Neuroprotective effect of 2-ethyl-6-methyl-3-hydroxypyridine succinate on the sciatic nerve and its segmental centers in experimental paclitaxel-induced peripheral neuropathy

Heraschchenko S. B., Ostrovskyi M. M., Kulynych H. B., Markiv I. M.
Ivano-Frankivsk National Medical University, Ivano-Frankivsk, Ukraine

Introduction

Up to 60% of patients suffer from the neurotoxicity of the chemotherapy drug Paclitaxel, namely paclitaxel-induced peripheral neuropathy (PIPN), during the treatment of breast cancer, ovarian cancer, and non-small cell lung cancer [16, 20]. The main symptoms of such neuropathy are burning pain and numbness in the hands and feet, loss of fine motor skills are so pronounced that up to 25% of patients require modification of the paclitaxel treatment regimen, including dose reduction, treatment postponement, or even discontinuation of therapy [2, 9, 20].

In vitro models using rat spinal cord neuron cell lines, human induced pluripotent stem cells, and in vivo models in rodents have identified a number of molecular pathways affected by Paclitaxel. The influence is not limited to axons of sensory neurons, but is present in other types of cells,
such as peripheral neuroglia, soma of segmental motor centers, cells of the immune system. These studies showed that Paclitaxel induces altered calcium signaling, the release of neuropeptides and growth factors, mitochondrial damage and the formation of reactive oxygen species, and has a direct effect on the disruption of microtubule transport [13, 21]. However, today there is still no sufficient neuromorphological data on the patterns of pathomorphogenesis of peripheral neuropathies caused by Paclitaxel. Previous attempts to use various neuroprotective agents in humans and in animal models have not shown sufficient effectiveness in preventing or significantly reducing the intensity of PIPN manifestations. The method of electroacupuncture, the action of magnetic fields, the use of cryotherapy and chylotherapy, the use of vitamin E, B vitamins, omega-3 fatty acids, glutathione, acetyl-L-carnitine, amitriptyline, progesterone, minoxidil and a number of other means have been tested, but this did not bring the desired result [6, 8, 11, 15, 19]. Therefore, it is very important to study and develop potential effective approaches during Paclitaxel chemotherapy to prevent and correct this complication. One of the possible ways to prevent damage to the nervous system during chemotherapy could be the use of metabolic drugs that have antioxidant, antihypoxic, and membrane-stabilizing properties. One of them is 2-ethyl-6-methyl-3-hydroxypyridine succinate (HS), which is quite widely used in endocrinology, neurology, and cardiology [4]. In particular, it was established that the use of HS in patients with diabetic neuropathy leads to a significant improvement in objective indicators and is accompanied by a reduction in its symptoms [17, 18].

The purpose of the study is to study the effect of the neuroprotective agent 2-ethyl-6-methyl-3-hydroxypyridine succinate on the morpho-functional parameters of the sciatic nerve and its segmental centers in experimental paclitaxel-induced peripheral neuropathy.

Materials and methods

The study was conducted on the basis of the Department of Histology, Cytology and Embryology of the Ivano-Frankivsk National Medical University. In the experiment, 56 white rats weighing 150-200 g were used. The animals were kept in vivarium conditions at a temperature of 21-24 °C, under a normal light regime (day-night) and on a diet with access to food and water ad libitum. The experiment was conducted in accordance with the recommendations of ARRIVE and EU Directive 2010/63/EU on the protection of animals used for scientific purposes, in accordance with the provisions of the "European Convention for the Protection of Vertebrate Animals Used for Experiments and Other Scientific Purposes" (Strasbourg, 2005), Law of Ukraine "On the Protection of Animals from Cruelty" (2006, Article 26), "General Ethical Principles of Animal Experiments", approved by the Fifth National Congress on Bioethics (Kyiv, 2013). The research was approved and confirmed by the bioethics commission of the Ivano-Frankivsk National Medical University (protocol № 122/21 - 09.06.2021).

Animals were injected intraperitoneally with Paclitaxel (Actavis, Romania) at a dose of 2 mg/kg of body weight after one day 4 times before reaching a total dose of 8 mg/kg according to the method of Polomano R. S. [14]. After that, the animals were divided into an experimental group - 24 animals, which were injected with 2-ethyl-6-methyl-3-hydroxypyridine succinate (the drug "Armadine", manufactured by Scientific and Production Firm "Microkhim" LLC), and a control group (24 animals, injection of water for injections). Neurophysiological indicators and electron microscopic picture of the norm were determined on 8 intact animals.

Neurophysiological studies were performed at 3-hour intervals on the 1st, 7th, 14th, and 28th days after the last administration of the HS drug. The hallmark of PIPN, mechanical allodynia, was determined as withdrawal of the hind paw of rats in response to stimulation with von Frey monofilaments using the "up-down" method [3, 24]. The main method of studying thermal hyperalgesia is the hot plate test. During its execution, rats were alternately placed on a metal plate heated to 55±1 °С. A stopwatch was used to measure the time from the moment the animal was placed on the plate to the end point of the test - licking the pads of the front and/or hind paws or jumping up. This time was the time of latent pain reaction. The maximum time the animals stay on the plate - 35 seconds [1, 10].

On the same day, the animals were removed from the experiment by applying ether anesthesia. The material for research (sciatic nerves, spinal nodes and the IX plate of the gray matter of the lumbosacral spinal cord (L2-S1)) was taken on 1st, 7th, 14th and 28th (6 animals for each period research) days after the last administration of HS. Electron microscopic research was carried out according to generally accepted methods and studied with the help of a PEM-125 K electron microscope, the image was photographed at a magnification of 4000-12000 times.

Results

Neurophysiological studies. Administration of Paclitaxel caused signs of peripheral neuropathy in the form of thermal hyperalgesia and mechanical allodynia in experimental animals.

When performing the von Frey test in intact animals, the mechanical pain threshold was 55.34±7.58 g. In animals of the control group, on the 1st day of the experiment, the mechanical pain threshold decreased to 26.08±3.39 g (p<0.05), and on the 7th day it dropped to minimum values and was caused by monofilaments with a pressure force of 18.12±2.03 g (p<0.001). From the next term of the experiment, we observed positive dynamics of the development of mechanical allodynia: on the 14th day, the pain threshold increased to 23.24±1.81 g (p<0.001), and on the 28th day it was caused by monofilaments with a
Fig. 1. Pronounced deformation of myelin nerve fibers, vacuolization of mitochondria in the axon, focal splitting of the myelin sheath, proliferation of Schwann cell mitochondria in animals of the control group on the 1st day of the experiment. Electron micrographs. a) x4800, b) x10000. Designation: 1 - myelin sheath, 2 - mitochondria, 3 - vacuoles.

Fig. 2. Layering of lamellae in the myelin sheath (a), disruption of the organization of the myelin sheath in the form of subaxonal inclusions (b) in the control group of animals on the 28th day of the experiment. Electron micrographs. a) x8000, b) x12000.
pressure force of 27.72±5.74 g (p<0.01). In the animals of the experimental group, which underwent HS correction, on the 1st day, the indicator was at the level of 35.43±4.28 g. On the 7th day, the lowest point was reached - the mechanical pain threshold was 28.32±3.65 g (p<0.05), which is 56.43 % better than the control group. Starting from the 14th day, we observed positive dynamics, which significantly exceeded the recovery indicators in animals of the control group. The threshold of pain sensitivity on the 14th day reached the level of 33.86±3.15 g (p<0.05) - 45.56 % higher compared to the control group, and on the 28th day it was 54.94±8.29 g (p<0.05), which differs by 98.17 % from the similar indicator in the control group of animals.

The duration of stay of intact animals on the "Hot Plate" was 17.20±0.93 s Animals of the control group on the 1st day after the last injection of the drug showed signs of thermal hyperalgesia: the indicator decreased to 12.44±1.25 s (p<0.01). On the 7th day, the lowest level of latent time of pain sensitivity was observed - 9.88±0.71 s (p<0.001). From the next term of the experiment, the indicator shows the dynamics of recovery to the original: on the 14th day, it was 10.28±1.01 s (p<0.001), and on the 28th day - 13.02±0.97 s (p<0.01). Thermal hyperalgesia was significantly less pronounced in animals of the experimental group: on the 1st day, the indicator was 14.13±1.31 s and on the 7th day - 13.82±0.72 s (p<0.001), which is 39.61 % better compared to the control group. Already on the 14th day, we observed the indicator approaching the initial level - 16.88±1.62 s (p<0.01), which is 63.95 % higher compared to the similar indicator of animals of the control group. On the 28th day, the time spent on the "Hot Plat" plate remained close to the initial level - 17.14±1.00 s (p<0.01).

**Morphological characteristic.** During the electron microscopic examination of preparations of the sciatic nerves of the control group of rats, it was established that on the 1st to 7th day of the experiment, the following are characteristic: deformation of myelin nerve fibers and their myelin sheath, disruption of its lamellae organization, mainly focal defibrillation of the myelin layers, swelling of the peri-axon space (Fig. 1a). We observe axon deformation, vacuolated mitochondria occur in the axoplasm (see Fig. 1b).

In numerous myelinated nerve fibers, we observe deformation of the myelin sheath and axon and an increase in the density of axoplasm. On the 14th-28th day of the experiment, the phenomena of destruction in myelin nerve fibers increase in animals of the control group. In most myelinated nerve fibers, the myelin sheath is thickened due to swelling of Schwann cells and intra-lamellae vacuolation (Fig. 2a). Quite often we observe myelinated nerve fibers with almost complete axon degeneration and severe swelling of the myelin sheath.

Manifestations of vacuolar transformation of membrane

![Fig. 3. Defibrillation of the myelin sheath (a), disorganization of neurotubules and neurofilaments (b) in the myelin nerve fibers of the sciatic nerves of animals on the 14th day after the last administration of HS on the background of PIPN. Electron micrographs. a) x6400, b) x96000. Designation: 1 - defibrillation of the myelin sheath, 2 - intra-lamellae vacuoles, 3 - vacuolar transformation of mitochondria, 4 - periaxonal vacuole.](image-url)
Fig. 4. Intra-lamellae vacuoles (a) and multiple flake-like inclusions against the background of swelling of the axon neuroplasm (b) of myelin nerve fibers of the sciatic nerves of animals of the experimental group on the 28th day of the experiment. Electron micrographs. 

(a) x9600, (b) x9600.

Fig. 5. Increased activity of the granular endoplasmic reticulum, hypertrophy of the Golgi complex in neurons of the anterior horn of the spinal cord. The term of the experiment is 7 days. Electron micrograph. 

(a) x6400, (b) x6400. Designation: 1 - autophagosome, 2 - lysosome, 3 - granular endoplasmic reticulum, 4 - nucleus, 5 - Golgi complex.
organelles are increasing. The vast majority of mitochondria are enlarged, with the phenomena of destruction of cristae, lightening of the matrix. On the 28th day of the experiment, pronounced disturbances in the structure of their axons were observed in individual myelinated nerve fibers, smaller and larger vacuoles, including periaxonal vacuoles, were found in the axoplasm (see Fig. 2b). At the same time, the axoplasm of individual axons contains polymorphic vacuoles, a moderate number of neurofilaments and neurotubules. The phenomena of myelinophagy of Schwann cells are frequent, remnants of axial cylinders are determined in their cytoplasm. In the specified terms, significant changes occur, which are manifested by a violation of axon transport systems, in particular, we see conglomerates of neurotubules and neurofilaments and accumulation of synaptic vesicles.

In animals of the research group that received correction, changes in myelin nerve fibers on the 7-28th day are less pronounced. In the sciatic nerves, we observed a polymorphic morphological pattern with the presence of both myelinated nerve fibers of a normal structure and with varying degrees of destruction and signs of structural restoration. In damaged myelinated nerve fibers, the myelin sheath also undergoes changes; it is unevenly thickened, in places of fiberization, but without gross violations of the lamellae structure. Axons contain expanded cisterns of agranular endoplasmic reticulum, vacuolated mitochondria. A distinctive feature of individual myelinated nerve fibers is the manifestation of distorted regeneration, namely the phenomenon of hypertrophy and disorganization of the myelin sheath. Intramyelin vacuoles, slight defibrillation of myelin plates are found in some of the nerve fibers (Fig. 3). Cytoplasmic vacuolation was observed in Schwann cells of myelinated nerve fibers, moderate edema was observed in the endoneurium. In some myelinated nerve fibers with preserved ultrastructure of the myelin sheath, phenomena of axonopathy were found, in particular, the presence of multiple fine-grained inclusions, accumulation of cisterns of agranular endoplasmic reticulum, uneven electron density of hyaloplasm (Fig. 4). Along with the destructively changed myelin nerve fibers, we also observed nerve fibers with pronounced signs of regeneration and the preserved ultrastructure of the myelin sheath.

In neurons of the spinal cord, a 10-day course of HS administration has a positive effect on the morphological state of mitochondria and activates synthetic processes in neurons. On the 7th-28th day after HS correction, an increase in the activity of the granular endoplasmic reticulum (Fig. 5a) can be observed in neurons, and the number of ribosomes attached to its cisternae increases. This ensures the biosynthesis of proteins to restore the protein microstructures of the neuron. Numerous ribosomes and polyribosomes are observed in the neuroplasm. At the same time, the hypertrophy of the Golgi complex, the presence of a sufficient number of lysosomes and autophagosomes in the neuroplasm, which creates the prerequisites for cleaning neurons from toxic and harmful products in order to accelerate their full regeneration (see Fig. 5b).

In the electron microscopic picture of the spinal cord of animals of the control group within 1-28 days after the last administration of HS, mostly neurons with light hyaloplasm (light neurons) are observed. The nuclei of such neurons are located in the center of the cell, their karyolemma contains a peripheral cluster of heterochromatin and clumps of heterochromatin in the karyoplasm against a background of euchromatin. In the cytoplasm of a sensitive pseudounipolar neuron, we observe signs of swelling, few mitochondria, some of which are vacuolated. Dissociated granular endoplasmic reticulum is located perinuclearily as a result of local central chromatolysis of Nissl bodies, and on the periphery of neurons we can see phenomena of chromatolysis and large transparent vacuoles (Fig. 6a).
In the cytoplasm of dark neurons, we see mitochondria of normal structure, Nissl bodies, a small number of smooth endoplasmic reticulum cisternae, ribosomes and polyribosomes.

On the other hand, in the spinal cords of animals with PIPN without HS correction, the majority of dark neurons had signs of pronounced vacuolar dystrophy (see Fig. 6b). Enlightenment of the matrix of mitochondria, disorganization and destruction of their cristae was noted. At the same time, in the soma, we observed an expansion of the cisterns of the granular endoplasmic reticulum and a decrease in the number of fixed ribosomes. Peripheral chromatolysis phenomena were observed in individual bright neurons. These changes occurred against the background of microcirculation disturbances in the spinal nodes: microclasmatosis phenomena were present in the lumen of capillaries, and an increase in the number of micropinocytotic vesicles was observed in the cytoplasm of endotheliocytes, which indicates an increase in transcapillary exchange.

**Discussion**

Our study was focused on testing 2-ethyl-6-methyl-3-hydroxypyridine succinate as a potential neuroprotector in experimental paclitaxel-induced peripheral neuropathy based on a complex morpho-functional analysis. The morphological changes of the sciatic nerve that we discovered from the beginning to the 28th day of the experiment in the control group of rats coincide with the processes described by other researchers in experimental PIPN, namely: focal destruction and swelling of myelin nerve fibers, pronounced changes in the lamellae of the structure of the myelin sheath, disturbances processes of division in Schwann cells with subsequent involvement in the pathological process of axons and accumulation of neurofilaments and neurotubules [12, 23]. In HS-treated rats, the destructive-dystrophic phenomena in the myelin nerve fibers of the sciatic nerves are less pronounced: edema and intralaminar vacuoles in the myelin sheath are visualized in the myelin nerve fibers. In individual fibers in the axon, phenomena of incomplete splitting of mitochondria with the formation of vacuoles filled with medium electron density contents are observed, and small young mitochondria with a matrix of increased electron density are also visualized.

Examining the motoneurons of the anterior horns of the spinal cord during the first 28 days after the use of HS for the correction of PIPN, we found pronounced regenerative and restorative processes, confirmed by the stable state of mitochondria and the appearance of their young forms in the neuroplasm, hypertrophy of the Golgi complex, and hyperplasia of the granular endoplasmic reticulum. Along with this, light neurons with signs of vacuolar dystrophy are observed. These changes occur against the background of restoration of the ultrastructural organization of the blood–neral barrier. Our data are consistent with the results of other researchers who proved the toxic effect of Paclitaxel on efferent neurons, in particular on axons and soma [5, 22]. The use of HS for the correction of paclitaxel-induced changes in neurons of the spinal cord showed a positive effect. During the first 14-28 days, we noted less pronounced destructive-dystrophic changes in the neurons of the spinal cord nodes, namely: central and peripheral chromatolysis of light and dark neurons and swelling of their cytoplasm, neurophagy phenomena in glial cells, hypertrophy of certain areas of the myelin sheath, with delamination and separation of myelin plates in the myelin nerve fibers of the posterior root of the spinal cord against the background of HS correction.

Therefore, the pathophysiological mechanisms of neurotoxicity when using Paclitaxel are: degeneration and demyelination of axons, impaired axonal transport, oxidative stress, mitochondrial dysfunction and immune-mediated reactions [7, 8]. The use of HS neutralizes these processes and leads to regenerative and restorative changes in the segmental centers of the sciatic nerves.

The results of the electron microscopic study obtained by us are fully consistent with our data of neurophysiological studies and indicate the possibility of using HS as an effective neuroprotector in PIPN.

**Conclusions**

1. After the correction of paclitaxel-induced peripheral neuropathy by 2-ethyl-6-methyl-3-hydroxypyridine succinate, the electron microscopic picture of the sciatic nerve, neurons of the anterior horns of the spinal cord, and most of the neurons of the spinal cord nodes showed marked regeneration processes within 1-28 days.

2. The use of 2-ethyl-6-methyl-3-hydroxypyridine succinate at a dose of 10 mg/kg of body weight within 10 days after the last administration of Paclitaxel significantly reduces the manifestations of peripheral neuropathy caused by the latter on the 7th, 14th and 28th that day of the experiment. The disappearance of manifestations of thermal hyperalgesia when using 2-ethyl-6-methyl-3-hydroxypyridine succinate is observed already on the 28th day of the experiment.

**References**


стало вивчення впливу нейропротекторного середника 2-етил-6-метил-3-гідроксипіридину сукцинату (ГС) на морфо-функціональні параметри сіднього нерва та його сегментарних центрів при експериментальній ПІПН. В експерименті використали 56 білих щурів, яким вводили внутрішньочеревно Паклітаксел у дозі 2 мг/кг маси тіла через одну добу 4 рази, після цього тварин поділили на дослідну групу - 24 тварин, яким вводили ГС і контрольну (24 тварини, введення води для ін'єкції) групи. Методом вивчення механічної апоплії були монофіламенти фон Фрея, термічну гіпералгезію вивчали тестом "гаряча пластина", а електронномікроскопічне дослідження здійснювали згідно загальноприйнятих методів і вивчали за допомогою електронного мікроскопа ПЕМ-125 К. Результати тестів "гаряча пластина" та використання монофіламентів фон Фрея показали, що застосування ГС достовірно знижує прояви ПІПН на 7-у, 14-у та 28-ту добу експерименту. У щурів, що отримували лікування ГС, деструктивно-дистрофічні явища в мієлінових нервових волокнах сіднього нерва є менш вираженими, а у окремих волокнах в осьових цилідронах спостерігаються явища незавершеного відщеплення мітохондрій з утворенням заповнених середньою електронною щільністю вакуолей, а також візуалізуються дрібні молоді мітохондрії.

Результати електронномікроскопічного дослідження повністю узгоджуються з даними нейрофізіологічних досліджень і вказують на можливість використання ГС як ефективного нейропротектора при ПІПН.

Ключові слова: паклітаксел, хіміотерапія, нейропатія, центральна нервова система, периферійна нервова система.

Author's contribution
Herashchenko S. B.: methodology and original project writing, project administration, software, supervision, data visualization. Ostrovskyi M. M.: conceptualization, research implementation, data visualization, formal analysis and validation. Kulynych H. B.: research, statistical processing, review writing and editing. Markiv I. M.: research, statistical processing, review writing and editing.