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Effect of Low-Molecular Fraction of Fish Peptide Albutrisan-NR on *In Vitro* Neurogenesis

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Abstract
The effect of the Albutrisan-NR substrate on the differentiation of the primary brain culture of aging rats and on the culture of the rabbit vibrissa dermal papilla was studied. The substance is a hydrolysate of fish tissue with a molecular weight of up to 5 kDa. It was found that adding 2% of the substance to the culture medium stimulates the growth and differentiation processes in rat brain cells. The possibility of using the substance as a growth factor for 3D cultivation of pluripotent dermal papilla cells and maintaining an uncommitted state was demonstrated. The use of Albutrisan-NR as a trophic factor in neuronal induction of dermal papilla cells was also shown.

Keywords: Brain cell culture; dermal papilla; multicellular spheroids; β3-tubulin; neuronal induction.

1. INTRODUCTION

Degenerative and traumatic disorders of the nervous system are among the key problems of modern medicine, as they can quickly and radically worsen the quality of life of patients. There are prerequisites for the search for substances of neuroprotective action among peptides obtained from natural sources—animal tissues. At the moment, from the point of view of pharmaceutical value, peptides from hydrolysates of many aquatic organisms, including fish, have been studied [1-3]. Since the waste from the fishing industry is a fairly affordable raw material, the search for areas of their medical and pharmaceutical applications is also important from the point of view of rational nature usage. In this research, we studied the effects of a low-molecular fraction (up to 5 kDa) of fish peptide hydrolysate (Albutrisan-NR) on isolated *in vitro* precursor cells of neural tissue. We also studied the effect of the medicine on the growth of neurons in adult rats as well as on the neural induction of progenitor cells of the neural crest taken from dermal papillae (DP) of the rabbit hair follicle. DP cells, a pool of cells that are derivatives of the neural crest, are a part of the hair follicle in adulthood and ensure its renewal. The possibility of culturing this type of cells *in vitro*, as well as their induction plasticity [4], was shown; in this regard, DP cells are considered as a promising source of cells for transplantation.

2. METHOD(S)

All experiments on animals were carried out in accordance with the Law of Ukraine "On the Protection of Animals from Cruelty" (No. 3447-IV of 02.21.2006), subject to the requirements of the institute's Bioethics Committee, consistent with the provisions of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (ETS 123 - Protection of Vertebrate Animals, 18.III.1986). A rat brain cell culture was obtained from a suspension of the central part of the brain containing the hippocampus—a part of brain with active adult neurogenesis [5]. A mechanically homogenized suspension was passed through a sieve with a pore size of 100 μm and then plated in a growth medium, DMEM/F12 (Biowest, France), with the addition of 10% fetal calf serum (FCS) (Biowest, France). Sowing was performed in petri dishes for cell cultures at a concentration of 20,000/ml. Cultivation was carried out in a CO_2 incubator. We have developed methods for 3D cultivation of DP cells based on the use of additives for serum-free cultivation. The cell culture of DP was obtained by the method of explants from Hair follicle (HF) neonatal rabbits. The monolayer was cultured under standard conditions.

In this experiment, for 3D cultivation, we used a commercial growth supplement for neuronal progenitor cultures—B27 (Sigma, USA) and the Albutrisan-NR substance (≤ 5 kDa fraction of fish tissue hydrolysate). DP cells were cultured as multicellular spheroids (MSs) for 21 days, after which they were sown on six-well plates and inducted in the neuronal direction. Induction was made by adding FCS 10^{-6} mmol of retinoic acid (Sigma, USA). Twenty-one days after the addition of the induction

medium, DP cells were stained with fluorescein isothiocyanate (FITC)-labeled monoclonal antibodies to express β3-tubulin (Sigma, USA), a neurofilaments protein expressed by mature neurons. T-test was used to assess the significance of differences between samples.

3. RESULTS AND DISCUSSION

Adhesive cultures were obtained from a rat brain containing a culture with homogeneous morphology (Figure 1a). Cells were cultured for 14 days; by this time the monolayer had reached 60-70% confluence. On day 14, the studied drug was added to the culture at a concentration of 2%; the drug was not added to the control variant. Three days after adding the drug, a visual analysis of the culture was performed. When studying the morphology of rat brain cell culture, the appearance of processes at the poles of cells in the culture to which the drug was added was noted. In the control variant, the growth of the processes was not observed (Figure 1b). Even 7 days later, cells with long processes at the poles and neuroblast-like morphology were found in the cell culture containing the studied drug (Figure 1c), but in the control the morphology of the cells did not change (Figure 1c). In addition, in the variant to which the studied drug was added, there were cells having the morphology of mature neurons—having long branched processes (Figure 1d). The data suggest that the formation of processes can indicate the induction of neurogenesis in cell culture [6]. There are data in the literature regarding the presence of peptides in the hydrolytes of fish tissue that activate the specialization of new neurons by activating neurotrophins at the transcription level.

When studying the culture of DP rabbit cells *in vitro*, their plasticity was confirmed by induction in neuronal, osteogenic, and adipogenic directions. A significant problem in the cultivation of progenitor cells is the preservation of their uncommitted state, since their spontaneous induction and loss of multipotent properties often occur. It is known that the common property of many progenitor cultures, including Neural crest (NC) derivatives, is the ability to form spherical colonies (MSs) [7].

MSs are a form close to tissues in the body, because they provide natural growth and microenvironment, which contribute to better maintenance of an undifferentiated state than in a monolayer. We have obtained MSs from DP cultures previously cultivated as a monolayer. We found that cultivation at a high concentration of cells (10^6 cells/ml) in a serum-free medium promotes the formation of MSs. As a comparison drug, a specialized supplement was used to support the growth of neural cultures of B27 (Sigma, USA). It was shown that cultivation in the DMEM/F12 medium with the addition of 2% Albutrisan-NR and without the addition of extra growth factors ensures the formation and growth of MSs similary to the medium with 2% of B27 supplement (Figure 2b). Upon cultivation with 2% PCS as growth supplement, a monolayer, not spheroids was formed (Figure 2a).

The literature contains data on the influence of growth factors on the quality of crops and the nature of their growth, which testifies in favor of the sensitivity of the culture to environmental signals inherent in progenitor cells [8]. It should be noted that most protocols for the induction of progenitor cells in the neuronal direction involve the use of retinoic acid and various growth factors [8].

Figure 1: Rat brain cell culture: (a) 14th day of growth (before the introduction of the drug), (b) 17th day of growth—negative control, (c) 17th day of growth—3 days after the introduction of the drug, intensive growth of processes; (d) 21 days of growth—7 days after application of the drug, a mature neuron. The scale bar is 100 microns.

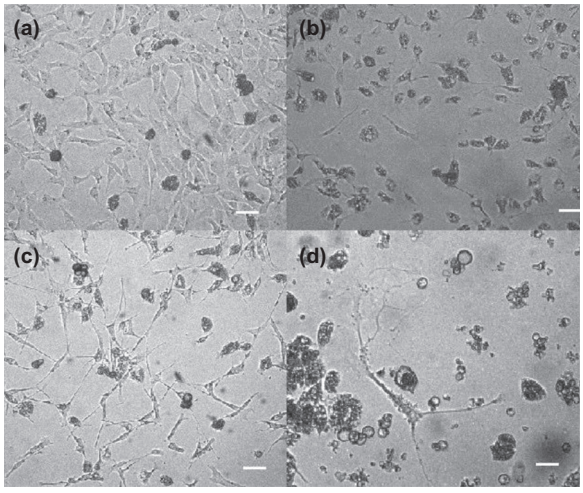


Figure 2: Cell culture—derivatives of NG taken from DP: (a) monolayer—cultivation in the presence of FCS; (b) multicellular spheroids—cultivation in the presence of Albutrisan-NR hydrolysate.

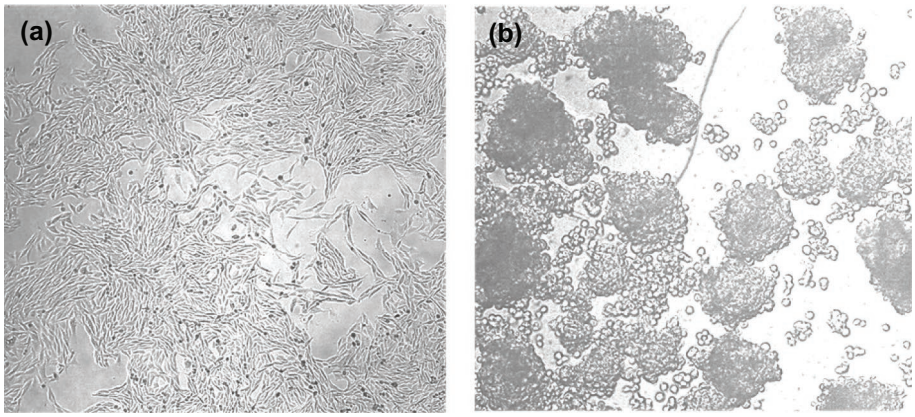


Figure 3: Comparing the monolayer filling rate during cell cultivation with the addition of 2% B27 and Albutrisan-NR in a medium containing 10% FCS.

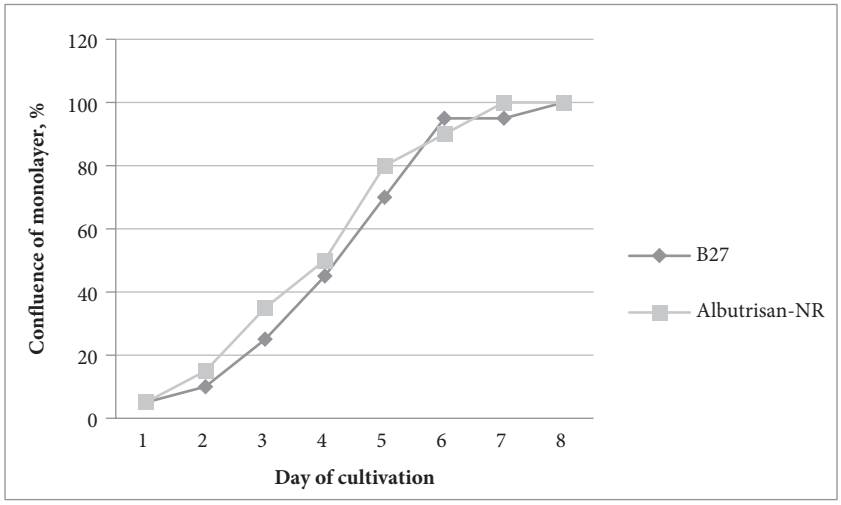
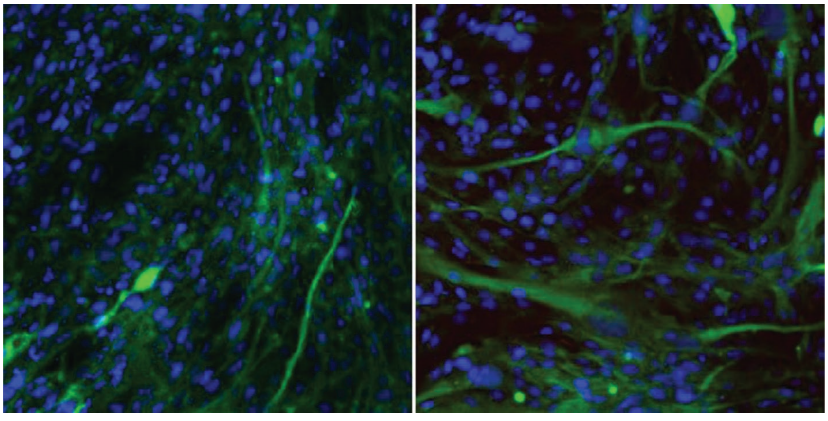


Figure 4: Neuronal-induced DP cell culture: (a) cultivation on growth additive B27; (b) cultivation on Albutrisan-NR hydrolysate. βIII-tubulin (staining – FITC-labeled monoclonal antibodies)



We compared the execution speed of a monolayer by DP rabbit cells when cultured in a medium supplemented with 10% FCS and growth additives B27 and Albutrisan-NR as additional cultivation factors. It was found that the speed of the monolayer does not differ significantly, but there is a tendency for faster growth, as a result of which the monolayer is confluent earlier—by 7 days, in comparison with B27—8 days (Figure 3).

As there are mechanisms providing the formation of MSs instead of a monolayer in serum-free conditions, we can assume a decrease in the concentration of signaling factors in the growth medium. Thus, it is known that high concentrations of Bone morfogenetic protein (BMP) and Fibriblast growth factor (FGF) [7, 9] present in fetal serum increase the bond with the substrate, increase the proliferation rate, and contribute to the development in the epidermal direction [8, 10]. The concentration of BMP in the environment determines which of the two categories, the neuronal or epidermal cells of the neural crest, will develop or retain an uncommitted state [11]. It is possible that a decrease in BMP concentration by excluding fetal serum from the medium reduces the likelihood of spontaneous induction of DP cells.

As a result, it was shown that both cultures express β3-tubulin and have morphological characteristics of neurons—polygonal bodies with outgoing long processes reaching 500 μm in length (Fig. 4). Thus, the use of the Albutrisan-NR substance for various neural crest–derived cell lines has shown its ability to enhance neuronal induction and maintain the multipotency of these cells in culture without the use of additional growth supplements. Probably, the peptides that the hydrolysate consists of are mimetics of neurotrophins and are able to enhance the growth and differentiation of neurons [11, 12].

Based on the obtained data, it can be assumed that the use of such a drug to enhance the reparative regeneration of nerve tissue in the body may be promising. There are various approaches to the hydrolysis process, as a result of which its products may have various properties, the biological spectrum of which is very wide and requires further study. In particular, data have already been accumulated on the valuable regenerative, regulatory, and antioxidant properties of low-molecular-weight peptides derived from fish tissue, which can also be used in further research in neurology.

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Conflict of Interest
None.

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