijing Eye Study. *Ophthalmology* 2007; **114**(2): 216–20.
2. Jnawali A, Beach KM, Ostrin LA. In vivo imaging of the retina, choroid, and optic nerve head in guinea pigs. *Curr Eye Res* 2018; **43**(8): 1006–18.
3. Ostrin LA, Wildsoet CF. Optic nerve head and intraocular pressure in the guinea pig eye. *Exp Eye Res* 2016; **146**: 7–16.
4. Cuenca N, Ortuño-Lizarán I, Sánchez-Sáez X et al. Interpretation of OCT and OCTA images from a histological approach: clinical and experimental implications. *Prog Retin Eye Res* 2020; **77**: 100828.
5. Hua R, Gangwani R, Guo L et al. Detection of preperimetric glaucoma using Bruch membrane opening, neural canal and posterior pole asymmetry analysis of optical coherence tomography. *Sci Rep* 2016; **6**: 21743.
6. Rao HL, Pradhan ZS, Suh MH, Moghimi S, Mansouri K, Weinreb RN. Optical coherencetomographyangiography in glaucoma. *J Glaucoma* 2020; **29**(4): 312–21.
7. Meyer JH, Larsen PP, Strack C et al. Optical coherence tomography angiography (OCT-A) in an animal model of laser-induced choroidal neovascularization. *Exp Eye Res* 2019; **184**: 162–71.
8. Leske MC, Heijl A, Hyman L et al. Predictors of long-term progression in the early manifest glaucoma trial. *Ophthalmology* 2007; **114**(11): 1965–72.

9. Nascimento E Silva R, Chiou CA, Wang M et al. Microvasculature of the optic nerve head and peripapillary region in patients with primary open-angle glaucoma. *J Glaucoma* 2019; **28(4)**: 281–8.
10. Zhou X, Qu J, Xie R et al. Normal development of refractive state and ocular dimensions in guinea pigs. *Vision Res* 2006; **46(18)**: 2815–23.
11. Zudaire E, Gambardella L, Kurcz C, Vermeren S. A computational tool for quantitative analysis of vascular networks. *PLoS One* 2011; **6(11)**: e27385.
12. Giannakaki-Zimmermann H, Kokona D, Wolf S, Ebner A, Zinkernagel MS. Optical coherence tomography angiography in mice: comparison with confocal scanning laser microscopy and fluorescein angiography. *Transl Vis Sci Technol* 2016; **5(4)**: 11.
13. Bowd C, Weinreb RN, Zangwill LM. Evaluating the optic disc and retinal nerve fiber layer in glaucoma. I: Clinical examination and photographic methods. *Semin Ophthalmol* 2000; **15(4)**: 194–205.
14. Patel N, McAllister F, Pardon L, Harwerth R. The effects of graded intraocular pressure challenge on the optic nerve head. *Exp Eye Res* 2018; **169**: 79–90.
15. Manalastas PIC, Zangwill LM, Daga FB et al. The association between macula and ONH optical coherence tomography angiography (OCT-A) vessel densities in glaucoma, glaucoma suspect, and healthy eyes. *J Glaucoma* 2018; **27(3)**: 227–32.
16. Williams D, Sullivan A. Ocular disease in the guinea pig (*Cavia porcellus*): a survey of 1000 animals. *Vet Ophthalmol* 2010; **13(suppl)**: 54–62.
17. Coster ME, Stiles J, Krohne SG, Raskin RE. Results of diagnostic ophthalmic testing in healthy guinea pigs. *J Am Vet Med Assoc.* 2008; **232(12)**: 18.

Figures

A

B

Figure 1

Analysis of OCTA parameters of the ONH at the age of 4 weeks using AngioTool software A. Retinal avascular layers segmentation mode was applied in ONH and selected by the inbuilt software of OCTA and were subsequently imported to the AngioTool software. B. The AngioTool software automatically measured the explant area (area within blue circles), vessel area (area within yellow circles), vascular density, total number of junctions (blue dots), junction density, total vessel length (red lines), average vessel length, and the total number of endpoints of vessels. OCTA, optical coherence tomography angiography; ONH, optic nerve head.

Figure 2

Morphological parameters of the optic nerve head in normal guinea pigs A. Cup-to-disc ratio: 0.38 ± 0.128 (3-week group) and 0.38 ± 0.128 (4-week group). B. Thickness of circumpapillary retinal nerve fibre layer: $55.4 \pm 16.27 \mu\text{m}$ (3-week group) and $51.3 \pm 13.45 \mu\text{m}$ (4-week group). C. Rim area: $0.76 \pm 0.217 \text{ mm}^2$ (3-week group) and $0.78 \pm 0.198 \text{ mm}^2$ (4-week group). D. Disc area: $0.91 \pm 0.211 \text{ mm}^2$ (3-week group) and $0.93 \pm 0.219 \text{ mm}^2$ (4-week group). E. Cup volume: $0.031 \pm 0.0173 \text{ mm}^3$ (3-week group) and $0.029 \pm 0.0184 \text{ mm}^3$ (4-week group). * $p < 0.05$.