Experimental Studies

https://doi.org/10.31288/oftalmolzh20254954

Morphometric analysis of retinal structural components in CBA/C57 mice in experimental diabetes mellitus

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Purpose. *To perform morphometric analysis of retinal structural components in CBA/C57 mice in experimental diabetes mellitus (DM) in an attempt to obtain measurable characteristics of possible retinal neurodegenerative changes required for comparison with those in the diabetic retina of other species.*

Material and Methods*. We retrospectively reviewed hematoxylin-and-eosin (H&E) stained specimens of eyes from eight CBA/C57 mice with diabetes duration of 6 months or less and healthy control animals. Total retinal thickness and thicknesses of the following retinal layers were measured: the photoreceptor layer (PRL), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion cell layer (GCL), and nerve fiber layer (NFL). The numbers of neural-cell rows in both nuclear layers were calculated visually.*

Results. *The mean total thickness of the retina was 197.2 ± 6.32 μm for healthy controls and 227.8 ± 6.2 μm for diabetic animals. In controls and diabetic animals, the mean thicknesses for particular retinal layers were as follows: PRL, 54.4 ± 2.49 μm and 54.9 ± 1.69 μm, respectively; ONL, 47.1 ± 1.73 μm and 49.8 ± 1.69 μm, respectively; OPL, 15.7 ± 1.08 μm and 16.5 ± 0.75 μm, respectively; INL, 24.6 ± 1.094 μm and 32.1 ± 1.46 μm, respectively; IPL, 41.6 ± 1.79 μm and 52.9 ± 1.73 μm, respectively; and GCL plus NFL, 13.8 ± 0.92 μm and 21.6 ± 1.05 μm, respectively. In addition, the numbers of neural-cell rows in the ONL were 9.1 ± 0.32, and 10.0 ± 0.38, respectively, and in the INL, 3.6 ± 0.13 μm, and 3.8 ± 0.16, respectively. In diabetic mice, thicknesses of all inner retinal layers (INL, IPL, and GCL plus NFL) were increased due to edema ($p < 0.01$ for all cases), resulting in a 30.6-μm increase in the total thickness of the retina. There was no microscopic evidence of neurodegeneration.*

Conclusion*: Microscopic images of the retina and measurements of thickness of individual retinal layers and numbers of neural-cell rows in retinal nuclear layers for mice with diabetes duration of 6 months or less indicated that a mouse model of streptozotocin-induced diabetes cannot be considered well suited for H&E staining assisted histological assessment of neurodegenerative changes.*

Key words:

diabetic retinopathy, diabetes mellitus, retina, morphometry, experiment, neurodegeneration

Introduction

Over 1 million deaths per year can be attributed to diabetes alone, making it the ninth leading cause of mortality [1]. Diabetic retinopathy (DR) is one of the most severe complications of diabetes mellitus (DM) and a common condition in ophthalmic practice. In 2012, there were approximately 93 million people with DR [2], 17 million with proliferative DR, and 28 million with vision-threatening DR worldwide [3].

It is important that, in patients with type 1 DM, DR can develop as early as 3-5 years after the onset of diabetes [4]. In 2017, approximately 462 million individuals were affected by type 2 DM. Additionally, the number of individuals with DM was projected to increase to about 600 million by 2035, and to 730 million in 2050, with an increase in the number of patients with DR [5-7].

Unfortunately, the prevalence of DM and DR in Ukraine is high similar to other countries, which is indicated by reports from the literature. Korobov [8] and

Chugaiev [9] reported that of the diabetic patients presenting to them, 25-27.5% were found to have DR. The number of people living with DM in the country was as large as 2.3 million for 2021 [10], with a contribution of 95% from type 2 DM [11]. Diabetes is still a major cause of blindness in adults older than 65 years of age [12], and individuals with diabetes are 25 times more likely to become blind than those in the general population [13].

This year, we substantiated the importance of conducting morphometric studies of neural cells in major species of laboratory animals (rats, mice and rabbits) and patients with DM, and clarified some outcome measures of these studies (e.g., measurements of the thickness of the total retina and individual retinal layers and numbers of neural-cell rows in the inner and outer nuclear layers in diabetic animals). We also concluded that, for Wistar rats with diabetes duration of 3 months, microscopic images of the retina and calculations of thicknesses of individual retinal layers and numbers of neural-cell rows in retinal nuclear layers provided no indication of neurodegenerative changes at this time point [14].

This conclusion is especially important, taking into account that DR initially affects not the retinal vasculature but neural retinal cells [15]. The current study continued the research in this field, but was conducted on animals of another species, CBA/C57 mice.

The purpose of this study was to perform morphometric analysis of retinal structural components in CBA/C57 mice in experimental DM in an attempt to obtain measurable characteristics of possible retinal neurodegenerative changes required for comparison with those in the diabetic retinae of other species.

Material and Methods

We retrospectively reviewed archival histological specimens of eyes, both from eight CBA/C57 mice with diabetes duration of 6 months or less and healthy control animals, stored in the Pathological Anatomy Laboratory, SI "The Filatov Institute of Eye Diseases and Tissue Therapy of the National Academy of Medical Sciences of Ukraine". Multiple low-dose streptozotocin (STZ) injections (40 mg/kg) were given for 5 days to induce diabetes in mice. All 5-µm histological sections were stained with hematoxylin and eosin. Taking into account that, in microscopic studies, the vitreoretinal structure of the mouse with two-month diabetes duration does not differ practically from that of the mouse with six-month diabetes duration (it is the histological sections of such animals that are stored in the laboratory archive), it was decided to consider only two groups of animals (a group of diabetic mice and a group of healthy control mice) in morphometic calculations.

These archival specimens have not been used previously for morphometic calculations. To perform morphometric calculations, we employed the classical histological methodology with the use of eyepiece micrometer. The actual size of each division of the eyepiece micrometer was determined with the help of the object micrometer at the specified magnification (objective magnification, x40, eyepiece magnification, x7) of the Laboval 4 microscope (Zeiss, Jena, Germany). Total retinal thickness was measured in micrometers, and the thickness of the following layers were measured in the neurosensory retina: the photoreceptor layer (PRL), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion cell layer (GCL), and nerve fiber layer (NFL). The numbers of neural-cell rows in both nuclear layers were calculated visually.

Statistical analysis was performed using JASP (Version 0.18.1; JASP Team, Amsterdam, The Netherlands). Mean and standard deviation (SD) values were calculated for each sample. The Student t-test was used to determine differences between mean values. P-values less than 0.05 were considered significant [16]. Additionally, the percentage contribution of each retinal layer to the total thickness of the retina was calculated and compared among the two groups.

Results

All the layers typical of the retina of a normal mouse could be seen in the retina of an untreated diabetic mouse (Figures 1-3). It should be, however, noted that CBA/C57 mice are not albino and have melanin inclusions in their retinal pigment epithelial (RPE) cells (note RPE in the top left of Fig. 2).

For normal control animals, the mean total thickness of the retina was 1972.2 µm (SD 37.92). In addition, the mean PRL thickness was 54.4 µm (SD 14.98), and the mean ONL thickness was 41.7 µm (SD 10.24) (Table 1). The ONL consisted of 9.1 neural-cell rows (SD 1.92), with a mean error of 0.32. The OPL thickness was 15.7 µm (SD 6.45). The mean INL thickness was 24.6 µm (SD 6.57),

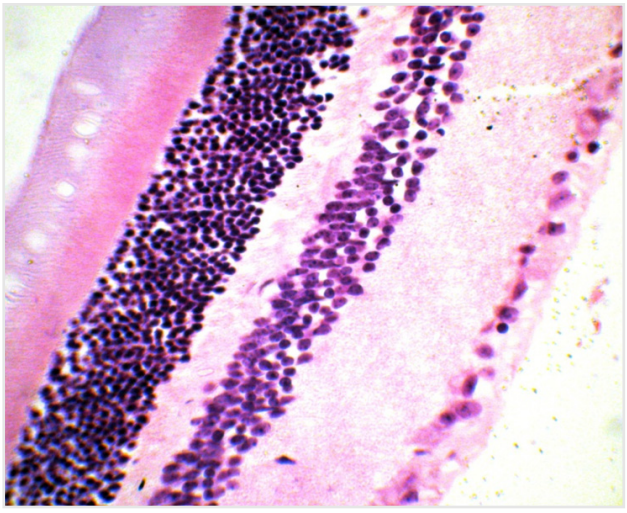

Fig. 1. Neural retina of a healthy control mouse. Hematoxylin and eosin staining. Objective magnification, x40; eyepiece magnification, x7.

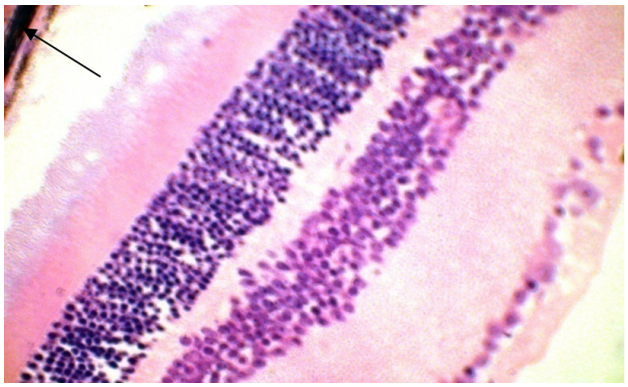

Fig. 2. Neural retina of a mouse with 2-month diabetes duration. Note retinal pigment epithelial cells in the top left of the image (arrow). Hematoxylin and eosin staining. Objective magnification, x40; eyepiece magnification, x7.

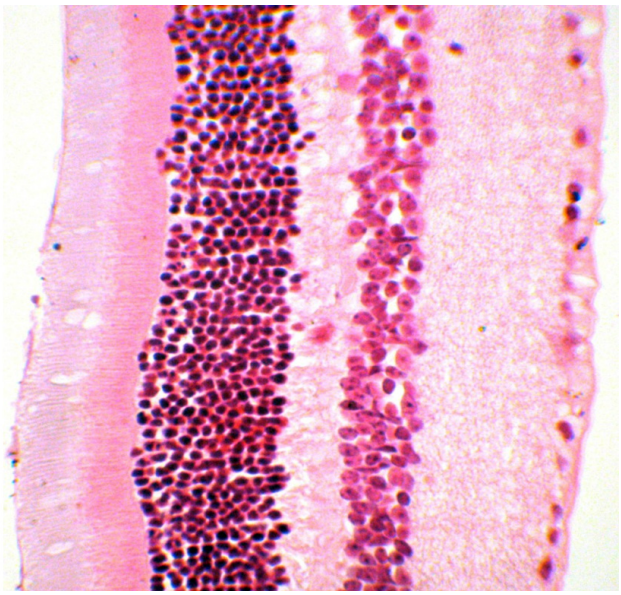

Fig. 3. Neural retina of a mouse with 6-month diabetes duration. Hematoxylin and eosin staining. Objective magnification, x40; eyepiece magnification, x7.

and the number of neural cells was substantially smaller in the INL than in the ONL. The INL consisted of 3.6 neural-cell rows (SD 0.81), with a mean error of 0.13. The IPL was 41.6 μm (SD 10.75) and was thicker than the OPL, and the mean thickness of the GCL plus NFL was 13.8 μm (SD 5.54)).

For diabetic animals, the mean total thickness of the retina was 227.8 μm (SD 51.97). In addition, the mean PRL thickness was 54.9 μm (SD 14.14), and the mean ONL thickness was 49.8 μm (SD 14.8) (Table 1). The INL consisted of 10.0 neural-cell rows (SD 2.89), with a mean error of 0.38. The mean OPL thickness was 16.5 μm (SD 6.26). The mean INL thickness was 31.1 μm (SD 12.21) and the number of neural cells was substantially smaller in the INL than in the ONL, similar to normal animals. The INL consisted of 3.8 (SD 1.22) neural-cell rows, with a mean error of 0.16. The mean IPL thickness was 52.9 μm (SD 14.52), and the IPL was thicker than the OPL, similar to normal animals. The mean thickness of the GCL plus NFL was 21.6 μm (SD 8.85), and was 1.6 times thicker than in controls.

In control mice, the percentage contribution of the PRL, ONL and OPL to the total thickness of the retina were larg-

Table 1. Comparison between diabetic mice and healthy controls in terms of thicknesses of individual retinal layers and the percentage contribution of each retinal layer to the total thickness of the retina

Retinal layers	Retinal later thickness (μm) mean ± error of mean (percentage contribution to the total thickness of the retina)		P-value
	Control group , n= 3	Diabetic animals, n= 5	
Photoreceptor layer	54.4± 2.49 (27.59 %)	54.9 ± 1.69 (24.1%)	>0.05
Outer nuclear layer	47.1 ± 1.73 (23.88%)	49.8 ± 1.69 (21.86%)	>0.05
Outer plexiform layer	15.7 ± 1.08 (7.96%)	16.5± 0.75 (7.24%)	>0.05
Inner nuclear layer	24.6 ± 1.09 (12.48%)	32.1 ± 1.46 (14.1%)	<0.01
Inner plexiform layer	41.6 ± 1.79 (21.09%)	52.9 ± 1.73 (23.22%)	<0.01
Ganglion cell layer plus nerve fiber layer	13.8 ± 0.92 (7.0%)	21.6 ± 1.05 (9.48%)	<0.01
Total	197.2±6.32 100%	227.8±6.2 100%	

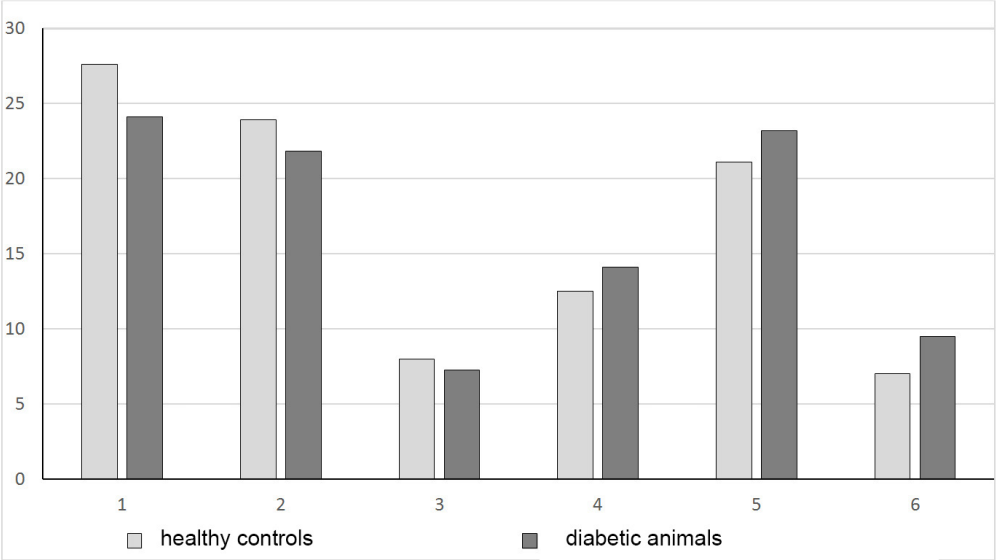

Fig. 4. Thickness of individual retinal layers and the percentage contribution of each retinal layer to the total thickness of the retina in healthy controls and diabetic mice. Note: 1, photoreceptor layer; 2, outer nuclear layer; 3, outer plexiform layer; 4, inner nuclear layer; 5, inner plexiform layer; 6, ganglion cell layer plus nerve fiber layer

er, whereas those of the ONL and OPL were smaller than in diabetic mice (Table 1). Additionally, in diabetic mice, the percentage contribution of the retinal GCL and NFL to the total thickness of the retina were larger than in control mice. Therefore, the PRL was the thickest layer in the mouse retina, followed by the ONL and OPL (Table 1). In total, these three layers accounted for more than 70 percent of the total thickness of the retina both in controls and in diabetic mice. The GCL plus NFL was the thinnest retinal layer, contributing 7% to the total thickness of the retina in a healthy CBA/C57 mouse. The percentage contribution of the OPL to the total thickness of the retina was less than 8% both in controls and in diabetic mice (Fig. 4).

Discussion

Microscopic studies of archival specimens allow a preliminary conclusion to be drawn on the absence of severe neurodegenerative changes in the retinal layers of CBA/C57 mice with STZ-induced diabetes. Certainly, the level of evidence increases with the use of morphometric analysis of the thickness of the retinal layers and calculation of the numbers of neural-cell rows in the INL and ONL. This is associated with the fact that a change that takes place in marked neurodegeneration and neural cell death is a substantial reduction in the thickness of relevant retinal layers and the numbers of neural-cell rows in the INL and ONL.

It can be easily seen from the above data that there were some significant differences in the retinal layers between a healthy mouse and a diabetic mouse, but not between a diabetic rat and a healthy rat [14]. These differences were, however, of a quantitative nature only. These included a reliably significant increase ($p < 0.01$) in the thickness of the INL, IPL, and GCL plus NFL (i.e., the layers of the inner retina), with a significant increase in the thickness of the total retina. The above phenomenon is likely to be caused by intrinsic vascularization of the layers of the inner retina in the mice. This is indicated also by the comparison of histological micrographs of the retina from mice with duration of diabetes of 2 months and 6 months (Figs. 2 and 3) with that of the retina from a healthy control mouse (Fig. 1).

We found that, in diabetic mice with duration of diabetes as long as 6 months, neither the thickness of retinal layers nor the numbers of neural-cell rows in the INL and ONL were significantly reduced compared to healthy controls. On the contrary, the thickness of the four inner retinal layers (INL, IPL, and GCL plus NFL) were increased due to edema ($p < 0.01$ for all cases), and the thickness of the outer retinal layers tended to increase ($p > 0.05$) compared to controls (Table 1). This proves the absence of signs of neurodegenerative changes in the retinal layers of diabetic mice at 6 months after disease onset. If neurodegenerative changes in the neuoretinal tissue were present, there would be a reduction not only in the number of neural cells and neural-cell rows in the INL and ONL, but also in the thickness of these layers. However, no such changes were detected. We believe that this may be explained using the analysis of the vascular bed of the mouse retina. In most mammals (including humans, rats and mice), the retina is vascularized, which is beneficial for its blood supply [15]. In the diabetic mouse, however, the internal retina becomes edematous, likely indicating that this species is not so capable of regenerating beta cells of the pancreas as the diabetic rat that may even develop a spontaneous diabetic remission [17, 18]. Rodent models of diabetes have been refined over time [19].

It should be taken into account that, it has been believed since the early 2000s that DR initially affects not the retinal vasculature but neural retinal tissue [20, 21] that undergoes neurodegeneration. Consequently, the mouse is not a species well suited for investigating diabetic retinal neurodegeneration with histological section staining. That is, we repeat the conclusion drawn in our previous paper with regard to investigation of the rat eye in diabetes [14]. The collection of the pathological anatomy includes also archival histological specimens of eyes from another laboratory species, the rabbit. Comparative morphometric studies similar to those performed in this work but using archival specimens of eyes from the rabbit will allow identifying the species with the most apparent signs of retinal neurodegeneration in DM. It is on these species that it would be reasonable to test potential medications for the treatment of DR.

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Disclosures

Received: 08.06.2025

Accepted: 22.07.2025

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Author Contributions: *: All authors meet the authorship criteria and contributed substantially to conception and design or acquisition of data, or analysis and interpretation of data, drafted or revised the version to be published. The authors are responsible for the content of the article. All authors state that they have not submitted and will not submit a similar paper for publication elsewhere in any language.*

Conflict of interest: *The authors state that they have no conflict of interest that could influence their views regarding the subject matter or materials described and discussed in this manuscript.*

Disclaimer: *The views presented in this article are those of the authors and do not necessarily represent those of their institutions or funding entities.*

Subjects of the study: *Animal experiments were performed in compliance with the Law of Ukraine on Protection of Animals from Cruel Treatment No. 3447-IV and European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes from the European Treaty Series (Strasbourg, 1986). The*

study was approved by the local Bioethics Committee of SI "The Filatov Institute of Eye Diseases and Tissue Therapy of the National Academy of Medical Sciences of Ukraine" (meeting minutes dated October September 14, 2024).

Data Availability Statement: *All the data obtained or analyzed during this study are reported in the article.*

This paper is part of the research project "To develop measures for early detection, immunocorrection and rehabilitation of patients with ocular tuberculosis, diabetes mellitus, and ischemic neuropathy under martial law" (state registration number, 0125U002071).

Abbreviations: *DM, diabetic mellitus; DR, diabetic retinopathy*