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### Ultrastructural changes in the rat retina in the presence of long-term opioid exposure

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**Purpose:** To determine the features of the ultrastructural reorganization in the rat retina by the end of week 4 and week 6 of experimental opioid exposure.

**Material and Methods:** Forty-eight adult male albino rats (weight, 200-250 g; age, 4.5 months) were used in this study. They received nalbuphine hydrochloride intramuscularly daily for 42 days. Particularly, the drug was administered daily at a dose of 0.212 mg/kg for weeks 1 and 2, 0.225 mg/kg for weeks 3 and 4, and 0.252 mg/kg for weeks 5 and 6. In this way, we experimentally created the conditions of chronic opioid exposure. Animals were divided into three groups. Group 1 (19 animals) received nalbuphine for 28 days, and group 2 (19 animals), for 42 days. Group 3 (control group) comprised 10 animals. Of these, 5 animals were treated with normal saline at a dose of 0.22 mg/kg intramuscularly daily for 28 days, and the rest were treated in a similar manner for 42 days. Transmission electron microscopy studies of the rat retina were conducted in a routine manner.

**Results:** By the end of week 4 of experimental opioid exposure, there was an increase in the number of retinal microvessels with signs of hyperemia and degenerative changes in retinal pigment epithelium (RPE) cells, increase in the destruction of membranous discs of photoreceptor outer segments, necrobiotic changes in the nuclei of individual photoreceptors, axonal degeneration in the outer and inner plexiform layers, degenerative changes in retinal horizontal neurons, and the appearance of necrotic structural changes in the cytoplasm of bipolar and amacrine cells. By the end of week 6, there was a further increase in hyperemia of retinal vessels and degenerative and necrotic changes in individual RPE cells and photoreceptor outer segments. In addition, we observed destruction and shortening of mitochondrial cristae of photoreceptor inner segments, necrotic nuclear changes in individual photoreceptors, degeneration of axons of the outer and inner plexiform layers, degenerative and necrotic changes in bipolar and amacrine cells, hypertrophic Müller cell processes, degeneration of ganglion cells, and vascular hyperemia and moderate perivascular edema in the outer and inner plexiform layers.

**Conclusion:** Therefore, in the current rat study, after a 4-week exposure to daily nalbuphine injections at a dose ranging 0.212 to 0.253 mg/kg, there was ultrastructural evidence of destructive processes in the RPE and photoreceptor outer segments, axonal degeneration in the outer and inner plexiform layers, degenerative and necrotic changes in bipolar and amacrine cells, hypertrophic Müller cell processes, ganglion cell degeneration and hyperemia due to an impaired retinal microcirculatory ultrastructure. At week 6 of the experiment, there was evidence of increased destructive and degenerative processes in structural components of the retina.

**Keywords:**

eye globe, retina, rat, opioid exposure, electron microscopy

## Introduction

The management of consequences of long-term use of opioid medications for medical indications and uncontrolled use of psychotropic and highly potent medications without medical indications is still an important problem. The use of opioids under combat conditions is a particularly contentious issue [1-4]. Domestic and foreign studies on the consequences of uncontrolled use of opioid medications in the clinical and experimental setting have reported on pathomorphological changes in body tissues, organs and systems in long-term use of opioids in various doses [5-10]. However, there are few reports on pathomorphological time-point-by-time-point changes in ocular structures under experimental exposure to various doses of opioids or at various frequencies and duration of experimental exposure to opioids [11-14].

Given the above, the long-term use of opioids is still a matter of concern due to the possible development of opioid-related retinopathy. Because the issues related to stabilization of pathomorphological symptoms in the retinal layers and blood microcirculation in experimental subchronic exposure to opioids are still to be clarified, we believe the current study is important from the point of view of both experimental morphology and practical ophthalmology.

The purpose of this study was to determine the features of the ultrastructural reorganization in the rat retina by the end of week 4 and week 6 of experimental opioid exposure.

## Material and Methods

Forty-eight adult male albino rats (weight, 200-250 g; age, 4.5 months) were used in this study. They received nalbuphine hydrochloride intramuscularly daily at 10 to 11 AM for 42 days. Particularly, the drug was administered daily at a dose of 0.212 mg/kg for weeks 1 and 2, 0.225 mg/kg for weeks 3 and 4, and 0.252 mg/kg for weeks 5 and 6. In this way, we experimentally created the conditions of chronic opioid exposure [14]. Animals were divided into three groups.

Group 1 (19 animals) received nalbuphine for 28 days, and their material was harvested at the end of week 4; group 2 (19 animals) received nalbuphine for 42 days, and their material was harvested at the end of week 6. Group 3 (control group) comprised 10 animals. Of these, 5 animals were treated with normal saline at a dose of 0.22 mg/kg intramuscularly daily in the morning (10 to 11 AM) for 28 days, and the rest were treated in a similar manner for 42 days.

Animals were maintained under standard vivarium conditions. All animal experiments were conducted in compliance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes from the European Treaty Series (Strasbourg, 1986), General Ethical Principles for Experiments in Animals, adopted by the First National Bioethics Congress (2001), and the Law of Ukraine on Protection of Animals from Cruel Treatment No.

№3447-IV dated 21.02.2006. This study was approved by the Bioethics Committee of the Danylo Halytsky Lviv National Medical University.

Prior to tissue harvesting, animals were euthanized by ether overdose. We have tried to preserve the topographic relationships between the ocular coats starting from performing enucleation and until embedding the eyes into paraffin blocks, prior to performing ultrastructural examination of the posterior pole of the rat eye. Electron microscopy studies were conducted at the Interdepartmental Laboratory for Electron Microscopy of the Danylo Halytsky Lviv National Medical University using a PEM-100-01 Transmission Electron Microscope (Selmi, Sumy, Ukraine). Histological material of the posterior pole of the globe was examined using a routine method [15].

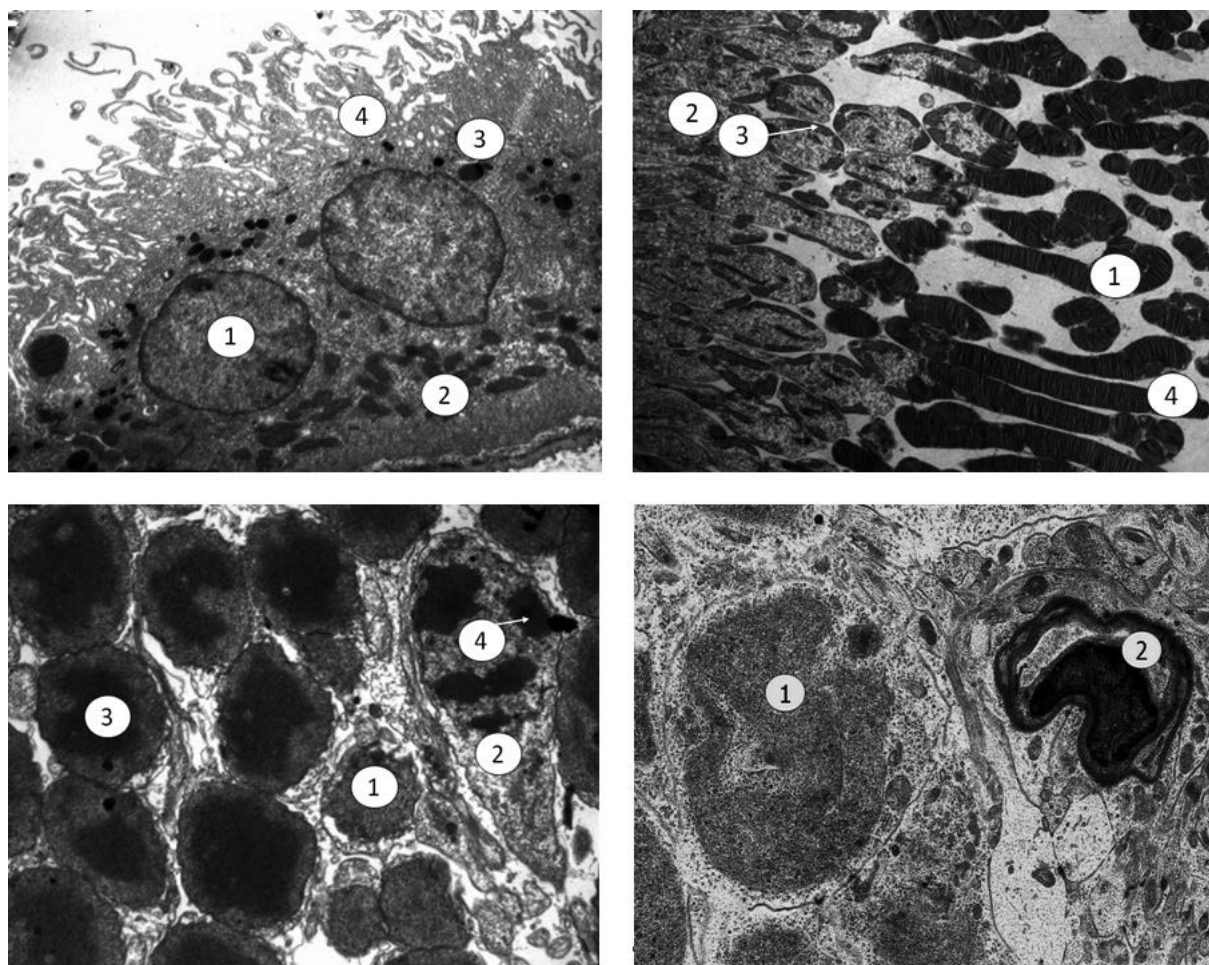
No statistical method was employed in this study.

## Results

The transmission electron microscopy analysis found that the retina of control rats had a structure similar to that of the human retina and is comprised of ten layers. The retinal pigment epithelium (RPE) layer is in contact with the basal choriocapillaris of the choroid; it consists of cells with a cytoplasm of a moderate electron density and an oval-shaped nucleus with a small amount of heterochromatin which attaches to the nuclear lamina and nucleolema. The cytoplasm contains a small number of organelles, mostly mitochondria, but also melanosomes varying in size and electron density. Processes are seen on the RPE cell surface close to rods and cones (Fig. 1A). The retinal photoreceptor layer consists of rods and cones (Fig. 1B). In rods the discs are discontinued and stacked within the plasmalemma. The outer nuclear layer contains the cell bodies of the photoreceptor cells, with nuclei varying in size and amount of heterochromatin (Fig. 1C). This layer is rather thin and formed by axons of the first neuron, dendrites of the second neuron and processes of radial gliocytes (Müller cells). Axons contain small granules and solitary mitochondria. The inner nuclear layer is formed by the cell bodies of the second neuron which contain nuclei varying in size and shape and a small amount of heterochromatin. Nuclei are surrounded by an electron-lucent cytoplasm with a small number of organelles. Cell bodies of horizontal cells and amacrine cells are seen.

The inner plexiform layer is wide and contains synapses formed by processes with an electron-lucent cytoplasm and a cytoplasm of a moderate electron density which contains electron-dense vesicles and mitochondria (Fig. 1D).

The ganglion layer contains neurons varying in shape and size (mostly large) with an electron-lucent cytoplasm and a moderate number of organelles, predominantly mitochondria. Nuclei of these cells have a small amount of heterochromatin (Fig. 1E) and an electron-dense nucleolus. The layer of optic nerve fibers is formed by axons of the third neuron with electron-lucent axoplasm with a small number of mitochondria. Blood capillaries are seen in this



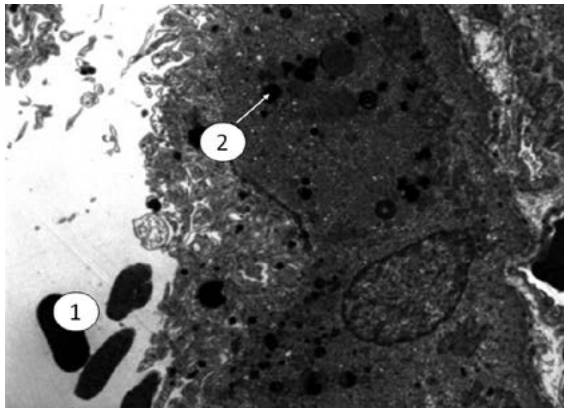
**Fig. 1.** Microphotographs of retinal fragments from control rats. (A) Ultrastructure of retinal pigment epithelial cells. Original magnification  $\times 3,000$ . Note: 1, RPE cell nuclei; 2, numerous mitochondria in the basal cytoplasm of RPE cells; 3, phagosomes in the apical cytoplasm; 4, apical microvilli. (B) Ultrastructure of retinal photoreceptor cells. Original magnification  $\times 1,900$ . Note: 1, photoreceptor outer segments; 2, photoreceptor inner segments; 3, mitochondria of photoreceptor inner segments; 4, contoured membranous discs of the outer segments of the first retinal neuron. (C) Ultrastructure of the retinal outer nuclear layer. Original magnification  $\times 3,000$ . Note: 1, nuclei of photoreceptor rod neurons; 2, nuclei of photoreceptor cone neurons; 3, heterochromatin in photoreceptor rod neurons; 4, accumulation of heterochromatin in photoreceptor cone neurons. (D) Neuron in the ganglion layer and blood capillary in the retinal nerve fiber layer. Original magnification  $\times 5,000$ . Note: 1, ganglion neuron nucleus; 2, blood capillary in the retinal nerve fiber layer.

layer, with their lumens filled with plasma. The internal limiting membrane appears like a homogeneous stripe and is formed by processes of radial gliocytes.

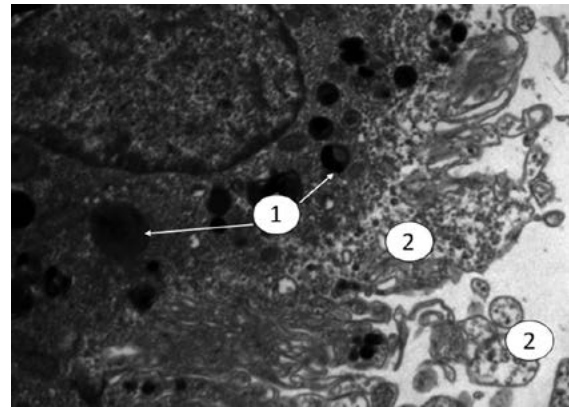
At four weeks after initiation of nalbuphine injections, transmission electron microscopy of rat eye wall samples showed degenerative changes in pigment epithelial cells, destruction of membranous discs of photoreceptor outer segments, degeneration of axons of the inner plexiform layer, and changes related to alteration in neuronal metabolism and structure in the outer and inner nuclear layers. Lumens of small arterioles and blood capillaries of the choroid appeared expanded, whereas endothelial cytoplasm appeared swollen and showed expanded canaliculi and vesicles of smooth endoplasmic reticulum. In some RPE cells, nuclei had signs of kariopyknosis and appeared displaced to the apical surface, and arranged

vertically. In individual RPE cells, nuclei appeared pear-shaped and the nuclear envelope was invaginated. Accumulation of phagocytized fragments of photoreceptor outer segments was seen in the apical zone of RPE cells. The RPE cytoplasm in treated rats was congested with numerous intensively osmiophilic phagosomes (developed due to the capture of shed discs of photoreceptor outer segments) (Fig. 2), well beyond the level of such inclusions in the RPE of control rats. This indicated the intensity of phagocytosis.

The basal plasmalemma of RPE cells showed structural heterogeneity, displayed microinfolds in some zones and appeared smooth at other zones. There was swelling and focal destruction of microvilli in the apical region of RPE cells (Fig. 3).



**Fig. 2.** Electron micrograph. Ultrastructure of the retina of a rat at the end of week 4 of experimental opioid exposure. Original magnification  $\times 1,900$ . Note: 1, fragments of photoreceptor outer segments on the apical surface of retinal pigment epithelial cells; 2, numerous intensely osmiophilic phagosomes in different cytoplasmic zones of RPE cells



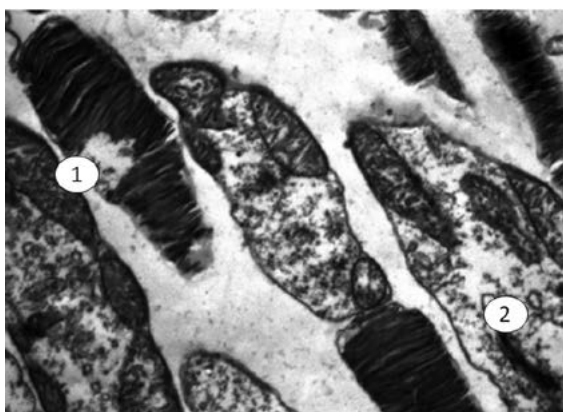
**Fig. 3.** Electron micrograph. Ultrastructure of the retina of a rat at the end of week 4 of experimental opioid exposure. Original magnification  $\times 4,500$ . Note: 1, numerous intensely osmiophilic phagosomes and primary and secondary lysosomes in the apical zone of RPE cells; 2, cytoplasmic edema and focal destruction of apical microvilli

Photoreceptor outer segments showed destructive changes with abnormal contour of outer segment membrane discs and collapse of some of these discs. Destructive changes resulted in the accumulation of fine granular material in the cytoplasm of the photoreceptor outer segments, and clear cytoplasmic zones were observed (Fig. 4).

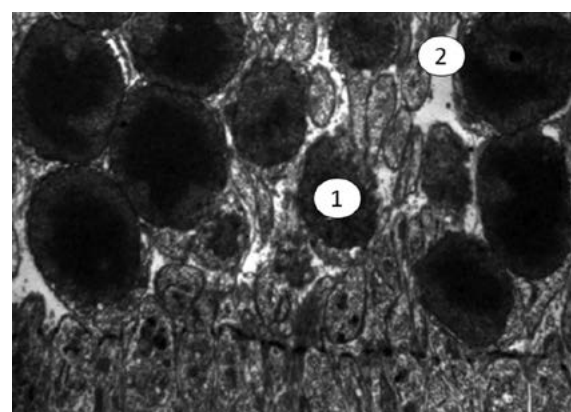
The cytoplasm of the photoreceptor inner segments was frequently clear (Fig. 4). In these zones, we observed heterogeneously dilated granular endoplasmic reticulum cisternae, destructed free ribosomes, and edematous inner mitochondrial matrix. Irregularly-shaped photoreceptor neuron nuclei, sometimes reduced in size, were seen in the outer nuclear layer (Fig. 5).

Some photoreceptor cell nuclei appeared displaced to the outer plexiform layer. Variation in electron density of photoreceptor axoplasm as well as a chaotic arrangement of synapses in the outer plexiform layer was noted.

There were also significant ultrastructural changes in bipolar neurons in the outer plexiform layer. A loss of ribosome binding to granular endoplasmic reticular membranes, increased clearance of mitochondrial matrix, and short mitochondrial cristae were observed. Some bipolar neurons showed an increased amount of heterochromatin in the nuclei. In addition, there were some bipolar neurons with pyknotic nuclei and apparently clear cytoplasm. Moreover, there were bipolar cells with well-preserved organelles and moderately clear cytoplasm.



**Fig. 4.** Electron micrograph. Ultrastructure of the retina of a rat at the end of week 4 of experimental opioid exposure. Original magnification  $\times 7,500$ . Note: 1, disorganization of membranous discs and destruction of the membranes of photoreceptor outer segments; 2, clear cytoplasmic zones in photoreceptor inner segments and mitochondrial cristae injury



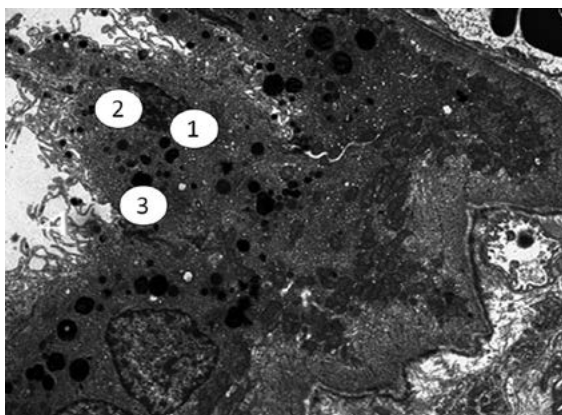
**Fig. 5.** Electron micrograph. Ultrastructure of the retina of a rat at the end of week 4 of experimental opioid exposure. Original magnification  $\times 3,000$ . Note: 1, individual nuclei of photoreceptor neurons appear reduced in size; 2, intercellular edema in the outer nuclear layer

Notable changes were seen in horizontal cells and amacrine cells (auxiliary retinal neurons). The perikarya of horizontal cells at the border between the inner nuclear layer and the outer plexiform layer were swollen and showed apparently dilated Golgi apparatus cisternae and smooth endoplasmic reticulum, thus indicating metabolic changes. The perikarya of amacrine cells in the outer nuclear layer showed changes such as variations in the electron density of the cytoplasm, and destruction and dissociation of free ribosomes. These cells showed increased nuclear heterochromatin which may indicate reduced transcription and, consequently, depressed metabolic activity (i.e., a sign of alteration in metabolism and structure in these cells). Radial gliocytes (Müller cells) were the glial cells mostly involved in changes; the glial changes were, however, mild, and likely, transitory.

In the inner plexiform layer, in addition to increased changes related to alteration in retinal microcirculation with the development of stasis and perivascular swelling, we noted electron-lucent axoplasm and breakage and non-compact arrangement of synaptic vesicles, which are the signs of gradually increasing swelling of the retinal layers. The ganglion neuronal cytoplasm was clear, showing dilated granular endoplasmic reticulum cisternae, individual destructed ribosomes, and short and broken mitochondrial cristae.

In the nerve fiber layer, there were broken mitochondrial cristae in the glial cytoplasm and expanded canaliculi with damaged zones of smooth endoplasmic reticulum.

Six weeks after initiation of nalbuphine injections, most changes in the structures under investigation tended to increase in severity compared to the previous time point (week 4). Retinal capillary lumen was dilated; in addition, we observed erythrocyte adhesion to the endothelial luminal surface and a reduced number of pinocytic vesicles at the



**Fig. 6.** Electron micrograph. Ultrastructure of the retina of a rat at the end of week 6 of experimental opioid exposure. Original magnification  $\times 1,200$ . Note: 1, nucleus appears reduced in size and arranged vertically in the edematous cytoplasm of retinal pigment epithelial cells; 2, a deeply invaginated nuclear envelope; 3, numerous intensively osmiophilic phagosomes

endothelial inner surface, which caused a reduction in the transendothelial transport processes. Variation in electron density of retinal pigment epithelial cells was noted. These cells showed increased nuclear heterochromatin, and some of them showed pyknotic nuclei. The apical region of pigment cells showed accumulated osmiophilic phagosomes, which occurred likely due to increased phagocytosis of photoreceptor membranous discs. Focal membrane destruction was observed in some photoreceptor disc fragments. Destruction of apical processes of RPE cells was seen in some zones (Fig. 6).

Compacted heterogeneous fragments of photoreceptor outer segments were occasionally observed at the apical surface of the RPE, in the interphotoreceptor space. Swelling, hypertrophy and occasionally destruction of RPE cell processes were seen (Fig. 7).

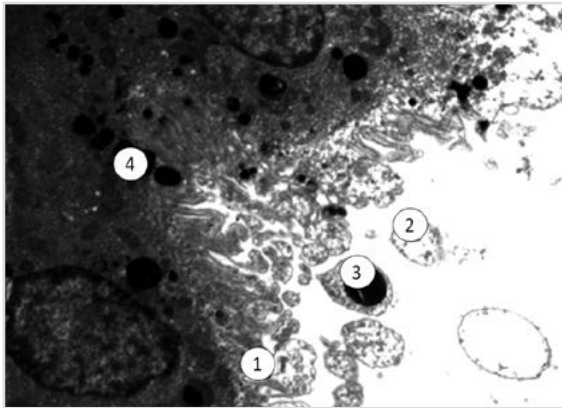
In many zones, photoreceptor outer segments were arranged somewhat loosely and chaotically, and membranous discs showed signs of loss of integrity or their fragments were seen. The photoreceptor inner segment cytoplasm showed clear spaces as a manifestation of edema process. Fragmented and short mitochondrial cristae were observed (Fig. 8). Individual photoreceptor cells showed changes in the structure of the inner segment/outer segment junctions. Edema processes were accompanied by the appearance of significant gaps between the nuclei of photosensitive cells. Some of these cells had irregularly-shaped nuclei with an invaginated nuclear envelope and signs of kariopyknosis.

The outer plexiform layer appeared thin due to migration of the nuclei of both photoreceptors and bipolar neurons. There was an electron lucent axoplasm of individual photoreceptor axons (Fig. 9), along with mitochondrial edema and dilated canaliculi of smooth endoplasmic reticulum. Bipolar cells had clear cytoplasmic zones. Changes of this type were seen in the cytoplasm of amacrine cells, along with the migration of their nuclei to the inner plexiform layer. Individual nuclei of amacrine and bipolar cells showed signs of kariopyknosis. Bipolar cell axons having edematous axoplasm and hypertrophic Müller cell processes were seen in the inner plexiform layer. The lumens of inner plexiform layer capillaries appeared dilated and filled with erythrocytes, there were no microvilli on the luminal surface and a small number of pinocytic vesicles at the inner surface of endothelial cells, and pericapillary zones were impregnated with electron-light masses of transudate, which indicated altered microcirculation.

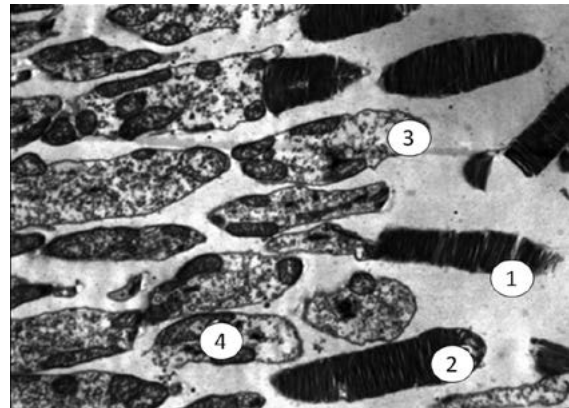
Nuclei of the ganglion layer cells were round, fragments of deeply osmiophilic heterochromatin closely adhered to the nuclear membrane, and dilated granular endoplasmic reticulum cisternae showed some destructed ribosomes. Changes similar to the above were observed in the glial cytoplasm.

### Discussion

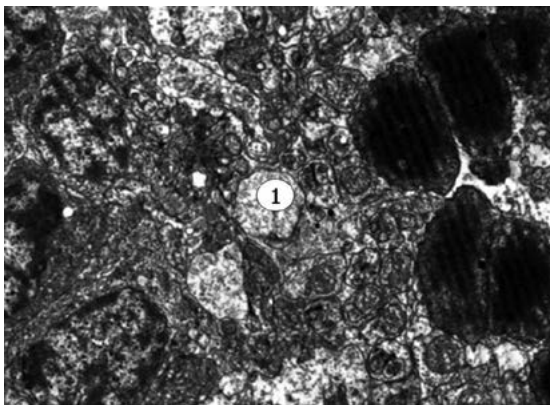
In the current animal study, there was ultrastructural evidence of changes in retinal neurons, glial cells, pigment



**Fig. 7.** Electron micrograph. Ultrastructure of the retina of a rat at the end of week 6 of experimental opioid exposure. Original magnification  $\times 3,000$ . Note: 1, microvilli of retinal pigment epithelium; 2, destruction of apical microvilli; 3, fragments of photoreceptor outer segments on the apical surface of retinal pigment epithelial cells; 4, osmiophilic phagosomes of various size in the cytoplasm of retinal pigment epithelial cells



**Fig. 8.** Electron micrograph. Ultrastructure of the retina of a rat at the end of week 6 of experimental opioid exposure. Original magnification  $\times 3,800$ . Note: 1, delaminated membranes in photoreceptor outer segment discs; 2, destruction of the membranes in photoreceptor outer segment discs; 3, edematous cytoplasm of photoreceptor inner segments; 4, destruction and shortening of mitochondrial cristae of photoreceptor inner segments



**Fig. 9.** Electron micrograph. Ultrastructure of the retina of a rat at the end of week 6 of experimental opioid exposure. Original magnification  $\times 2,200$ . Note: 1, electron-lucent axoplasm of axons in the outer plexiform layer

cells and retinal microcirculation components over 4 to 6 weeks of daily nalbuphine injections at a dose ranging 0.212 to 0.253 mg/kg. Pathomorphological changes were accompanied by an increase in manifestations of vascular hyperemia, degenerative changes in RPE cells and membranous discs of the photoreceptor outer segments, necrobiotic changes in the nuclei of individual photoreceptors, alteration in metabolism and structure in axons of the outer and inner plexiform layers, degenerative changes in retinal horizontal neurons, and the appearance of necrotic structural changes in bipolar and amacrine cells. It is likely that the above changes resulted from an increase in the severity of hypoxic phenomena.

It should be noted that in our previous study (with the drug administered daily at a dose of 0.212 mg/kg

for week 1 only), there was both ultrastructural and light microscopic evidence of less severe morphological changes in the retinal neurons and RPE cells than those observed in the current study, but, in the previous study, that changes maintained for a long period of time [13].

In an experimental hyperthyroidism and hypothyroidism study by Shchur and colleagues [16], the changes in the rat retina were similar to those that we observed under nalbuphine exposure. Thus, Shchur and colleagues [16] reported on the disintegration of the membranes of rods and cones, disorganization of RPE cell processes, vacuolization of the mitochondrial matrix with disorganization of mitochondrial cristae, and presence of neurons with a clear cytoplasm and reduced number of organelles.

In an experimental study by Molchaniuk [17], there was electron microscopy evidence of changes in the rat choroid, RPE cells and retinal neurons 3 hours to 7 days after a single injection of alcohol mixture (40% ethanol and 100% methanol), with these changes being similar to those observed in the current study. Molchaniuk [18] demonstrated that a long-term toxic effect of consuming a small dose of methanol resulted in severe pathologic changes in and poor reserve potential of the cells of the chorioretinal complex, which was reflected in slow repair processes during the period from 1-month to 3-month time points.

We found that the negative effect of 6-week daily nalbuphine injections on the structural components of the retina and retinal microcirculation in the current study was somewhat similar to that of alcohol mixture in studies by Molchaniuk [17, 18].

Pidvalna [11] experimentally investigated the effects of opioid exposure on the choroidal components in the rat, and demonstrated a reduction in the size of lumen of choroidal

blood capillaries, whereas in the current study, there was a gradual increase in the size of lumen of blood capillaries. We believe that this disagreement may be explained by differences in the dose and duration of effects of the analgetic. There are reports on the morphological changes in the microcirculation structures and other components of the eye (e.g., iridocorneal angle structures [10]), central nervous system structures [19], and periodontal tissues [5] following exposure to a nalbuphine dose similar to that used in the current study. Novytsky and colleagues [8] demonstrated negative effects of codterpin, a codeine-based medication, on the optic nerve, with the ophthalmoscopic picture varying from the absence of pathological changes to papillitis and optic nerve atrophy.

The results of our experimental study on the effects of nalbuphine injections on the retina complement the results of studies by others [11, 17, 18] on the effects of various medications on the eye components. The lesion to the retinal microcirculation component which we found is likely to be the major trigger of the pathological processes in the retina, whereas ultrastructural changes in RPE cell processes in the presence of edema of the intra-photoreceptor space may trigger the development of multifocal areas of focal retinal detachment, and destruction of retinal neurons will cause impaired conduction of nerve impulses to the visual centers of the brain and block light perception.

Therefore, in the current rat study, after a 4-week exposure to daily nalbuphine injections at a dose ranging 0.212 to 0.253 mg/kg, there was ultrastructural evidence of destructive processes in the RPE and photoreceptor outer segments, axonal degeneration in the outer and inner plexiform layers, degenerative and necrotic changes in bipolar and amacrine cells, hypertrophic Müller cell processes, ganglion cell degeneration and hyperemia due to an impaired retinal microcirculatory ultrastructure. At week 6 of the experiment, there was evidence of increased destructive and degenerative processes in structural components of the retina.

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## **Disclosures**

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**Author Contributions:** *YP: Conceptualization, Experiment and Data Analysis, Writing – original draft, Writing – review & editing; ZM: Writing – original draft, Writing – review & editing; ICh: Experiment and Data Analysis, Writing – original draft, Writing – review & editing; OD: Writing – original draft, Writing – review & editing; KhS: Writing – original draft, Writing – review & editing; MShch: Writing – original draft, Writing – review & editing.*

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