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Neuroprotective effect of erythropoietin in amyotrophic lateral sclerosis (ALS) model *in vitro*. Ultrastructural study

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Abstract

Erythropoietin (EPO) is a chemokine hormone that is widely distributed throughout the body including nervous system. For last years its role as cytokine involved in many physiological processes out of the bone marrow has been suggested. Moreover, it plays a very important role in CNS as potential neuroprotective agent. There is much evidence that EPO protects neuronal cells in vitro *and* in vivo *models of brain injury, independently of its erythropoietic action. The aim of this study was to determine the potential neuroprotective effects of erythropoietin on the glutamate-mediated injury of motor neurons (MNs)* in vitro*. The study was performed on organotypic cultures of the rat lumbar spinal cord subjected to glutamate uptake blocker, DL-threo-beta-hydroxyaspartate (THA) and pretreated with EPO. Ultrastructural study evidenced that the spinal cord cultures pretreated with EPO exhibited less severe neuronal injury. The cultures exposed to EPO + THA showed inhibition of early MNs degeneration, including various mode of degenerative changes caused by THA, whereas in the later period the typical postsynaptic necrotic changes of neuronal cells occurred. However, the ultrastructural characteristics of apoptotic MNs changes were not observed during the whole period of observation. The results of this study indicate that, in the model of chronic glutamate excitotoxicity, EPO exhibits the neuroprotective ability mainly through prevention of apoptotic neuronal changes.*

Key words: erythropoietin, neuroprotection, excitotoxicity in vitro*, motor neuron degeneration.*

Introduction

Erythropoietin is the well known primary regulator of erythropoiesis, being produced by the kidney in response to hypoxic stress; it stimulates bone marrow to produce erythrocytes. For last years however, its role as cytokine involved in many physiological processes out of the bone marrow has been documented [27]. There is much evidence that EPO exerts neurotrophic and neuroprotective abilities independently of its erythropoietic action [6].

Amyotrophic lateral sclerosis (ALS) is one of the neurodegenerative diseases of upper and lower motoneurons, leading to death usually within 3-5 years, mainly because of respiratory insufficiency and hypoxemia. There is no efficient, safety and well

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tolerated drug discovered till now, which can stop the progression of the disease. That is why many experimental and clinical trials have been performed for years trying to find out the real pathomechanisms of the disease and secondly to reach any mediators which could potentially effect on the course of the degenerative process [23]. Multifactoral pathogenic mechanisms, including inflammation, attenuated survival signals and enhanced death signals are suggested to be involved in ALS. Glutamate excitotoxicity is the well-documented pathogenic factor of neurodegeneration that is considered to contribute to the development of neuronal cell death in ALS.

EPO is suggested to be one of compounds, that might play a potential neuroprotective role in ALS. There are many data showing that EPO helps neurons, exposed to damaging agents, to survive. It is especially involved in apoptotic mechanism of cellular death. As a neuroprotective agent, erythropoietin has many functions: antagonizing glutamate cytotoxic action, enhancing antioxidant enzyme expression, reducing free radicals production rate and affecting neurotransmitter release. It works indirectly through restoration of blood flow or directly by activating transmitter molecules in neurons. Although apoptosis is not reversible, early intervention with neuroprotective therapeutic procedures such as erythropoietin administration may reduce the number of neurons that undergo this mode of cell death [12,19]. Previous studies have shown that EPO exerts the neuroprotective effect as preconditioning and post injury adding agent. In contrast to its antiapoptotic effect, EPO has not shown any activity in preventing necrosis in the nervous system [29].

The exact mechanism leading to progressive motor neurons (MNs) loss in the course of ALS remains unclear, but glutamate-mediated mechanism is thought to be responsible. Our previous ultrastructural studies performed on a model of ALS *in vitro*, based on chronic glutamate excitotoxicity, revealed the coexistence of neuronal degeneration and astroglial abnormalities and suggested an active role of astrocytes in MNs degenerations [22]. The studies evidenced also subset of morphological features characteristic to different modes of MNs death, including apoptotic, autophagic and necrotic degeneration [20,21].

The aim of this study was to determine the effect of erythropoietin on ultrastructural characteristics of the MNs in organotypic cultures of rat lumbar spinal cord chronically exposed to specific glutamate uptake blocker DL-threo-beta-hydroxyaspartate (THA), pretreated with erythropoietin.

Material and methods

The study was performed on organotypic cultures prepared from lumbar spinal cord obtained from 8-day-old rat pups. The explants were placed on collagen-coated cover glasses with two drops of nutrient medium (consisting of 25% inactivated fetal bovine serum and 75% Dulbeco Modified Eagle's Medium supplemented with glucose to a final concentration of 600mg% and with antibiotics), sealed into Maximow double assemblies and kept at 36.6°C.

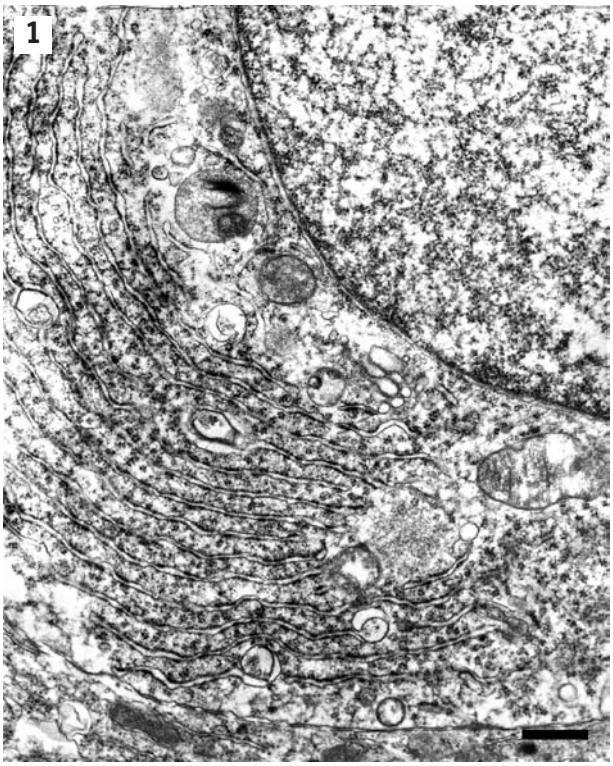
On the 10-14th *in vitro* (DIV), the well-differentiated cultures were incubated with 100 μM of specific glutamate uptake blocker, DL-threo-beta-hydroxyaspartate (THA, Sigma) and erythropoietin in three experimental groups: 1) group incubated with 5 U/ml of EPO, 2) cultures treated with 100 μM of THA, 3) cultures incubated with medium containing THA but pretreated with EPO (5 U/ml).

After 24 hours, 3, 5, 7, 9 and 14 days post exposition the cultures were processed for electron microscope. They were fixed in a mixture containing 0.8% formaldehyde and 2.5% glutaraldehyde for 1 hour, postfixed in 15 osmium tetroxide, dehydrated in alcohols in graded concentrations and embedded in EPON 812. Ultrathin sections were counterstained with uranylacetate and lead citrate and examined in JEOL 1200EX electron microscope.

Results

Spinal cord cultures exposed to EPO alone, displayed quite well preserved perikarya and cellular processes of MNs as well as astroglial cells, likewise the spinal cord cultures maintained in medium without additional compounds (Figs. 1 and 2). Normal appearance of MNs was characterized by round nucleus with evenly dispersed nuclear chromatin, usually prominent nucleolus and large perikaryal cytoplasm with rich granular endoplasmic reticulum, typically arranged in parallel profiles, well maintained mitochondria and Golgi apparatus.

The cultures treated with THA showed early changes even 3 hours after THA exposure. The MNs showed dispersion of rough endoplasmic reticulum, peripheral swelling and slight microvacuolization of perikaryal cytoplasm (Fig. 3). Swollen cytoplasm of



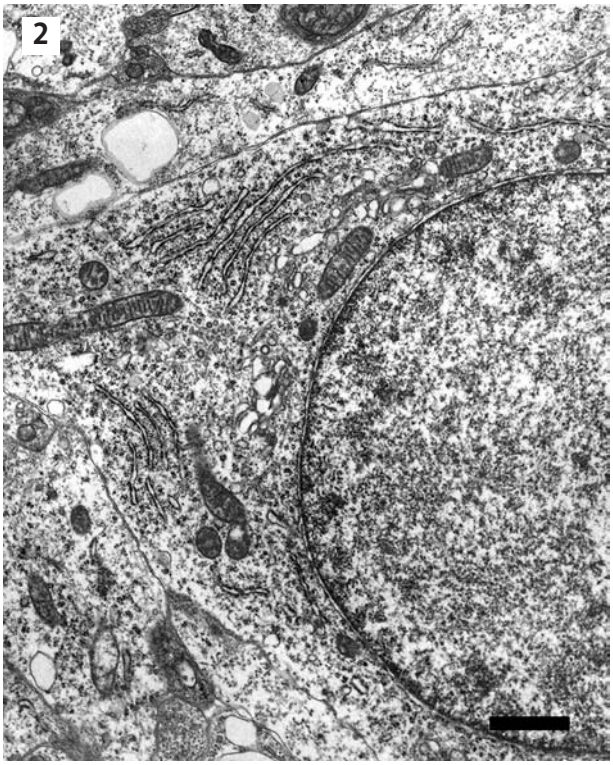
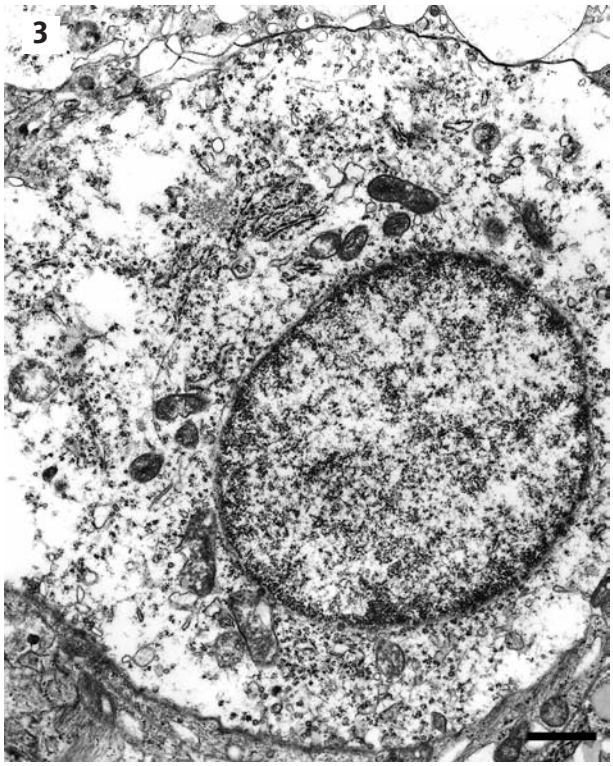
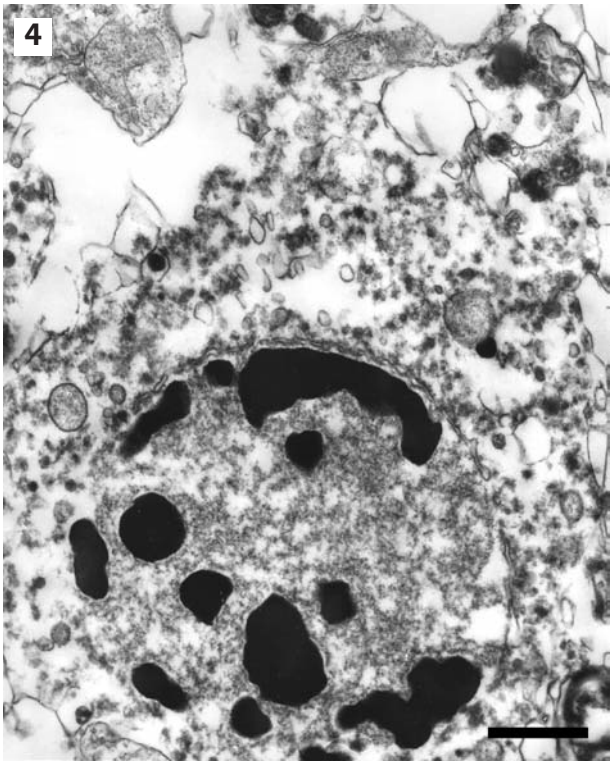


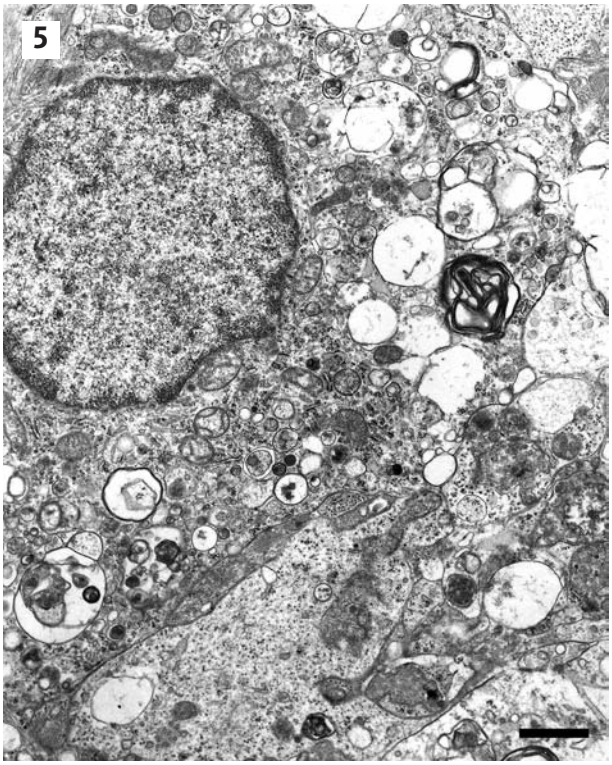
Fig. 1. Cell body of normal MN in lumbar spinal cord culture at 29 DIV. Bar – 500 nm.

Fig. 2. Normal perikarya of MN with large eu-chromatic nuclei and well preserved neuronal and glial processes in neuropil. 5 days of 5 U/ml EPO incubation. Bar – 1 μm.

Fig. 3. MN with swollen cytoplasm and depletion of rough endoplasmic reticulum. 3 hours of 100 μM THA incubation. Bar – 1 μm.







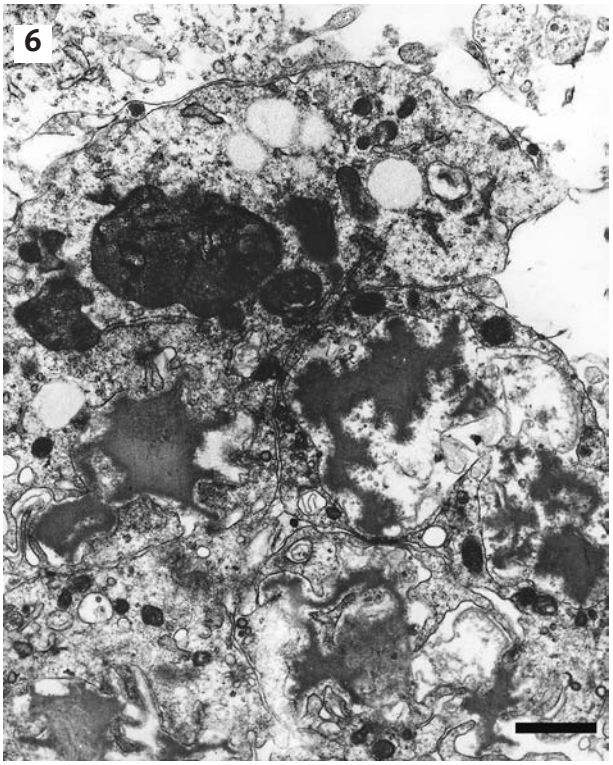
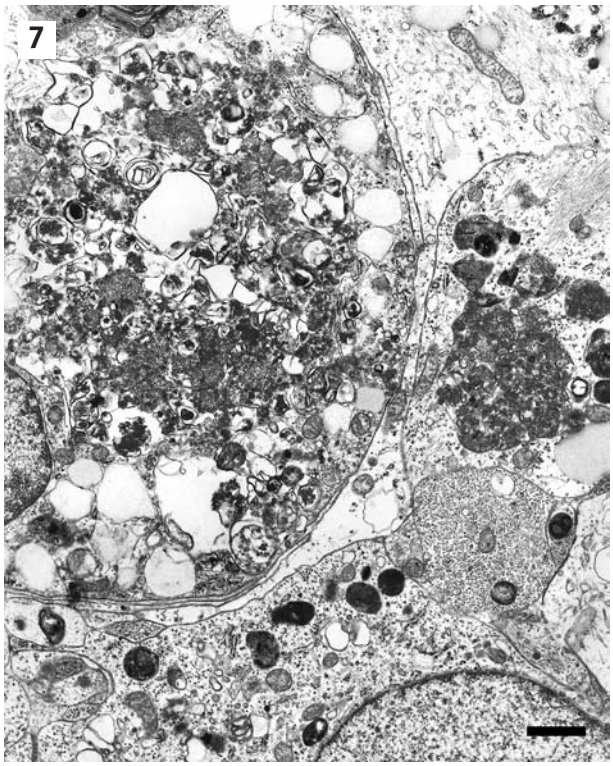


Fig. 4. Dying neuron with swollen necrotic cytoplasm and apoptotic-like pattern of nucleus. 3 days of 100 μM THA incubation. Bar – 1 μm.

Fig. 5. MN with cytoplasm containing numerous autophagic vacuoles. 9 days of 100 μM THA incubation. Bar – 1 μm.

Fig. 6. Various apoptotic bodies and heterolysosomes within cytoplasm of phagocytic cells. 5 days of 100 μM THA incubation. Bar – 1 μm.



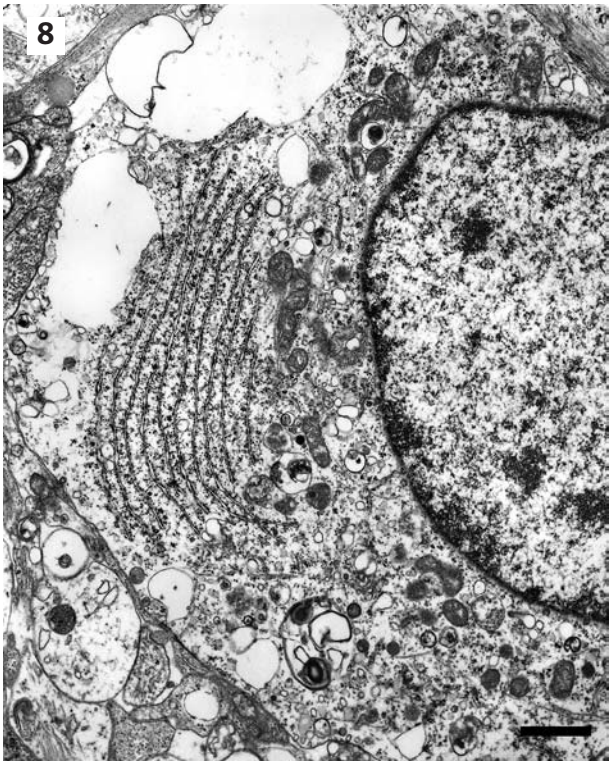
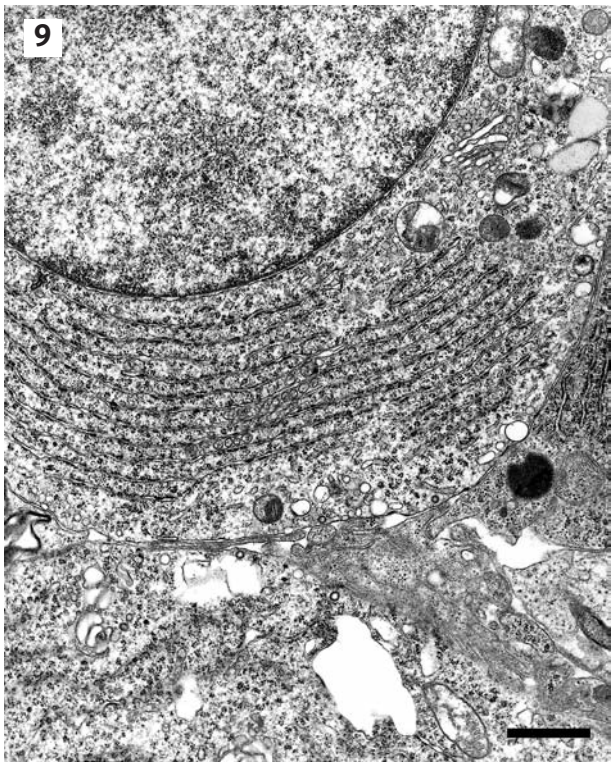


Fig. 7. Degenerating cellular debris enclosed by processes of astroglial and phagocytic cell. 7 days of 100 μM THA incubation. Bar – 1 μm.

Fig. 8. Quite well preserved MN with cytoplasm showing vacuolar changes but well maintained long profiles of granular endoplasmic reticulum and other organelles. 3 hours of 5 U/ml EPO + 100 μM THA incubation. Bar – 1 μm.

Fig. 9. MN with well preserved nucleus and cytoplasm containing regular long profiles of granular endoplasmic reticulum. Slight vacuolar changes are seen in cytoplasm of a neighboring MN. 3 days of 5 U/ml EPO + 100 μM THA incubation. Bar – 1 μm.



postsynaptic dendrites was also observed. At 3-7 days after THA exposure there were numerous damaged MNs, exhibiting predominantly ultrastructural features of necrotic and apoptotic mode of cell death (Fig. 4). Later, 7-9 days after THA exposure, some MNs showed autophagic degenerative changes within the cytoplasm (Fig. 5). Advanced degeneration of MNs was accompanied by numerous scattered membrane-bound apoptotic bodies and fragments of degenerative cellular debris, ingested by cytoplasm of glial and phagocytic cells (Figs. 6 and 7). The cultures exposed to THA and pretreated with EPO uncommonly exhibited damaged MNs during early period of observation and increase of delayed necrosis of MNs at 7-9 day of experiment. The majority of MNs were well preserved in cultures examined at 3 hour (Fig. 8), 3 day (Fig. 9) and 5 day after EPO + THA (Fig. 10). Many MNs showed normal ultrastructural appearance of nuclei and regular arrangement of long profiles of granular endoplasmic reticulum in the cytoplasm. Mostly observed slight changes consisted of membrane-bound vacuoles located in peripheral part of cytoplasm or fine vesicular changes. The majority of glial cells and cellular processes in neuropil were also well preserved (Fig. 11). Occasionally, advanced swelling of cytoplasm and/or necrotic or autophagic type of changes were observed in individual neuronal cells (Fig. 12). At 7-9 days after EPO + THA treatment, the typical postsynaptic necrotic changes of neuronal cells occurred (Figs. 13 and 14) and were more common than during earlier period of observation. At the same time, many postsynaptic dendrites and MNs showed undamaged ultrastructure (Figs. 15 and 16). During the whole period of observation, MNs with ultrastructural characteristics of apoptotic changes were not occurred.

Discussion

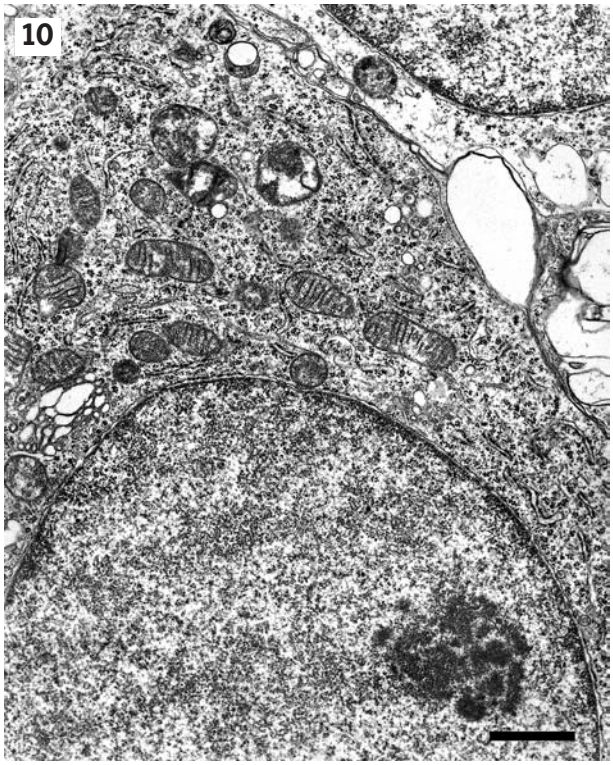
The term neuroprotection refers to mechanisms which protect neurons from degeneration in various types of brain injury or chronic neurodegenerative diseases. The real mechanism of many neurodegenerative processes remains still unknown, but one of them seems to be related with the glutamate excitotoxicity.

Previous studies have suggested that EPO might protect neurons from glutamate toxicity *in vitro* [25]. Erythropoietin is a chemokine hormone that is widely distributed throughout the body. EPO and its receptors (EPOR) are also present in the nervous system [7]. It plays a very important role in CNS as potential neuroprotective agent acting via direct modulation of neuronal excitability and as trophic factor for neurons both *in vivo* and *in vitro* in variety of physiological and pathological states of the nervous system.

Preclinical findings show, that EPO synthesis is triggered in response to cellular hypoxia. The erythropoietin administration occurred to protect nerve cells from hypoxia-induced glutamate toxicity [5,25] induced by many other agents as excitotoxins, glucose deprivation, kainate, serum deprivation, anoxia, NO, NMDA, glutamate and chemical hypoxia. It was shown that neurons within the penumbra would undergo apoptosis unless exposed to EPO within 3 hours after injury [30]. The expression of EPO and EPOR is highly induced by various stressors in both, animal models and human diseases [11]. It has been documented that EPO administration is also associated with marked decrease in proinflammatory cytokines within hemisphere undergoing infarction. The important role of EPO in reducing immune response was established on a model of experimental autoimmune encephalomyelitis [2]. EPO has been also shown to reduce injury and enhance recovery in compression injuries of peripheral nerves [8,9,13,28]. Moreover, erythropoietin improves hippocampus dependent memory by modulating plasticity, synaptic connectivity and activity of memory-related neuronal networks [1,10].

The mechanisms of action of EPO and other potentially neuroprotective agents were identified on different, molecular, cellular and clinical levels [24]. The neuroprotective effect of EPO has been demonstrated in different animal models [26] and preclinical studies of certain nervous system diseases. This chemokine hormone might inhibit apoptosis of neurons in spinal cultures induced by kainate [31]. Systemically delivered recombinant human EPO (rhEPO) can cross blood brain barrier and inhibit neuronal death and inflammatory processes. The mechanism of neuroprotective action of EPO seems to be *via* stimulation of neuronal function and viability *via* activation of Ca^{2+} channels [16].

Recently, the treatment with erythropoietin is proposed in cerebrovascular events and different neurodegenerative diseases or chemotherapy-induced peripheral neuropathy [4]. EPO has been tested in clinical trial in aspect of its potential effect in stroke, schizophrenia and ALS.



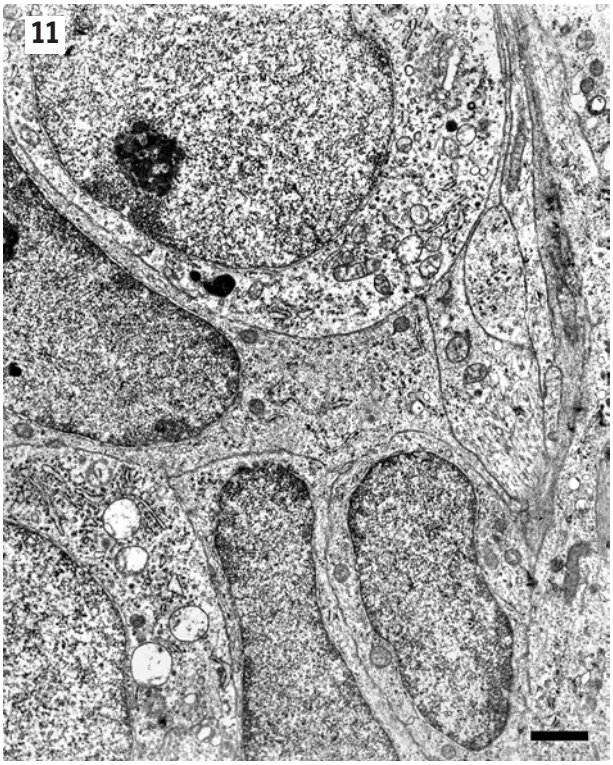
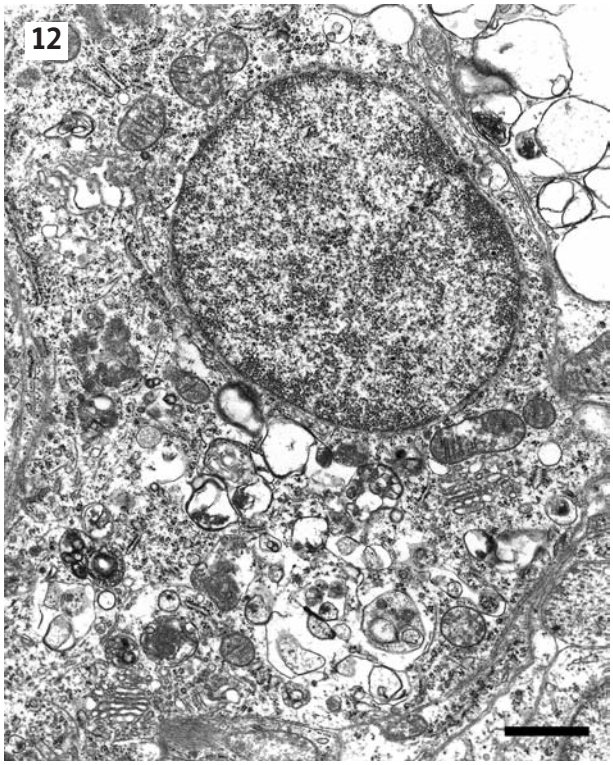
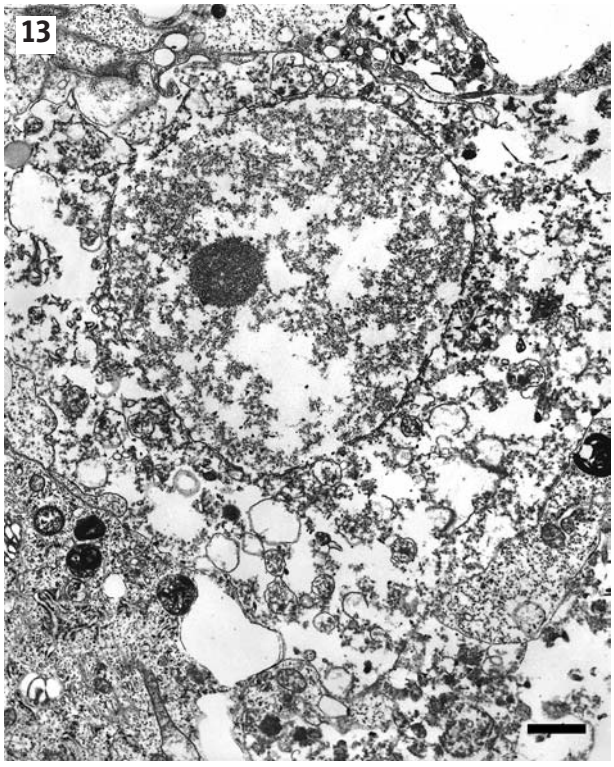


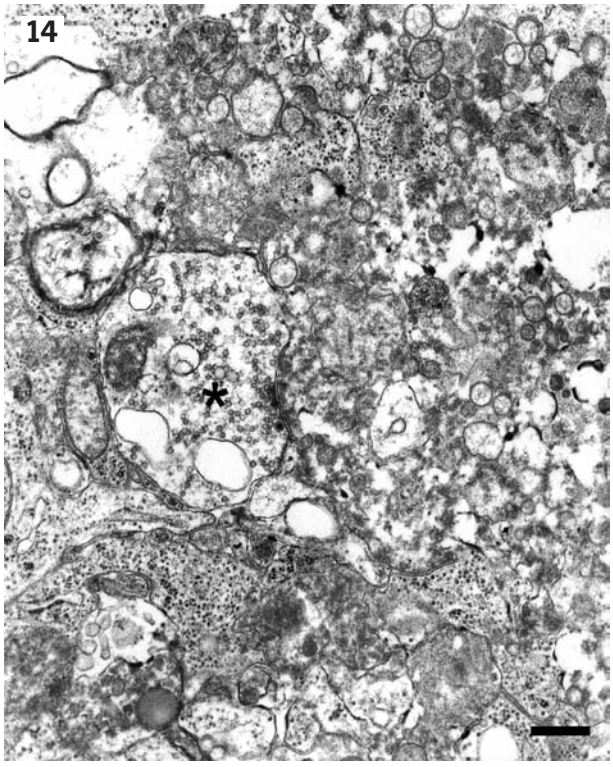
Fig. 10. Normal appearance of a MN with a large euchromatic nucleus, prominent nucleolus and well preserved organelles in cytoplasm. 5 days of 5 U/ml EPO + 100 μM THA incubation. Bar – 1 μm.

Fig. 11. Well preserved neuronal and astroglial cells. 5 days of 5 U/ml EPO + 100 μM THA incubation. Bar – 1 μm.

Fig. 12. MN show many autophagic vacuoles in the cytoplasm. Nucleus appears normal. 5 days of 5 U/ml EPO + 100 μM THA incubation. Bar – 1 μm.







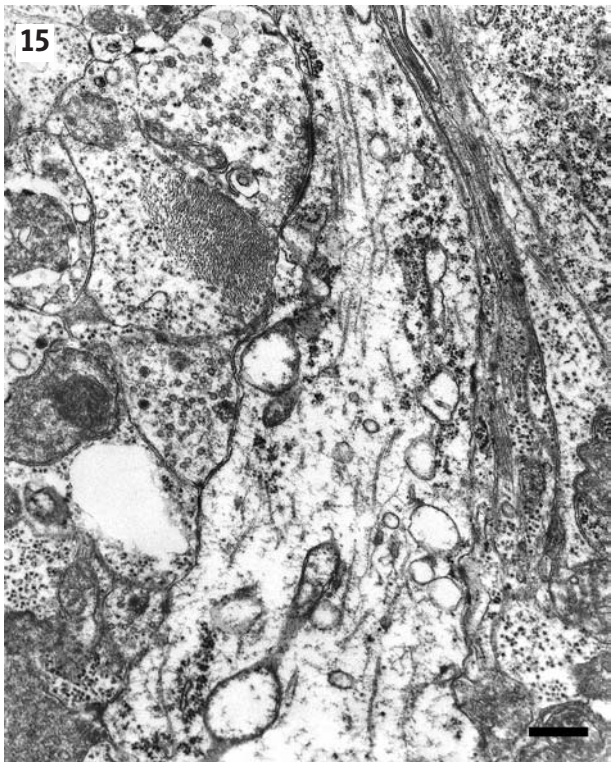


Fig. 13. Typical feature of necrotic death of neuronal cell. 9 days of 5 U/ml EPO + 100 μM THA incubation. Bar – 1 μm.

Fig. 14. Presynaptic ending (asterix) with well preserved synaptic vesicles and postsynaptic neuronal cells with necrotic changes. 9 days of 5 U/ml EPO + 100 μM THA incubation. Bar – 500 nm.

Fig. 15. Normal appearance of presynaptic endings forming synapses with a well maintained postsynaptic dendrite of the motor neuronal cell. 9 days of 5 U/ml EPO + 100 μM THA incubation. Bar – 500 nm.

Recombinant human EPO (rhEPO) has been shown to be relatively safe drug, as many patients have received it over the last decade for treatment of anemia. Well known side effects of EPO includes increased blood pressure and risk of thrombosis, especially in patients who are not anemic. Thus, the use of EPO in chronic diseases associated with neuronal apoptosis or neuroinflammation, such as Parkinson's disease, Alzheimer disease or multiple sclerosis, would be possible only if the erythropoietic effect was blocked. In clinical trials the recombinant human erythropoietin was proposed to be used for treatment in patients with ALS [15,17]. This compound occurred usually well tolerated and safe. There have been also studies to access the effect of rhEPO in animal models of ALS, the results of which show that the treatment significantly prolongs the onset of clinical symptoms, preserves motor neurons, enhances survival signals and attenuates inflammatory signals in dose dependent manner [15].

This study support the evidence of the role of EPO in neuroprotection in model of ALS *in vitro*. Our previous ultrastructural study evidenced that THA exposition induces selective degeneration of MNs characterized by various types of morphological changes, including necrosis, apoptosis and autophagocytosis. The cultures pretreated with EPO exhibited less severe neuronal injury. The cultures exposed to EPO + THA showed inhibition of early MNs degeneration, including various mode of degenerative changes typical for THA effect. However, in later period (7-9 days) of observation the delayed necrotic changes of MNs, typical for excitotoxicity, were seen. The results of this study indicate that EPO exerts neuroprotective effect in the investigated model of chronic excitotoxicity mainly through prevention of apoptotic neuronal changes.

Our results as well as collected previous data [14,18] suggest that EPO may be promising therapeutic drug in different neurological diseases, including ALS.

Acknowledgements

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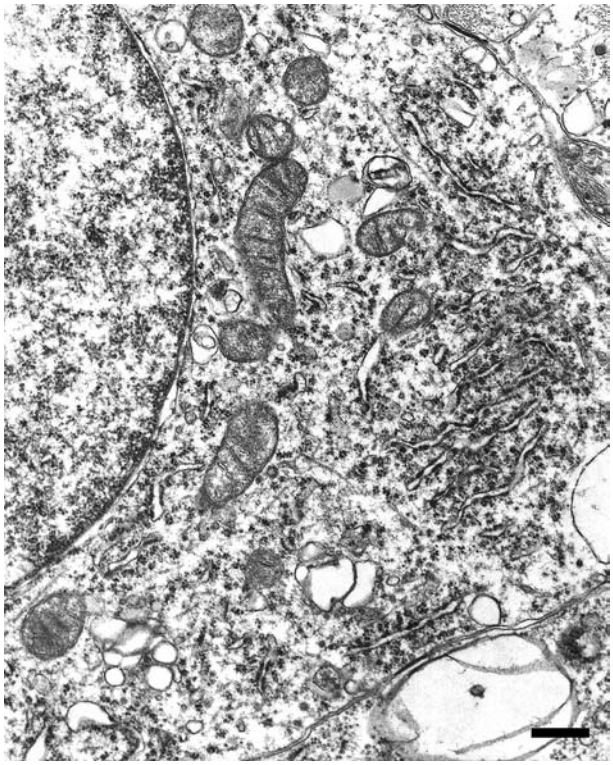

Fig. 16. MN with well preserved cell body. 9 days of 5 U/ml EPO + 100 μM THA incubation. Bar – 500 nm.

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