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The influence of *Hericium erinaceus* extract on myelination process in vitro

Мієлінові оболонки, які вкривають аксони, виконують деякі важливі функції, а саме: прискорення проведення нервового імпульсу, підтримка аксонів, їх захист, ізоляція та живлення. Пошкодження компактної структури мієліну призводить до порушення нормального функціонування нервової системи та тяжких нервових захворювань. Тому необхідним є пошук речовин з регуляторною та захисною дією щодо нормальної мієлінізації, а також зі стимулювальним ефектом на процес відновлення мієлінових оболонок після їх руйнування. Нещодавно було показано, що екстракт гриба Hericium erinaceus *має активуючу та стимулювальну дію на нервову тканину. У зв'язку з цим метою нашої роботи було дослідити вплив цього екстракту на клітини мозочка та процес мієлінізації in vitro. Згідно з отриманими результатами, нервові та гліальні клітини розвивалися нормально у онаявності екстракту протягом культивування. Екстракт не викликав патологічних змін і не демонстрував токсичної дії щодо клітин. Ультраструктура клітинних елементів була інтактною і не відрізнялась від такої, що спостерігається у клітин мозочка in vivo. Процес мієлінізації при наявності екстракту починався раніше і проходив швидше, порівняно з контролем. Таким чином, екстракт* H. erinaceus *сприяв нормальному розвитку та росту культивованих клітин мозочка і демонстрував регулювальну та стимулювальну дію щодо процесу мієлінізації in vitro.*

INTRODUCTION

Myelin sheath of the nerve fibers is a special multilamellar concentric structure, which is formed by membranes of myelinating glial cells: oligodendrocytes (OL) in the central nervous system (CNS) and Schwann cells in the peripheral nerve system [17]. Formation of this sheath begins when axon reaches the certain diameter, and includes signalling between myelinating cell and axon; "recognition"; movement and myelin-specific components synthesis [5, 18, 19]. Composition of the myelin sheath differs from the membranes of the other cell types due to a high content of lipids (70–85 %) and rather low level of proteins (15–30 %) [4]. The high lipid content in a myelin sheath defines its functions, namely: insulation of axons and prevention of electric contacts in the tightly packed nerve bundles, as well as acceleration of the nerve impulse conduction through saltation between Ranvier nodes [2]. Damage of the myelin compact structure leads to a severe CNS impairment and underlies some neurodegenerative diseases (Alzheimer disease, multiple sclerosis, etc.). Therefore, discovery of protective, activating or regulatory properties of some substances on the myelin genesis is of a special interest.

It was recently shown, that extracts of some edible mushrooms have physiological and pharmacological properties [7]. Extract of one of these mushrooms, *Hericium erinaceus*, was demonstrated to have anti-tumour action and was used for cancer therapy and prevention [7, 8]. Besides, this extract was shown to have stimulating immune system and neurotropic effects as well as to activate the synthesis of the nerve growth factor (NGF) [8, 11]. Further investigation of the properties of *H. erinaceus* extract is rather promising in order to reveal its effect on the nerve tissue and myelination process. Presumptive positive effect could be pos-

sibly used to prevent some neurodegenerative disorders (Alzheimer disease, etc.).

An appropriate and widely used technique to study the process of myelination as well as an influence of different physiological factors on the myelin genesis is cell culture [1]. Its main advantages are, that were used in present work, the possibility of vital microscopic investigation of neurons and glia to analyse their morphological and functional changes at growth or after an influence of biologically active substances.

In order to test an influence of *H. erinaceus* extract on the myelin genesis we performed the detailed structural and ultrastructural study in vitro of the stages of myelination in presence of this extract.

MATERIALS AND METHODS

Cell culture. Cerebellum of newborn WISTAR rats was used and cultured as described previously [3, 9]. In brief, cerebella were taken out and dissociated mechanically using Pasteur pipettes. Then cell suspension was plated on the plastics (aclars) in the Petri dishes. The aclars were previously coated with 1 % water solution of poly-L-lysine. Suspension contained $2x10^5$/μl cells. Culture was maintained for 21-31 days in vitro (DIV). Nutrient medium consisted of Dulbecco's Minimal Essential Medium (70 %), Gibco (USA), Cat. No. 10331084; *H. erinaceus* extract (10 %); fetal bovine serum (10 %), Intergen Cat. No. 1020-75; horse serum (10 %), Gibco (USA), Cat. No. 26050-070; glucose (4,5 g/L); penicillin-streptomycin (200 u/ml), Sigma (USA). *H. erinaceus* extract was absent in a control culture medium. Cells were incubated under an atmosphere of 5 % CO_2 and 95 % air at 37°C. The nutrient medium was changed every 5 days.

H. erinaceus extract preparation. To prepare extract of *H. erinaceus* its powder was dissolved in 96 % ethanol in ratio 1:10 [13] (the powder was kindly given by Weser-Champignon, Germany). In 10 days soluble part was separated from insoluble sediment. Received extract was placed into thermostat at 40^0C. After evaporation was complete, the same volume of the distilled water was added. Concentration of this water extract was taken as 100 %. Then extract was diluted with the nutrient medium to get 10 % solution. Ethanol content in water extract did not exceed 0,01 % and was not toxic for cultivated cells. The extract was kept at 4^0C for 10 days.

Electron microscopy. Cell cultures (21, 26 and 31 DIV) were washed with 0.1 M phosphate buffer (PB) and fixed with 1,5 % glutaraldehyde solution in 0,1 M PB. After washing out, cells were fixed with 1 % OsO_4 solution in 0.1 M PB and dehydrated in ethanol series: 30, 50, 70, 80, 95 and 100 %. Then cells were embedded in Epon-Araldit mixture. The method of "upturned" capsules was used. Capsules were filled with embedding medium. Then they were turned over and placed onto definite sites of the aclars, which were chosen before using light microscope.

After thin sectioning and double post-staining (in 2 % uranyl acetate solution in 70 % ethanol and the lead citrate solution) the specimens were viewed with an Jem-100CX (JEOL) electron microscope.

Statistic analysis. The nonparametric two-tailed Kolmogorov-Smirnov test was used to assess the differences between analysed samples ($P<0,01$ was considered to indicate statistical significance).

RESULTS

Light microscopic analysis of cerebellar cells growth in culture. Following mechanical dissociation the resulting cell suspension of *H. erinaceus* extract-treated and control cultures were observed under a phase contrast microscope as rounded cell bodies without processes during 1st DIV, although some cells had initial segments of processes. Cell adherence to the surface took place within 4-8 hours after cell plating. By this term the whole surface of a culture substrate was covered with cell mass layer. Compact cell aggregates, forming small "islands" in the culturing cell mass, were quite often observed (Fig. 1, a). They were interconnected by separate fibres or their bundles during cultivation. The processes of a renewal or formation de novo were observed for next 48 hours. No morphological distinction

could be made between neurons and glia during the first few days. By the 5-7th DIV neurons (usually with pyramidal or round bodies) and glial cells were identified due to different refraction under phase contrast microscope. After 2-week cultivation dense interlacing network made by well-developed nerve and glial processes was observed. Cell processes strongly ramify on a distance from the cell body. Some of the processes grouped into bundles of different thickness. Then single processes and their bundles formed the network. All morphological types of neurons, described previously [17], were detected, namely: multipolar neurons with short and ramified or long and direct dendrites, as well as the neurons with one thick dendrite, which branches on some distance from the cell body. Cultivation lasted 21-31 DIV. Culture maturation (cell density increase and nerve processes growth) has been shown to occur within during at period (Fig. 1, b).

According to a light microscopic analysis, no difference in the cell growth and the development

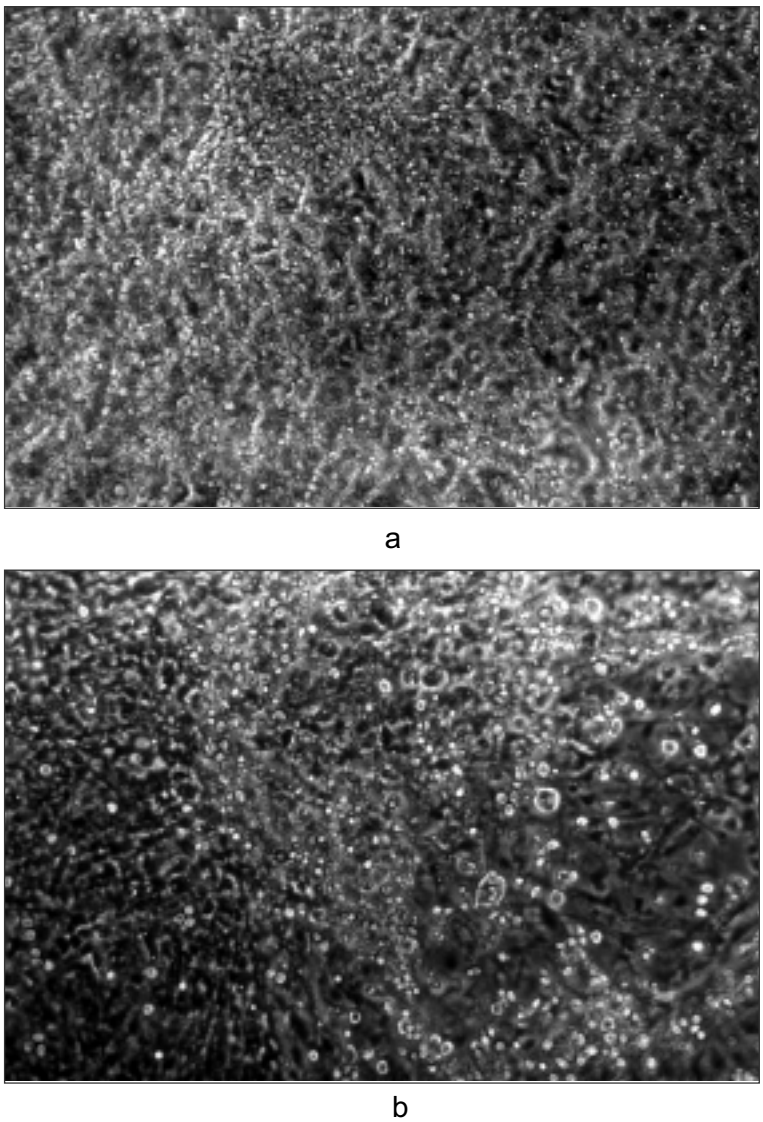


Figure 1. Dissociated culture of the rat cerebellar cells treated with *H. erinaceus* extract, 1 DIV (a) and 26 DIV (b); phase contrast microscopy, x 125

of both treated and control cultures was detected.

Electron microscopic analysis of myelin sheath development in culture. Three stages of myelination in the presence and absence of *H. erinaceus* extract (21, 26, 31 DIV) were examined.

Myelin sheaths of axons in both treated and control cultures were already formed by the 21st DIV (Fig. 2, a). The sheaths numbered 6-7 lamellas on average in the treated cultures and 5-6 lamellas in control ones. Axolemma was outlined clearly and usually adjoined to the inner surface of a myelin sheath, although some small local spaces between the axon and the sheath were sometimes observed. The contents of the axon included neurofilaments and seldom microtubules, membrane structures and dense bodies. The cytoplasm on the inner side of myelin sheath was often absent, but it was available on the outer one. In general, the structure of the myelin sheath was similar to that observed in vivo. The lamellas, consisting of the protein and lipid layers (dark and light lines on electronnograms, respectively), were well distinguished and were characterized by an integral concentric structure. But the most of lamellas were arranged loosely and included thin cytoplasm layer between them. Consequently, the myelin sheath of the most of axons was not compact. It could be concluded, that myelin was immature by this cultivation term.

By the 26th DIV the myelin sheathes looked more mature (Fig. 2, b). The lamellas joined each other tightly and formed a compact spiral structure, which was similar to that observed in vivo. No local or the whole space between an axolemma and a myelin sheath was found. The myelin sheaths of the axons contained 10-11 lamellas on average in the treated cultures and 7-8 in control ones.

By the 31st DIV the myelin sheaths had 10-11 lamellas on average in both treated and control cultures (Fig. 2, c). The structure of the sheath did not change significantly as compared to the two previous stages. However, it should be noted, culture was undergone some degeneration by this term. It was characterized by the local degradation of an axon and/or axoplasmic organelles. Sometimes small local voids within the myelin sheath and spaces between the axolemma and the sheath were found.

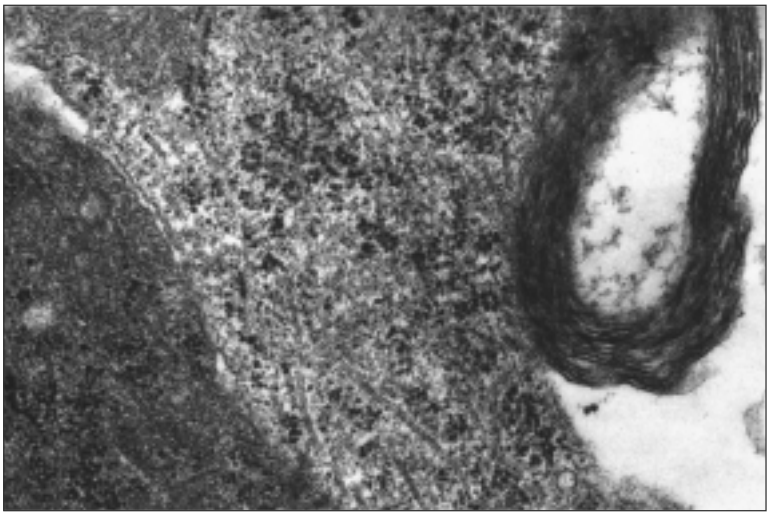
Statistical analysis of a number of lamellas in the myelin sheaths during myelination in the cerebellar cell culture was performed. Stepwise increase in the lamellas amount during the myelin sheath development has been revealed. By the 21st DIV number of lamellas averaged 6,44±0,13 and 5,57 ± 0,21, by 26 DIV – 10,03 ± 0,15 and 8,07 ± 0,24 and by 31 DIV – 10,37 ± 0,19 and 10,52±0,24 in treated and control cultures, respectively ($P<0,01$) (Table, fig. 3).

Electron microscopic analysis of cerebellum cells development in culture. Electron microscopic examination of ultrathin sections of the culturing cells has revealed typical cerebellar cell types during cultivation in both treated and control cultures. The cells were intact and mature and had a intact ultrastructure.

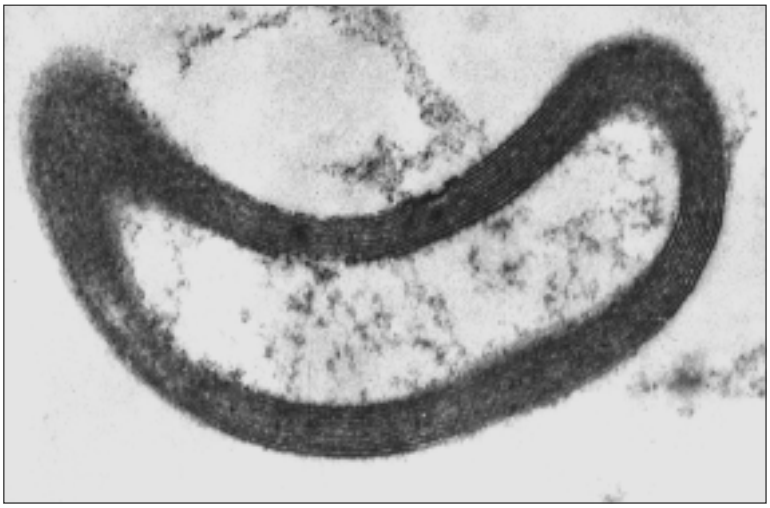
It has been shown, that OL were pictured as moderately dense cells. This feature was used to differ them from other cell types. The nucleus usually lies in an eccentric position. Consequently, its part appeared to be surrounded by thin rim of cytoplasm. The most of OL had homogenous karyoplasm and chromatin lumps, which were localised nearby the nuclear envelope. The granular endoplasmic reticulum and the Golgi apparatus were well developed. Ribosomes, multiple small granules, inclusions and dense bodies were well observed in the cytoplasm. As far as culture matured by 31st DIV the vacuoles were found in the cytoplasm.

Statistical analysis of a number of lamellas in the myelin sheaths during cerebellar cell cultivation in the presence and absence of *H. erinaceus* extract (n=200, P<0.01): 1 – control, 2 – *H. erinaceus*

Cultivation term	21 DIV	26 DIV	31 DIV
Number of lamellas			
(treated cultures)	6,44±0,13	10,03±0,15	10,37±0,19
(control cultures)	5,57±0,21	8,07±0,24	10,52±0,24



a



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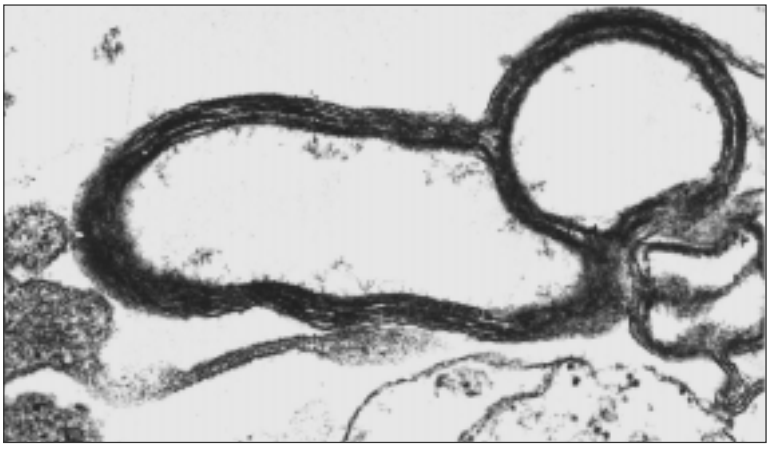

c

Figure 2. The myelinated fibres in the culture of the rat cerebellar cells treated with *H. erinaceus* extract, 21 DIV (a), 26 DIV (b) and 31 DIV (c); x 20 000

Neurons were often observed. They had the large oval nucleus (usually with nucleolus). The most types of neurons were characterized by a homogenous karyoplasm (molecular and ganglionar layers of the cerebellum), the rest of them had a heterogeneous one with chromatin clumps (granular neurons and granular layer of the cerebellum). Lucent cytoplasm contained free ribosomes, granular endoplasmic reticulum, Golgi apparatus, mitochondria, microtubules, neurofilaments, lysosomes, multivesicular bodies, lipofuscin granules and fibrillar inclusions.

The cytoplasm of the neuronal processes (dendrites and non-myelinated axons) is characterized by a presence of mitochondria, granular and agranular endoplasmic reticulum (before the first arborisation and after the second one, respectively), free ribosomes, large amount of microtubules (axons) and neurofilaments (axons and dendrites).

Astrocytes were often found. They were characterized by a homogenous karyoplasm in fibrous astrocytes and by a presence small chromatin lumps nearby the nuclear envelope in protoplasmic astrocytes. The cytoplasm of cell body and processes contained multiple filaments, ribosomes, endoplasmic reticulum, Golgi apparatus, some mitochondria, lysosomes, microtubules and granules.

By the 31 DIV the ultrastructure of some cells was characterized by aging alterations. The cytoplasm looked more dense and contained dark granules and vacuoles of different size and density.

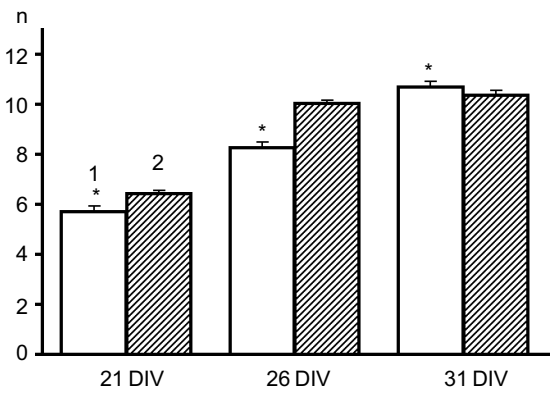

Figure 3. Comparative diagram of the course of myelin sheath formation in control and treated with *H. erinaceus* extract cerebellar cells in vitro.

Number of organelles in axoplasm somewhat decreased, the axolemma was locally invaginated. Thus, the culturing cells demonstrated aging degeneration by this cultivation term.

DISCUSSION

Dissociated culture of rat cerebellum cells allowed us to monitor the basic stages of the myelin genesis in vitro and to study influence of *H. erinaceus* extract on this process. 21, 26 and 31 DIV were considered as the optimal cultivation terms to investigate the myelin sheath formation in vitro by following reasons. First, according to the biochemical data, described previously [14, 16], myelin-specific proteins appearance were detected on the 19 DIV in the nerve tissue culture. Consequently, 21 DIV was the most appropriate cultivation term to describe the beginning of myelination process in vitro. Second, our own light microscopic data demonstrated that treated and control cultures entered the ageing phase and had some degenerative alterations after 31 DIV. Myelin sheaths could either lose partly their laminar structure or include the voids or holes. It should be noted that the similar events were observed in myelin sheaths of the normal aged animals [10]. Thus, this cultivation term was chosen as the last stage of myelin genesis in vitro.

An intensive myelination in rat cerebellum is known to start on 5–7 postnatal days [1]. So the cerebellum tissue of a newborn rat did not contain myelinated fibres. Native myelin sheaths were formed during cultivation. Our results have shown, that the course of myelination in vitro is very similar to that, revealed in vivo. Myelin sheaths wrapped both single and grouped axons. It should be noted that each of the grouped axons had a separate myelin sheath. Thus, it is suggested, that single axons of cultivated neurons were myelinated first. Then some of these processes grouped into a bundle and was wrapped by additional common myelin sheath. Axon grouping in culture can be explained by a tendency of the cells to aggregate and group their fibres into bundles during culture growth and/or by unilateral growth of the neighbouring axons during network formation.

Two sera (horse and fetal bovine) were used to culture cerebellum cells. Our choice was dictated by following reasons. The horse serum is known to promote a survival and a development of the nerve and some glial cells where as the fetal bovine serum is necessary for OL growth in vitro [9]. Our own experiments confirmed these data. OL were absent in the cerebellar cells cultures when the cells were cultured in the presence of the horse serum only.

According to our results, treating with *H. erinaceus* extract promoted normal development of the cerebellar cells during the whole period of cultivation. The cells in treated and control cultures grew and matured in the same way. Electron microscopic observation of cells, has revealed, that the ultrastructure of the cells in both cultures was similar on the 21st, 26th and 31st DIV. It can be concluded, that the cells treated with the extract developed normally during four weeks of cultivation. Afterwards they entered ageing stage. Thus, the extract of *H. erinaceus* in 10 % concentration neither evoked noticeable changes in cerebellar cell structure during development nor caused pathological alterations during cells cultivation.

However ultrastructural analysis of the myelinated fibres in treated and control cultures has shown that the extract of *H. erinaceus* influenced the process of myelination in vitro. Number of lamellas in the myelin sheaths in the nerve fibres in treated cultures was higher by the 21st and the 26th DIV as compared to those in control cultures. Thus, it could be concluded, that myelin genesis began earlier and proceeded faster and more intensively in the presence of extract. By the 31st DIV quantity of lamellas in the sheaths in treated cultures did not increase and remained approximately the same. Similar number of lamellas was counted in the myelin sheaths of the fibres in the control cultures. Thus, myelin sheath formation in the treated cultures seemed to be completed by the 26th DIV.

As the extract of *H. erinaceus* is able to activate the synthesis of NGF [8, 11], it could be suggested, that cultivated cells expressed NGF more intensively after adding of the extract, and, thus, they developed NGF-rich nutrient medium. One of the functions of NGF is a support and control of the survival of the nerve cells during their development. Therefore, availability of the extract of *H. erinaceus* in the cultures could intensify growth of the nerve cells and accelerate their maturation during cultivation. An earlier neuron maturation resulted in earlier myelination.

An influence of NGF on myelinating cells in CNS, OL, has not been clear yet and it is somewhat controversial. It was reported, that NGF receptors were found in the OL membrane. Binding of NGF to its receptors could promote OL better survival as well as initiated their apoptosis [6, 12, 15]. According to our results, OL in the treated cultures did not demonstrate any pathologic alterations. The apoptosis in OL was not observed either. Moreover, the rate of myelin sheath formation in treated cultures was higher as compared to control cultures. Since our results are in accordance to the data about the survival of the OL after NGF influence [12, 15], it can be proposed, that extract of *H. erinaceus* could support normal development of the OL and, probably, promote their maturation and accelerate synthesis of myelin-specific molecules. The latter could be the reasons for earlier and more intensive myelination in vitro.

CONCLUSION

1. Extract of *H. erinaceus* in culture promoted the normal cultivating the nerve and glial cells. Their ultrastructure was similar to that observed in the cerebellum cells in vivo.

2. Process of the myelin sheath formation in the presence of extract *H. erinaceus* proceeded faster and was completed by 26 DIV (in controls, it was finished by 31 DIV). Thus extract of *H. erinaceus* demonstrated regulatory action on rate and duration of myelination in vitro.

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THE INFLUENCE OF *HERICIUM ERINACEUS* EXTRACT ON MYELINATION PROCESS IN VITRO

Myelin sheathes, wrapping axons, perform the following important functions: support, protection, feeding and isolation. Injury of myelin compact structure leads to an impairment and

severe illness of the nerve system. Exact mechanisms underlying the myelination process and myelin sheaths damage have not established yet. Therefore search for substances, which provide regulatory and protective effects on the normal myelination as well as stimulating action on the remyelination after myelin damage, is of special interest. Recently it was shown that extract from mushroom *Hericium erinaceus* had activating action on the nerve tissue. So the aim of the present work was to study an influence of an extract from *H. erinaceus* on the cerebellar cells and the process of myelination in vitro. Obtained data revealed the normal growth of the nerve and glial cells with extract at cultivating. No pathologic or toxic action of the extract has been found. The cell ultrastructure was intact and similar to that observed in vivo. The process of myelination in the presence of the extract began earlier as compared to controls and was characterised by a higher rate. Thus, extract of *H. erinaceus* promoted normal development of cultivated cerebellar cells and demonstrated a regulatory effect on the process of myelin genesis process in vitro.

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