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Dovga Natalia
Department of Histology, Cytology and Embryology Ivano-Frankivsk National Medical University Ukraine

Sergey Gerashchenko
Department of Histology, Cytology and Embryology Ivano-Frankivsk National Medical University Ukraine

Deltsova Elena
Department of Histology, Cytology and Embryology Ivano-Frankivsk National Medical University Ukraine

Correspondence
Dovga Natalia
Department of Histology, Cytology and Embryology Ivano-Frankivsk National Medical University Ukraine

Effect of 2-ethyl-6-methyl-3-hydroxypyridine succinate on the retina with correction of paclitaxel-induced retinopathy in the experiment

NZ Dovga, SB Gerashchenko and OI Deltsova

Abstract
In the experiment 72 white rats were injected with intraperitoneal Paclitaxel (Actavis, Romania) in the dose of 2 mg/kg body weight 4 times a day, the total dose of 8 mg/kg, followed by 48 animals injected intraperitoneally with 2-ethyl-6-methyl-3-hydroxypyridine succinate (armadin) in the dose of 10 mg/kg (in the control group 24 rats were treated with water for injection intraperitoneally). It was established that in case of paclitaxel-induced retinopathy corrected with armadin the morphometric parameters (thickness of the whole retina and its layers) from the 1st to the 28th day reveal a stable tendency to normalization, indicating the improvement of its morpho-functional state, the restoration of the layer the rods and cones, and the thickness of other layers approaching the standard indices. From the 60th day the positive influence of armadin on the retina fades, a gradual decrease in the thickness of the layer of rods and cones, outer nuclear, plexiform and ganglionic layers takes place. On the 120th day there may be observed signs of swelling in the outer and inner nuclear layers as well as the layer of nerve fibres. The thickness of the retina increases.

Keywords: Paclitaxel, retinopathy, armadin

1. Introduction
Ophthalmopathies as a manifestation of the side effects of taxanes in chemotherapy that is widely used to treat cancer patients, attract all the more attention [5, Modi]. The authors report vision problems during or after receiving a course of Paclitaxel [4, Ito], which are manifested by such complications as cystic maculopathy [7, Rahimy]. Recently the method of optical coherence tomography has allowed to state that after the cessation of systemic antiblastic Paclitaxel chemotherapy the patients' retina thins significantly and remains such for the following 3 months [2, Bakbak]. In view of the above, the question of preventing or correcting the negative impact of Paclitaxel becomes of great importance [3 Ishbashi]. According to researchers, the problem of retinotoxicity caused by chemotherapy should be considered in the overall context of retinotoxicity, which will contribute to the development of innovative neuroprotective strategies [6, Park].
The aim is to study morphometric indices and morphological picture of retina in Paclitaxel-induced retinopathy corrected by using 2-ethyl-6-methyl-3-hydroxypyridine succinate.

2. Material and Methods
In the experiment 80 random bred white rats weighing 150-200 g were given intraperitoneal injections of Paclitaxel (Actavis, Romania) in the dose of 2 mg/kg body weight 4 times a day, the total dose of 8 mg/kg by R. S. Polomano *et al*. [1, Polomano], after which the animals were randomly divided into I (48) and II (32) research groups. In research group I the animals were injected with intraperitoneal 2-ethyl-6-methyl-3-hydroxypyridine succinate (Armadin, LTD., scientific-production firm "micro CHEM", Ukraine) in a dose of 10 mg/kg in 0.5 ml of water for injection. The control group of animals was treated intraperitoneally with water for injection (equivalent volume). 2-ethyl-6-methyl-3-hydroxypyridine succinate is considered to be a cell membrane protector, antioxidant, with the strong influence on nervous system. Normal morphometric indices were identified for 10 intact animals. In the experiment the Animal testing regulations have been observed. Research material (eyeballs) was taken in 1, 7, 14, 21, 27, 60, 90 and 120 days after the last injection. As a fixative 10% neutral-buffered formalin was used and then the sections were embedded in paraffin according to common rules. The sections were stained in hematoxylin and eosin and measured for the thickness of retina and its layers.

For the measurement we used software UTHSCSA Image Tool® for Windows® (version 3) in interactive mode with the use of the microscope Axioskop and digital camera Toupcam UHCCD5100KPA with the software ToupView production of Touptek Photonics Co. Ltd. For the statistical processing we used electronic table Microsoft Excel 2000 and Biostat and Statistica for Windows.

3. Research and Discussion

It was found out that in intact animals the thickness of the layer of rods and cones is (22,64 ± 0.19) μm, outer nuclear (40,98 ± 0,24) μm, outer plexiform (6.08 ± 0.09) μm, inner nuclear (18,92 ± 0.16) μm, inner plexiform (28,04 ± 0,25) μm, ganglionic (5,33 ± 0.06) μm, of nerve fibers (3.02 ± 0.08) μm. In general, the thickness of the retina reaches (125,01 ± 0.15) μm. In the control group the morphometric indices of the retina in all terms of the observations displayed no substantial difference from that present in the intact animals.

1 day after the introduction of armadin (11 days after the last input Paclitaxel) layer of rods and cones has a uniform thickness, and between the outer segments of photoreceptor neurons there are scattered extensions. The morphometric study reveals its thickening (16,97 ± 0,24) μm, in animals of Group I – (10,15 ± 0.15) μm, $p<0.05$. The rods are of different thickness with narrowings and widenings in length. There are dystrophic and sometimes also destructive changes in the inner segment of rods, the connecting cilium is often destroyed. One can observe swelling, deformity of the outer segments, disorganization and vacuolization of the membranous discs of the rods. The membranous disc are vaguely delineated. The intersegmental spaces are unevenly expanded. Between the disks of the destroyed outer segments there can be observed isolated melanosomeses as well as their groups. Outer nuclear layer contains slightly swollen areas between groups of nuclei and has the thickness of (30,86 ± 0.27) μm, in animals II Group – (32,95 ± 0.21) μm, $p>0.05$. In the perikaryon of photoreceptor cells the neuroplasm has swollen, the organelles are identified with difficulty. Outer plexiform layer has thickened to (6,40 ± 0.13) μm, in animals of II Group – (4,06 ± 0.07) μm, $p<0.05$. Here from the outer nuclear layer shifted the nuclei of photoreceptor neurons. There can be seen differently sized processes of photoreceptor and bipolar cells and the dendrites' axolemma and neurilemma become more noticeable; sometimes also the spherules (areas of synaptic contacts of rods with horizontal and bipolar neurons) can be seen. The thickness of the inner nuclear layer is (22,79 ± 0.28) μm, which is 8.8% more than in the intact animals, $p>0.05$. In the neuroplasm of the bipolar neurons of this layer, mitochondria have shortened cristae, there are vacuoles and extended cisternae of the endoplasmic reticulum. Inner plexiform layer thickens to (38,48 ± 0.32) μm – by 5.5%, compared to animals of Group II, > 0.05. Between the processes of neurons there are small sized blisters. Ganglionic layer has become thinner by 45.1% comparing to the index of group II, $p<0.05$. The neurons there are uneven with wide gaps between them. The nuclei of some ganglion neurons lost the roundness and the neuroplasm swollen. Ganglion neurons are found in different morpho-functional state, mainly in the state of swollen neuroplasm. The nerve fiber layer became thicker by 18.59%, $p<0.05$. The thickness of the retina, in general, was by 0.9% less from animals' retina in Group II, comprising respectively (121,66 ± 0.23) μm and (122,76 ± 1.17) μm, $p>0.05$. In the capillaries of the retinal choroid plexus the lumen is narrow, often filled with erythrocytes; the endothelial layer is thin, the basal membrane is not expanded.

The morphometric picture on the 7th and 15th day of the experiment presented the biggest changes in the layer of rods and cones. The surface inverse to the pigment layer is smooth. Very few nuclei have moved here from the outer nuclear layer. The thickness of the layer of rods and cones on the 7th day has risen to (20,01 ± 0.13) μm, (9.81 ± 0.09) μm in animals II group, < 0.05, at the expense of the stretched outer segments of the photoreceptor cells.

The pigment layer cells are activated: they contain numerous melanosomeses, their apical processes seem to be shorter than those in control. At the same time they became less numerous near the outer segments of the photoreceptor cells. Membranous disks of the rods have clear outlines; large vacuoles cannot be observed. Spaces between the disks expanded in the outer segments, which are located near the processes of pigment cells.

Outer nuclear layer extended to (36,44 ± 0.15) μm (16.32% from the 1st day of the experiment). In this layer, the nuclei of photoreceptor cells are placed loosely. The volume of neuroplasm in their perikaryons increases, the mitochondria have thin cristae and undamaged inner and outer membranes.

Outer plexiform layer has obscure outlines due to the fact that the neurons from the adjacent layers move in here; it became slightly narrower – (6.29 ± 0.07) μm, compared to the 1st day (6,40 ± 0.13) μm, $p>0.05$. The spherules increase in number and in size, contain mitochondria and have clearly visible plasmalemma. We often observe the synaptic contacts between neurons and their processes in the outer plexiform layer.

The inner nuclear and the ganglionic layers have expanded, compared to the previously taken data. Neurons of these layers contain large rounded nuclei. In bipolar neurons the nuclei are delineated clearly. In their cytoplasm, groups of mitochondria have been identified, as well as the cisternae of granular endoplasmic reticulum, free ribosomes and polysomes.

Inner plexiform layer has narrowed down to (32,72 ± 0.15) μm, compared to the previous term. The thickness of the retina has risen to (137,11 ± 0,69) μm, whereas in animals of group II the value of this indicator decreased in comparison with the previous period of the experiment.

In the capillaries of the retinal choroid plexus the inner lumen widens, and red blood cells sit there freely. In the cytoplasm of endotheliocytes, near lumenal plasmalemma, micropinocytotic vesicles are concentrated, indicating the directed movement of substances from the lumen of the capillaries into the basal plasmalemma.

14th and 21st days were represented by incremental steps towards stabilization and normalization of the morpho-functional condition of the retina. This is especially noticeable in the layer of rods and cones, the thickness of which amounted to, respectively, (21,30 ± 0.27) mm (24,60 ± 0.35) μm; in the outer nuclear (39,76 ± 0.41) μm (UAH 40.01 ± 0.53) μm; in the nuclear (20,47 ± 0.23) μm (20,24 ± 0.57) μm and ganglion cell (10,11 ± 0.19) μm (8,16 ± 0,37) μm layers of the retina. The thickness of the retina in Group I was, respectively, (136,02 ± 0.08) μm (136,32 ± 0.18) μm versus (111,56 ± 0,78) μm (± 113,60 0,54) μm in case of uncorrected retinopathy, $p<0.05$.

On the 27th day, the changes were moving in different directions, although the indicators of the thickness of the retinal layers only slightly differed from the norm. The

thickness of the layers of rods and cones, outer nuclear and outer and inner plexiform layers as well as the one of nerve fibers reduced, whereas the inner nuclear and ganglion cell layers began to grow. Retinal thickness decreased (131,53 ± 0.18) μm), in animals of Group II – (116,26 ± 0,92), normal state being (125,01 ± 0.15) μm.

On the 21st - 27th day, the condition of capillaries of the basal lamina of capillary choroid improves. The components of Bruch's membranes (basal complex) become thinner, the thickness of their basal lamina of capillary comes to norm.

The invaginations of basal plasmalemma in pigment cells have become deeper, and the processes of the apical plasmalemma have become shorter. In their cytoplasm one can distinguish mitochondria, Golgi complex, the cisternae of granular endoplasmic reticulum and the decreased number of melanosomes, which are located exactly in the apical pole near the outer segments of the rods and cones. The latter are placed orderly, have clear outlines of their outer plasmalemma and membranous discs. Vacuoles are rare and present the expansion of the disc cavities.

The nuclei of photoreceptor neurons show no deviations from the norm. In the outer plexiform layer there can be identified their processes and those of bipolar neurons, both possessing membranes. Spherules of different size are closely adjacent to the plasmalemma of horizontal neurons, forming with them numerous synaptic contacts. The spherules contain mitochondria. Among them radial glial cells with mitochondria of normal structure (except for a few that have shortened cristae and swollen matrix) can be located. Ultramicroscopic structure of bipolar ganglion neurons and structures in the inner plexiform layer and the layer of nerve fibers has returned to normal indices.

From the 60th to the 90th day of the experiment the vector of morphometric changes of the retina in group II animals goes to reducing the thickness of its layers, at the exception of inner and outer plexiform layer. The layer of rods and cones is, accordingly, (21.62 ± 0.16 μm) and (20,38 ± 0.15) μm; outer nuclear (38,49 ± 0.19) μm (36,09 ± 0.14) μm; inner nuclear (20.69 ± 0.14) μm (17,79 ± 0.19) μm; ganglionic – (9,71 ± 0.11) μm (8,92 ± 0.09) μm. All in all, the thickness of the retina has decreased from (125,76 ± 0.14) μm on the 60th day to (123,89 ± 0.13) μm on 90th day. At the same time, the thickness of the outer plexiform has grown from (5,97 ± 0.11) μm on the 60th day to (6,36 ± 0.07) μm on 90th day. In animals with uncorrected Paclitaxel-induced retinopathy the thickness of the outer nuclear layer, on the contrary, increases from 60th to the 90th day by 6.37%; outer plexiform – by 6.96%; inner nuclear – by 11.85%; inner plexiform – by 11.51%. Instead, layers of rods and cones, ganglionic and of nerve fibers remain thinned. All in all, the thickness of the retina of animals in Group II decreased from (137,02 ± 1,34) μm on the 60th day to (112,06 ± 0.64) μm on a 90th day, $p<0.05$.

On the 60th day period the experiment the fibrous layers in Bruch's membrane expanded, the elastic layer has become uneven. The basal laminae of capillaries and pigment epithelium are loosened. The invaginations of plasmalemma on the basal pole of pigment cells have deepened. There are numerous mitochondria with dense and tightly packed cristae. Closer to the apical pole there have appeared a lot of melanosomes. Apical plasmalemma demonstrates long processes directed to the outer segments of the rods and cones. It is in these parts where melanosomes can be noticed. The outer segments of the photoreceptor cells have uneven thickness – their narrowings are interspersed with extensions. The intersegmental spaces have enlarged. Membranous discs acquire extensions up to vacuoles of different sizes.

Perikaryons of photoreceptor cells contain a small number of other organelles. In the outer plexiform layer the synaptic contacts have become less distinct than in the previous period the experiment – the area of axon endings of rods and cones and that of dendritic endings in bipolar neurons diminishes. The capillaries of the outer capillary plexus of the retina (between outer and inner nuclear layers) demonstrate widened lumen. The endotheliocytes of their wall have a thinned cytoplasm. In the plexiform layer the cross sections of processes of the neurons belonging to the adjacent layers are deformed, their membranes are not well shaped. Neurons in the ganglionic layer are presented in the "light" and "dark" form. The "light" neurons have large nuclei, swollen neuroplasm with bloated yet smal-sized mitochondria. "Dark" neurons have thickened cytoplasm, mitochondria with quite long cristae, cisternae of rough and smooth endoplasmic reticulum, free ribosomes and polysomes.

In 90 days after the last armadin injection pigment cells have elongated nuclei with coarse peripheral chromatin condensation. In their cytoplasm there are slightly swollen mitochondria; Golgi complex contains expanded saccules and tubules. Near them one can often observe melanosomes at different stages of development; their content is fine granular, ranging from light grey to black in color. Autophagic structures appear, too. Invaginations of basal pole cytolemma are shallow. The basal membrane of pigment epithelium is loosened. The deformation of the outer segments of the rods and cones continues. When approaching the outer segment of the pigment cell, melanosomes move in the projections of its apical plasmalemma and destroy the nearest membranous discs. The membranous discs are blurry. In the outer nuclear layer the volume reduction of perikaryons of photoreceptor neurons and the simultaneous worsening of their dystrophic processes attract attention. In the outer plexiform layer the dendrites of bipolar neurons contacting with photoreceptor neurons are slightly swollen, deformed. The Synaptic contacts have blurred pre- and post-synaptic membranes. The cytoplasm of bipolar neurons is swollen, they are closely adjacent to each other. In the capillaries of the inner capillary plexus there can be observed a significant thickening of the basal membrane, in the cleavage of which there are pericytes on quite a large area along the perimeter of the capillary. On the lumenal surface of the endotheliocytes there can be observed sail-shaped processes.

On the 120th day after the introduction of armadin, compared with the 90th day, the thickness of the layer of rods and cones is still decreasing; the outer nuclear layer has little changed; inner and outer plexiform, ganglion layers have thinned; the inner nuclear and the nerve fiber layer are enlarged. The thickness of the retina thus slightly increased to (128,66 ± 0,24) μm. Increasing the thickness of the retinal occurred mainly due to the swelling of the inner nuclear layer.

The detected morphological manifestations of positive influence of armadin on the retina can be associated with the pharmacological properties of the medicine, which is the inhibitor of free radicals, a cell membrane protector, produces antihypoxic effect, improves microcirculation and rheological properties of blood; causes strengthening of countervailing activation of aerobic glycolysis and reducing the degree of oppression of oxidizing processes in the Krebs cycle in case of hypoxia with increased content of adenosine triphosphate (ATP) and creatine phosphate, activation of energy-producing

functions of mitochondria, the stabilization of cell membranes. The results received confirm the idea that Armadin (2-ethyl-6-methyl-3-hydroxypyridine succinate) contributes to the preservation of the ganglion cells of the retina and the optic nerve fibers in progressing neuropathy, the consequences of which are chronic ischemia and hypoxia.

4. Conclusion

1. Paclitaxel induces retinotoxicity manifested by morphological and morfometric changes in the retina. Armadin correction of Paclitaxel-induced retinopathy showed that the morphometric parameters (thickness of the separate layers and retina in general) within the period from 1st to 28th day reveal stable improvement of morpho-functional condition of the retina and approaching the thickness of its layers to metric standards. Within 7th-14th day occurs the activation of pigment cells, the outlines of membranes of membranous discs in rods and cones become clear, the number of vacuoles in the discs reduces; mitochondria have well shaped membranes and cristae; synaptic contacts between neurons and their processes in the outer layer of the plexiform (spherules) are clearly delineated. In the retinal capillaries in the cytoplasm of endotheliocytes near lumenal plasmalemma micropinocytotic vesicles are located. The structures of the Bruch's membrane are normalized.
2. On the 60th day begins a gradual levelling of the positive influence of the armadin on the retina. Lack of armadin effect is presented by the loosening of the basal membrane of capillaries and the pigment epithelium, deformation of the outer segments of photoreceptor cells, extension of intersegmental spaces, the formation of vacuoles in membranous discs, swelling of neurons' neuroplasm.
3. Starting with the 90th day, the effect of armadin weakens and the neurons acquire dystrophic changes; to the 120th day the membranes of outer discs of photoreceptor cells are still destabilized; the mitochondria reveal damages of the inner membrane, the radial glial cells become very active. The retina contains the signs of edema and thickening.

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