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EFFECT OF A WATER SOLUBLE DERIVATIVE OF FULLERENE C_{60} ON THE FEATURES NEURAL PROGENITOR CELLS *IN VITRO*

ABSTRACT

We studied the effect of a water soluble derivative of fullerene C_{60} on the behavior of cultured neural stem/progenitor cells. Addition of 20 nM of metal fullerenolate C_{60} (*NaFL*) into the cell culture increased the population of the cells almost twice in comparison with the control and also suppressed the formation of neurospheres. The obtained data allow us to suggest that *NaFL* has a positive effect on the proliferative activity of neural progenitors. The water-soluble fullerene nanostructures such as *NaFL* promoting the proliferation of neural stem cells might have numerous beneficent applications in cell biology and biotechnology.

KEYWORDS: neural stem cells, *NaFL*, cell proliferation, neurospheres, *FGF-2*, immunocytochemical staining, electron microscopy.

One of the perspective areas in cell and combined gene-cell therapy is the selection, identification of stem cells, and obtaining of stem clones, especially pluripotent cells; and their subsequent differentiation into specialized cells [1].

Neural stem cells (*NSC*) can differentiate into neurons, astrocytes and oligodendrocytes, providing required amount of cells in the brain during its development and pathology. The study of *NSC* properties in culture conditions plays a huge role in the research of central nervous system (*CNS*) diseases, neurons development, its differentiation and regeneration.

Detection of neural stem cells and their identification in the brain of embryos and adults transformed the conception about the *CNS* plasticity. To replace the postulate that the neural cells do not renew and divide, there appeared the statement that the functions of damaged neurons in the adults are renewed by *NSC*. At present there has already been collected quite a large number of experimental data on the possible use of the *NSC* in various neurodegenerative diseases.

At present, the researchers' attention has been attracted by the problem of isolation, multiplication, long-term storage of the *NSC*, and the study of their differentiation during the cultivation and after transplantation into the brain. In the adult stem cell number is much less than in the embryonic and early postnatal period. Therefore, there is not enough donor material for transplantation. Their ability to proliferate *in vitro* is used to increase the amount of stem cells.

The main advantage of cultivated cells is a capability for *in vivo* monitoring of cells using a microscope. Essential is that, working with cell cultures in the experiment, healthy cells only are used, and they remain viable throughout the experiment. It is possible to ensure that, testing cell culture periodically. Moreover, it is easy to estimate the relative number of viable cells.

In the culture, the *NSC* maintained as self-renewing in clones, immature multipotent cells with a set of specific phenotypic markers. After proliferation blocking, they differentiate into neurons, oligodendrocytes and astroglia [2]. *In vitro* cultivated *NSC* is a widest source of cells for transplantation, drug screening, gene therapy and neuronal development studies [3-6].

At present there is clearly identified regulatory and selective effect of some growth factors on *NSC* properties both, *in vivo* and *in vitro* [7]. However, despite the early successes in the study of the *NSC*, there are still many issues and problems related to the study of these cell properties, development of methods for their isolation, growth, and differentiation.

At present, nanotechnology is considered the most promising of all the technologies, because there is a qualitative leap from the manipulation of the material to the manipulation of individual atoms [8, 9]. One of perspective directions is the use of carbon nanostructures — derivatives of fullerene (C_{60}). Fullerene — is a molecular compound that belongs to the class of the allotropic forms of carbon, which are convex closed polyhedral composed of an even number of three-coordinated carbon atoms. In molecules of fullerene, carbon atoms are positioned at corners of regular hexagons and pentagons. The surface of a sphere or ellipsoid is composed of them.

Recent studies point to an antioxidant [10-18], neuroprotective [19-23] and nootropic [24] properties of C60 fullerene derivatives [10-24]. The authors also report about radioprotective [17], oncoprotective [25, 26],

antiallergic [27], antibacterial, and antiviral [28-36] properties of water-soluble C_{60} fullerene derivatives.

Despite such an impressive list of biological effects of fullerenes, we have to recognize that the mechanisms of many of them still have not been decrypted. There is a lack of knowledge of investigated nanoparticles pharmacodynamics and pharmacokinetics. There are not schemes of treatment for pulmonary, oncological, neurodegenerative and other diseases by fullerenes. Therefore, this trend requires more detailed research.

In this context, the aim of this research is to study the properties of the *NSC* by the action of water-soluble *metal fullerenolate* C_{60} (*NaFL*).

MATERIALS AND METHODS

ISOLATION OF NEURAL PROGENITORS AND ANALYZE OF THEIR VIABILITY

Under sterile conditions hippocampuses from the brain of *FVB* mice (17-18 days of embryonic development) were isolated. Then they were mechanically dissociated in a *Neurobasal* medium (*Gibco*, USA). Received cell suspension was passed through a 40 mcm nylon cell strainer (*Falcon*, USA). Purified fraction of *NSC* was prepared by centrifugation of cell suspension in a density gradient (22% *Percoll*). The percentage of viable cells in the suspension was determined by flow cytometry, using a cell sorter *FACSAria* (*Becton Dickinson*, USA), after incubation of cells with 7-amino-actinomycin *D* (*7-AAD*).

CULTIVATION OF NEURAL PROGENITORS SUPPLEMENTED WITH *NAFL*

Neural progenitors were plated on *Matrigel*-coated glasses in 24-well plate ($8 \cdot 10^4$ cells in each well) and cultivated in a CO_2 incubator (t = +37 ^{0}C, 5% CO_2). The first 2 days the cells were cultured in a standard *Neurobasal medium* (*Gibco*, USA) supplemented with *FGF-2*, as a growth factor that affects the proliferation of neural progenitors. In the absence of *FGF-2* in the culture medium, the cells begin to differentiate spontaneously or form neurospheres.

Neural progenitors were cultivated under different conditions:

1 – standard conditions, *FGF-2* is present in the culture medium;

2 – standard conditions + 20 nM *NaFL*;

3 – *FGF-2* is not added in the culture medium;

4 – culture medium without *FGF-2* + 20 nM *NaFL*.

The cells have been cultivated for 9 days after plating, 7 days in the presence of *NaFL*.

ANALYZE OF PROLIFERATIVE ACTIVITY OF NEURAL PROGENITORS, STATISTICAL PROCESSING OF THE DATA

It is known that the proliferative (mitotic) activity of NSC depends on the presence of certain growth factors (*FGF-2, EGF, NGF, BDNF, NT-3, NT-4*) in the medium [7, 37-39]. Microenvironmental factors, in most cases, determine the fate of neural progenitors.

Proliferative activity of neural progenitors isolated from the hippocampus of 17-18-day mouse embryos was observed, using an inverted light microscope (*Zeiss Telavar 31*, Germany). On the 9th day after plating, the maximum cell density was observed in the wells to which 20 nM concentration of metallofullerenol C_{60} was added.

Then, the cell culture was fixed with 4% paraformaldehyde for 30 minutes, washed in 0,1 M phosphate buffered saline (*PBS*) and enclosed into balsam.

Quantitative analysis of neural stem cells and neurospheres were performed, using an inverted microscope with a relief phase contrast (*Olympus IX 71*, Japan).

IMMUNOCYTOCHEMICAL STAINING OF CULTIVATED NSC

To identify the neural progenitors and potential way of their differentiation there was used a double immunocytochemical staining for *Nestin, β-tubulin III*. Nestin is a specific marker of neuronal stem progenitors, post-mitotic neurons, and early neuroblasts [40]. *β-tubulin* III is a specific marker of mature neuronal cytoskeleton.

Cell cultures were fixed with 4% paraformaldehyde for 30 minutes, washed with a 0,1 M PBS, and treated with 0,3% *TritonX-100*, 0,5% bovine serum albumin. During the following 24 hours they were incubated in a mixture of murine monoclonal Nestin (1:300), rabbit polyclonal *β-tubulin III* (1:750) of primary antibodies.

After washing off 0,1 M *PBS*, cell cultures were treated with a mixture of secondary anti-mouse *Alexa Fluor 488* (*Invitrogen*, USA) 1:1000 and anti-rabbit *Alexa Fluor 647* (*Invitrogen,* USA) 1:1000 antibodies for 1 hour.

Then the cells were washed in *PBS* and enclosed in the medium *FluoMount*.

Fluorescence was visualized using selective filters (488 nm for *Alexa Fluor 488*, 647 nm for *Alexa Fluor 647*). This method allowed us to detect the presence of proliferating neural progenitors, and observe the morphological characteristics of neurospheres. Optical studies were performed using a laser scanning confocal microscope *FluoView™ FV1000* (*Olympus*, Japan).

ELECTRON MICROSCOPIC RESEARCH OF CULTIVATED HIPPOCAMPAL SC

Cultures of neural progenitors were fixed with 2,5% glutaraldehyde solution and 2% paraformaldehyde for 1 hour at room temperature. Subsequently, cell cultures were washed with 0,1 M *PBS*, and fixed in 1% OsO_4 solution for 1 hour; dehydrated in ethanol (30%, 50%, 70%, 80%, 96% absolute alcohol) and in 100 % acetone. Culture cells were enclosed in epoxy resins. The final step was a polymerization at 56 °C.

SC preparations, enclosed in a resin, were attached to the flat surface of the blocks with an adhesive *«Cyanopan»* (Poland). Ultrathin sections (500 A^o) were cut on a microtome (*LKB Bromma 8800*, Sweden). They were contrasted with solutions of uranylacetate and lead citrate. Morphological characteristics of neural progenitors were evaluated with a transmission electron microscope *JEM-100 CX* (*JEOL*, Japan).

STATISTICAL PROCESSING OF THE DATA

Statistical processing was carried out with the Student t-test, significance of the difference was taken at $p<0.001$.

RESULTS AND DISCUSSION

Using flow cytometry method there was determined that the percentage of viable cells after incubation with 7-amino-actinomycin (*7-AAD*) ranged from 89,8% to 93,5%.

Neural progenitors have been cultured for 9 days after planting, and with *NaFL* — for 7 days with and without *FGF-2*.

It is established that *FGF-2* causes proliferation of multipotent cells, which are the progenitors of neurons and glia in the subventricular zone of the lateral ventricles of the brain. *FGF-2* together with NGF (neuronal growth factor) can maintain the generation and viability of neurons, which are cell derivatives of the subventricular zone. This way they promote neurogenesis in the adult human brain, as due to its natural reserves, or by implantation of progenitor cells [41]. In pharmacokinetics study of *FGF-2* has been established that, after subcutaneous injection of 5 ng/g of body weight dose into rats, its concentration in blood and brain liquor rapidly increases, indicating

its free passing through haemathoencephalic barier. In adult animals it increased the amount of *NSC* mitoses in the subventricular zone and olfactory tracts, regulating current neurogenesis with particular humoral method [38]. Therefore, *FGF-2* performs the complex regulation of the de novo generation of brain cells [7]. During cultivation of multipotent stem cells, growth factor causes intense proliferation; and its removal from the medium induces their differentiation into neurons and glia. [39]

In our case, the neural progenitors, which were cultured with the addition of *FGF-2*, remained proliferative potential without formation of neurospheres **(Fig. 1, A)**. In the wells without *FGF-2* a small amount of neural progenitors was remained. Also there was observed an appearance of neurospheres. That indicates the loss of neural progenitor's proliferative potential **(Fig. 1, B)**.

Quantitative microscopic analysis of *NSC* under various conditions of cultivation showed that in the control, where in the culture medium *FGF-2* was present, 543 ± 61 cells per mm^2 appeared **(Fig. 1, A; Fig. 2)**; at the same time, the amount of *NSC*, adding 20 nM concentration of *NaFL*, remained practically unchanged — 530 ± 66 cells per mm^2 **(Fig. 1, B; Fig. 2)**.

In those wells, where the cells were cultured without *FGF-2*, the appearance of neurospheres was observed, the number of neural progenitors was 200 ± 77 cells per mm^2 ($p<0.001$) **(Fig. 1, C; Fig. 2)**. By adding 20 nM concentration of *NaFL* (missing the growth factor in the culture medium), we observed a significant increase of *NSC* - 426 ± 101 cells per mm^2 ($p<0,001$) **(Fig. 1, D; Fig. 2)**.

These data indicate that in the presence of 20 nM concentration of *NaFL* in the culture medium, but without growth factor, the number of neural progenitors is about 2 times higher than in control wells.

It can be assumed that *NaFL* increases the proliferative potential of neural progenitors by still unknown mechanism of action.

Method of immunocytochemical staining of cell culture has given us the ability to visualize neural progenitors and neurospheres in different types of cultivation. Neural progenitors at cultivation in the usual medium and the medium with 20 nM concentration of *NaFL* were morphologically identical and showed practically the same proliferation activity **(Fig. 3, A-E)**.

This result allows us to assume that 20 nM concentration of *NaFL* in this version of cultivation is not toxic, but did not affect the activity of *NSC* proliferation.

In immunocytochemical analysis of *NSC* cell culture, which was cultured in medium without *FGF-2*, we observed the formation of neurospheres, as well as a small number of neural progenitors. Currently it is not considered something exceptional to obtain the culture growth by type formation of neurospheres. This opens new possibilities for a thorough examination of cells within a neurosphere, and their subtyping [42]. It is obvious that, during the formation of neurosphere, more complex functions of undifferentiated neural cells appear than at cultivation in adherent mediums. Because the genesis of such a unique multi-cellular structures as a neurosphere requires the *NSC* ability to complex cellular interactions [43].

It is remarkable that today the *NSC* is the only type of stem cells, which possibility of such self-sustaining colony formation in promitotic environment is described [7].

Our study demonstrated that the addition of 20 nM concentration of *NaFL* in culture medium, which contained no *FGF-2*, increases the number of neural progenitors, and decreases the number of neurospheres **(Fig. 4, D-E)**. This result confirms that the 20 nM concentration of *NaFL* increases the proliferative potential of *NSC*.

The electron-microscopic analysis showed a mitotic activity during the cultivation of neural progenitors in the medium, which contained *FGF-2*. This is characteristic to this cell type **(Fig. 5, A)**. But, when we added *NaFL* into the culture medium, by the 7th day it was found not only outside the cells, but was localized near the cell membrane, in the membrane itself, and in the cytoplasm **(Fig. 5, B-E)**.

Electron-microscopic investigations have shown that metal fullerenolat C_{60} may not only be present in the solution, but also penetrates into the cells directly across the cell membrane without damaging it; and localizes itself in the cytoplasm of cells, though this substance did not show any specific tropism for neural progenitors organelles.

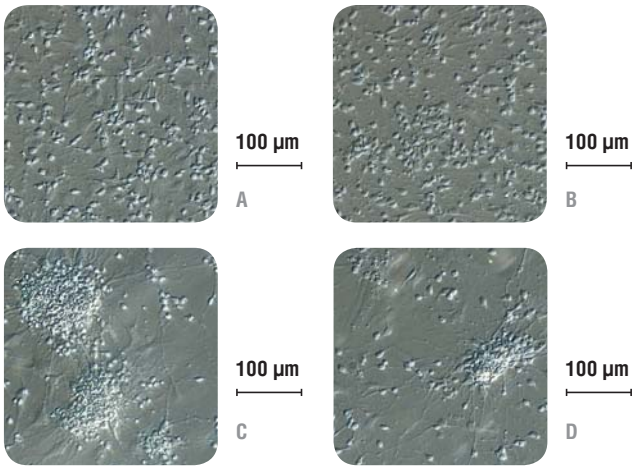

Fig. 1. Neural stem cells and neurospheres, 7days in culture.
A – control (*NSC + FGF-2*);
B – *NSC + FGF-2* with the addition of 20 nM concentration *NaFL*;
C – *NSC* without *FGF-2* - neurospheres control;
D – *NSC* cultivated without *FGF-2 + 20 nM NaFL*.

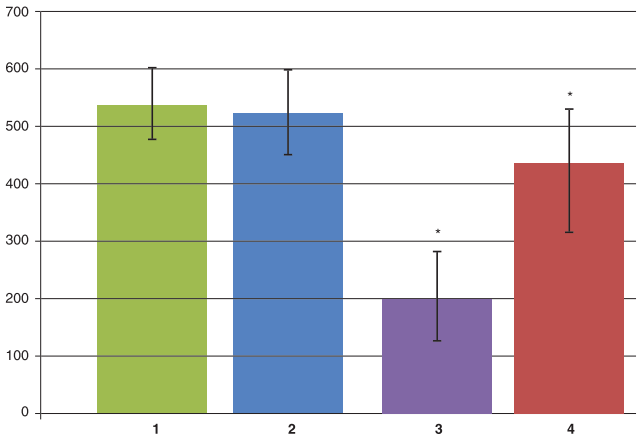

Fig. 2. Quantitative analysis of *NSC* under different culture conditions, 7 days:
1 – standard conditions, *FGF-2* is present in the cultivation medium;
2 – standard conditions + *20nM NaFL*;
3 – the cultivation medium without *FGF-2*;
4 – cultivation medium without *FGF-2 + 20 nM NaFL*.

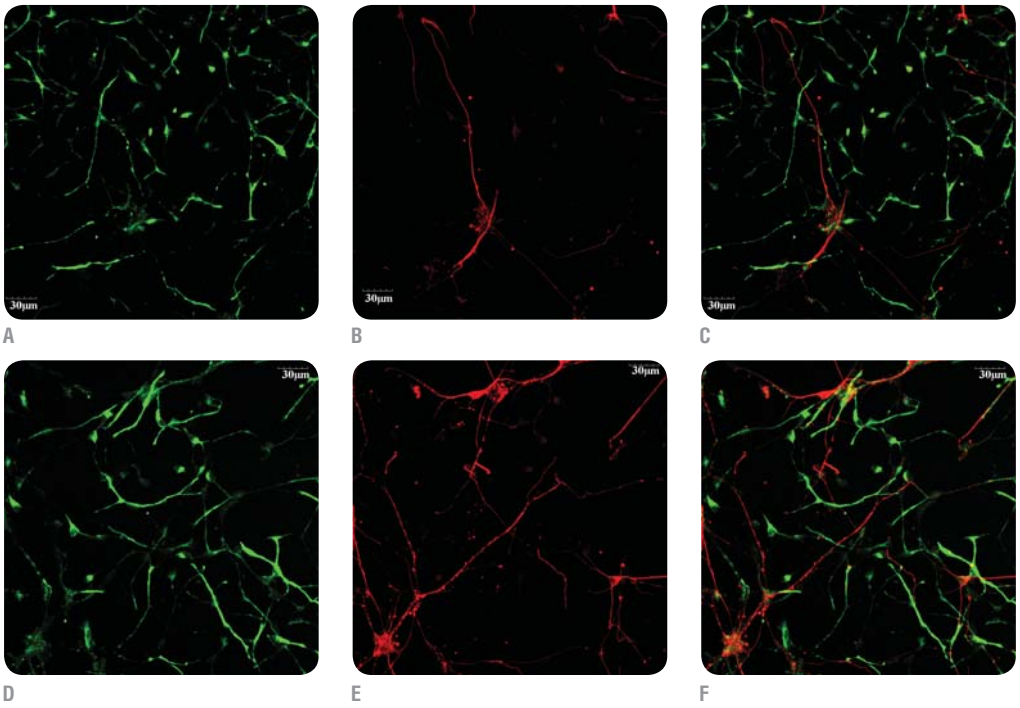

Fig. 3. The immunocytochemical staining of neural progenitors cultured in medium containing *FGF-2*, 7 days (x40):
A – control, *Nestin*-positive cells (green);
B – control of *β-tubulin III*-positive cells (red);
C – *Nestin + β-tubulin III*, control,
D – NSC + NaFL ,Nestin-positive cells (green),
E – *NSC + NaFL β-tubulin III*-positive cells (red), and
F – *Nestin + β-tubulin III*, *NSC + NaFL*.

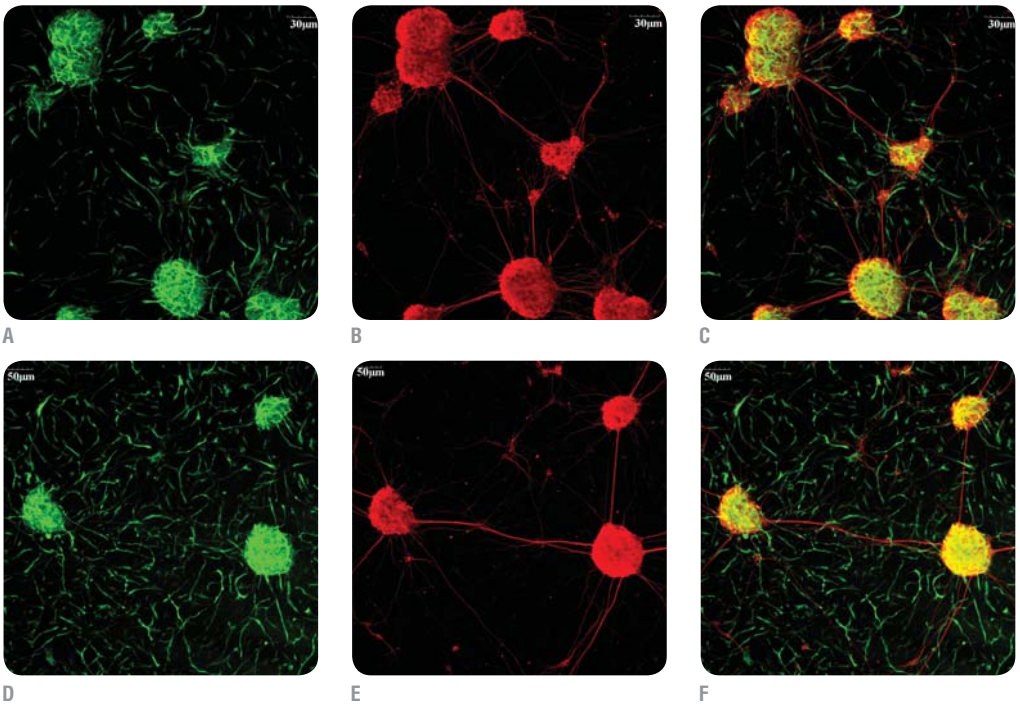

Fig. 4. The immunocytochemical staining of neural progenitors and neurospheres cultured in medium without *FGF*-2 7days (x20).
A – control (*Nestin*, green);
B – control (*β-tubulin III*, red);
C – *Nestin + β-tubulin III*, control;
D – *NSC + NaFL* (*Nestin*, green);
E – NSC + NaFL (*β-tubulin III*, red);
F – *Nestin + β-tubulin III*, *NSC + NaFL*.

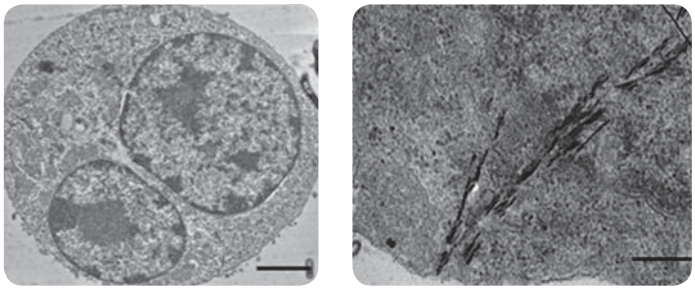

Fig. 5. Electron-microscopic images of cultured *NSC*:
A – dividing cell, control;
B – localization of *MC60(OH)x* in the membrane and in the cytoplasm of cells;
C – localization of *NaFL* outside the cell processes;
D – localization of *NaFL* in cytoplasm;
E – localization *NaFL* near the membrane and in the cytoplasm.
Scale bar A — 0,7 μm, B, C, D — 0,3 μm.

CONCLUSIONS

IT HAS BEEN SHOWN THAT UNDER THE CONDITIONS IN VITRO THE NUMBER OF CULTURED *NSC*, ADDING 20 NM CONCENTRATION OF METAL FULLERENOLAT C_{60}, INCREASES ALMOST 2 TIMES, COMPARED WITH THE CONTROL. THEIR PROLIFERATIVE ACTIVITY REMAINS THROUGHOUT THE PERIOD OF CULTIVATION, AND ALSO THE FORMATION OF NEUROSPHERES DECREASES. ULTRASTRUCTURAL ANALYSIS INDICATED THAT *NAFL* ENTERS THE CELL DIRECTLY THROUGH THE CELL MEMBRANE, WITHOUT DAMAGING IT; AND LOCALIZES IN THE CELL CYTOPLASM.

THUS, THE RESULTS OF THIS STUDY LEAD TO THE HYPOTHESIS THAT THE *NAFL* AFFECTING THE PROLIFERATION OF NEURAL STEM CELLS, MAY BE PERSPECTIVE FOR ITS USE IN CELL BIOLOGY AND BIOTECHNOLOGY.

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