The study of the remyelinating effect of leukemia inhibitory factor and melatonin on the toxic cuprizone model of demyelination of murine cerebellar cells culture in vitro

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ABSTRACT

The cuprizone model of toxic demyelination in vitro is widely used to study de- and remyelination in the CNS, as well as to address the issues of finding potential compounds that affect myelination of neuron axons.

THE AIM OF THE STUDY was to investigate the role of recombinant human leukemia inhibitory factor (rhLIF) and melatonin in remyelination, using the cuprizone demyelination model in vitro.

METHODS. To study the features of the demyelination and remyelination processes of neuronal axons, the culture of dissociated cerebellar cell culture of the 7-day-old FVB/N lineage mice was used. To detect the myelin sheaths, a histochemical staining with a Sudan Black B was used. To identify oligodendrocytes, immunocytochemical staining of 28-30-day old cerebellar cells cultures for oligodendrocytes marker Olig2 was performed.

RESULTS. The direct effect of the demyelinating factor of cuprizone and remyelination agents (rhLIF and melatonin) on oligodendrocytes in vitro was confirmed. The remyelinating effect of LIF and melatonin on the restoration of myelination processes in dissociated cerebellar cell culture using histochemical and immunocytochemical staining has been revealed.

CONCLUSIONS. Cuprizone-induced demyelination in vitro is associated with the death of Olig2+ oligodendrocytes and loss of myelin formation. rhLIF and melatonin prevented the loss of oligodendrocytes and, consequently, reduced the destruction of myelin membranes.

KEY WORDS: cerebellum; cuprizone; demyelination; remyelination; rhLIF; melatonin
shown [6]. The anti-apoptotic effect of LIF on oligodendrocytes is asso-
associated with a decrease in the manifestations of neuroinflammation due to inhibition of proinflammatory cytokine production of TNF-alpha [15]. In
neural progenitors and newly formed neurons, LIF inhibits caspase-
mediated apoptosis [16]. Improvement of myelination of the axons of
brain neurons was shown after intraperitoneal administration of LIF at a
dose of 25 μg/kg or 50 μg/kg to inbred mice with an experimental model of
demyelination [17]. Earlier in our studies, the neuroprotective effect of
rhLIF on the mice with cuprizone-induced model of toxic demyelination in
vivo depending on the age of the animals was shown [18].

The effectiveness of the reparative processes in the brain in the
pathologies of the nervous system depends on the influence of factors of
macro environment, in particular hormones [19]. Melatonin exhibits a
wide range of biological activity, in particular, its participation in antiox-
diant protection of the organism is shown. This hormone, one of the stron-
gest direct antioxidants, absorbs endogenous free radicals (hydroxyl radi-
cal, superoxide radical anion, singlet oxygen, nitric oxide) and protects
cells macromolecules (proteins, lipids, nuclear and mitochondrial DNA)
from oxidative damage. Melatonin also acts as an indirect antioxidant,
stimulating the activity of antioxidant enzymes in the brain of animals with
neurodegenerative diseases [20-22].

It is known that antioxidants are able to prevent cell death induced by
various toxins on the proteins of the mitochondrial pores. It is free
radicals that lead to the opening of mitochondrial pores, through which
cytochrome C that activates cytoplasmic proteolytic proteins (caspases) is
released from the mitochondria in the cytosol. This leads to enhanced
proteolysis of cellular protein mediated by caspases, which plays a signifi-
cant role in the development of apoptosis. The direct suppressive effects
of melatonin on the pores of mitochondria is shown [21]. In the study of
 Kashani I. R. et al., the restoration of myelination of axons of brain
neurons after intraperitoneal administration of the melatonin to mice with
an experimental toxic cuprizone model of demyelination, with an analysis
of the expression of genes of mitochondrial proteins of myelin-producing
cells and ultrastructural changes in mitochondria was demonstrated [23].
There is data on amplification under the influence of melatonin of the pro-
iferative potential of neural stem cells (NSCs) in the brain and their dif-
fentiation in the neurons [24, 25]. There are studies suggesting that the
pineal hormone melatonin suppresses the development of Parkinson’s
disease or parkinsonism in experimental and clinical use [26, 27].

To study the features of the processes of myelination, demyelination and
remyelination of neuron axons, the culture of dissociated cerebellar
cells of the 7-day-old FVB/N mice (n = 27) was used. Euthanasia of new-
born mice was performed by ether overdosing. All studies with exper-
imental animals were carried out in compliance with the legislation and
principles of bioethics: the Law of Ukraine «On the Protection of Animals
from Cruelty» (dated February 21, 2006), «European Convention for the
Protection of Vertebrate Animals Used for Experimental and Other Scien-
tific Purposes» (Strasbourg, 1998).

Isolation of the cerebellum was carried out on ice, in sterile condi-
tions. The cerebellum was placed in a medium containing 90 % of DMEM
medium, 10 % horses serum, inactivated at 56 °C for 30 minutes, 6 g/L
glucose, 100 U/mL penicillin, and 100 μg/mL streptomycin (all reagents –
Sigma, USA). Dissociated cells were obtained by mincing the cerebellum
with scissors in 0.25 % trypsin solution (Sigma, USA) after pre-washing
samples in a phosphate buffered saline (PBS) with antibiotics PenStrep
(Sigma, USA). The resulting suspension was left for 5 minutes at +37 °C
with constant shaking, followed by mechanical dissociation using pipettes
of different diameters. The dissociated cerebellar cells were counted and
transferred to 35 mm cell culture dishes precoated with poly-L-lysine at
a seeding density of 2•10^6 cells/cm². Cell cultivation was carried out in
a complete culture medium that contained 90 % DMEM, 10 % horse se-
rum (Sigma, USA), 6 g/L glucose, bovine insulin (Sigma, USA) 10 μg/mL,
100 U/mL penicillin, and 100 μg/mL streptomycin under standard condi-
tions in a CO₂ incubator at a temperature of +37 °C and in a humidified
atmosphere with 5 % of CO₂.

In our previous studies, it was found that a significant death of Olig2-
oligodendrocytes compared with control cultures was observed only with
the use of cuprizone on the 18th day of cultivation of the dissociated cul-
ture of the cerebellar cells [28]. Therefore, on the 18th day of cultivation,
cuprizone (Sigma, USA) at a dose of 25 μM was introduced in the culture
for 48 hours. After 48 hours, a medium containing recombinant human
LIF (20 ng/mL) or melatonin (10 μg/mL), was introduced into the cell cul-
ture during three days. The cultivation was carried out during 28-30 days
with the replacement of the growth medium twice a week. Assessment of
condition of cultured cells were performed with an inverted microscope
IX71 (Olympus, Japan) in phase contrast mode.

To detect myelin sheaths, a histochemical staining with Sudan of
black B was used. 28-30-day cultures of the cerebellar cells were washed
with PBS and fixed with 1 % paraformaldehyde solution on 0.1 M PBS
for one hour. Dehydration was then carried out in 25 %, 50 % and 70 %
ethanol solutions for 5 minutes in each solution. The stained sections
were examined using an inverted microscope IX71 (Olympus, Japan) in
phase contrast mode.

To identify oligodendrocytes, the culture was immunocytochemically
stained for oligodendrocyte marker Olig2. Cultures of 28-30 days were fixed
with 4 % paraformaldehyde solution for 30 minutes. After washing, the cell
culture was blocked in 0.1 M PBS (pH = 7.4) with 0.5 % bovine serum al-
bumin and 0.3 % Triton X-100 (Sigma, USA). Within 48 hours at +4 °C, cell
culture was incubated with primary rabbit monoclonal antibodies to Olig2
(Chemicon, USA) in dilution 1:200. Primary antibodies were visualized with
secondary anti-rabbit AlexaFluor 488-conjugated antibodies (Invitrogen,
USA) in dilution 1:1000. Cell nuclei were stained with Hoechst 33342 dye
(Invitrogen, USA) in dilution 1:5000. Microscopy was performed using an
inverted fluorescence microscope Axio Observer A1 equipped with a digital
camera AxioCam ERC 5s with ZEN 2012 and AxioVision 4.8 software, using
the fluorescence filters 21HE (for Hoechst) and S6NE (for AlexaFluor 488)
(Carl Zeiss, Germany). Cell counting was performed in 5 random fields of
view of the culture of each experimental group.

To clone the human LIF gene, we used the technology for obtaining the
cDNA gene with a reverse transcriptase reaction with subsequent
amplification of cDNA using a polymerase chain reaction [12]. It has
been established that human and mice LIF exhibit up to 80 % homologous
sequences; human LIF is capable of binding to LIF receptors on mouse
cells and activating them [29].

Statistical analysis was performed using the Statistica 7.0 (StatSoft
Inc., USA) software. The differences between the groups were assessed
using Student’s t-test. The results are presented as means and standard
error of mean (M ± m). The difference between the parameters was con-
sidered statistically significant at a value of p < 0.05.

MATERIALS AND METHODS

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RESULTS AND DISCUSSION

Our previous studies confirmed the process of myelination, which is
impossible without the presence of mature differentiated oligodendrocytes
and demyelination under the influence of the cuprizone in vitro [28]. It was
found that cuprizone-induced demyelination is associated with a loss of oli-
godendrocytes with subsequent inhibition of the myelin formation. During
this work, which continue the previous studies, we studied the direct action
of biologically active agents (rhLIF and melatonin) on remyelination using the cuprizone-induced model of toxic demyelination in vitro [30].

We have found that rhLIF without previous cuprizone introduction into the culture increases the total number of nucleated cells in the dissociated cerebellar cells culture in comparison to the control cultures, but the number of oligodendrocytes does not change under the influence of this factor (Fig. 1). However, the addition of melatonin to the culture medium did not lead to significant changes in both the amount of Olig2+ and the total number of nucleated cells compared to control cultures (Fig. 1). Thus, it can be assumed that only the multifunctional cytokine LIF affects the proliferative potential of neural cells under normal conditions.

At the same time, the addition of rhLIF to the culture for 3 days at a concentration of 20 ng/mL or 10 μg/mL melatonin after the cuprizone treatment resulted in an increase in both the number of oligodendrocytes and the total amount of nucleated cells compared to the cultures treated with cuprizone. Moreover, the treatment of cultures with 25 μM cuprizone for

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Fig. 1. Microphotographs of the dissociated culture of the cerebellar cells on the 28-30th days of cultivation: control cultures (A), cultures with 20 ng/mL rhLIF (B) or 10 μg/mL melatonin (C). Fluorescent microscopy, immunocytochemical staining for Hoechst 33342 (turquoise) and Olig2 (green). Light microscopy: staining of axon myelin sheaths with Sudan black B, x200. Histograms of the total number of nucleated cells by Hoechst and Olig2+ oligodendrocytes (D).

Note: * – p < 0.05 compared with control group.
48 hours followed by the addition of rhLIF or melatonin for 3 days induced an increase in the total number of cells in 4.2 and 6.4 times, respectively, compared to cultures treated with cuprizone only (Fig. 2).

In addition, the total number of cells was significantly higher (p < 0.05) in cultures treated with cuprizone and melatonin compared to cultures under the influence of cuprizone + rhLIF and control cultures. Perhaps, this is due to the effect of melatonin on the activation of antioxidant enzymes, which, on the one hand, reduces damage to brain neurons, and, on the other hand, provides conditions for reparative processes in the brain involving T-lymphocytes. The study of Abdurasulova I. and Klimenko V. showed that T-lymphocytes play a double role in the action on the nervous tissue and manifestation of their damaging effects on the brain neurons are related precisely to the components of oxidative stress and proinflammatory cytokines [31]. At the presence of rhLIF or melatonin, the number of oligodendrocytes in cultures pre-treated with cuprizone increased by 4.4 times (from $62.5 \pm 18.8$ to $279.8 \pm 26.8$ cells) and 8.4 times (from...
62.5 ± 18, 8 to 528.2 ± 115.4 cells), respectively (Fig. 2). Since Olig2 is a transcription factor that is required for myelin activation, specifically for the expression of the myelin regulatory factor (MyRF), which in its turn regulates myelin-associated proteins [32, 33], our studies have shown the recovery of myelin membranes. Thus, rhLIF and melatonin prevented the loss of oligodendrocytes and, consequently, reduced the destruction of myelin sheaths. The cuprizone model of demyelination in vitro is a relatively simple and useful model for studying the processes associated with de- and remyelination in the central nervous system (in particular, in the cerebellum), and it solves the problem of finding remyelinating factors in order to restore myelination of axons. The results of restoration of myelin production in the demyelinated culture of the cerebellar cells under the influence of rhLIF and melatonin allow to assess the state of remyelination of the cerebellar neuron axons, which is important for the study of the demyelinating diseases pathogenesis, in particular multiple sclerosis, and to expand the list of drugs with remyelinating effect.

CONCLUSION

There was an increase in the total number of cells in the culture of murine cerebellar cells without cuprizone treatment after the addition of recombinant rhLIF. The addition of rhLIF or melatonin did not affect the amount of Olig2+ oligodendrocytes. In the culture of the murine cerebellar cells treated with cuprizone, when the pineal hormone melatonin was added, the total number of cells was significantly higher than with rhLIF application. At that, the amount of Olig2+ oligodendrocytes, reduced under the impact of the cuprizone, was restored.

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