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Obtaining and characterization of rat fetuses neural cells aggregates/spheroids

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Aim. Search for optimal conditions for the formation of aggregates/spheroids by neural cells (NC) isolated from the brain tissue of rat fetuses. Study of behavioral characteristics of NC in culture as a part of three-dimensional aggregates/spheroids. **Methods.** Isolation of NC from fetal rat brain tissue; cell viability assessment; cultivation; immunocytochemical staining; NC proliferative activity assessment. **Results.** It was found that the conditions for the robust formation of aggregates are: 1) the presence of 10 % adult rat's blood serum in the cell culture medium, 2) the seeding cell density — 1–4×10^6 cells/ml and 3) the initial cell viability higher than 20 %. **Conclusions.** The isolated rat fetuses brain cells, when cultured in the presence of 10 % serum and at concentrations above 1×10^6 cells/ml, spontaneously form multicellular aggregates. The structure of aggregates formed during short-term cultivation depends on the initial cell viability, and the size of aggregates depends on the initial viability and seeding densities of NC. The rat fetuses NC aggregates/spheroids contain stem/progenitor cells that can proliferate and differentiate. NC aggregates/spheroids form three-dimensional structures, in which favorable conditions are developed for the survival and adequate functioning of the committed and stem/progenitor NC.

K e y w o r d s: rat fetuses, neural cells, aggregates, spheroids, cultivation.

Introduction

Neurological disorders such as Alzheimer's and Parkinson's, schizophrenia, amyotrophic lateral sclerosis, stroke and brain injuries affect about one billion people worldwide [1]. For many of these diseases there are still no effective treatments. Several factors contribute to this, including the limitations of the experimental instruments used.

Much of our knowledge of how the brain functions has been gained from research of

animal models. One of the most commonly used systems for studying the characteristics of neural cells (NC) functioning *in vitro* was a two-dimensional (2D) cell culture. However, the cells that are grown in 2D conditions do not fully reproduce the three-dimensional (3D) characteristics of the nervous tissue. Furthermore, in 2D cell culture, the nonoptimal mechanical cues of hard plastic or glass surface, as well as the lack of the three-dimensional extracellular matrix support, can significantly affect the behavior, functioning, growth, and morphology of NC [2–6].

Thereby, two-dimensional models do not allow the reconstruction of elementary architectural components and 3D microenvironment of the intact brain. Therefore, the efforts of recent research were focused on developing three-dimensional mammalian brain cell culture systems that can fill the gap between *in vivo* natural and *in vitro* artificial conditions and provide highly productive and reproducible research results [6]. In such three-dimensional cell systems, intercellular interactions and physiological signals from the extracellular matrix create the conditions for cells that are similar to those *in vivo*.

The aim of our work was to find the optimal conditions for the formation of aggregates/spheroids by the primary suspension of NC isolated from the brain tissue of rat fetuses. The work is also aimed at studying the characteristics of the behavior of NC in culture as a part of three-dimensional aggregates/spheroids.

Materials and Methods

Isolation of NC. Cells were isolated from brain tissue of rat fetuses (15-16 embryonic day of development, ED15-16) by mechanical and enzymatic-mechanical methods. When isolated by a mechanical method, the brain tissue was washed with DMEM/F12 (Sigma, USA) from blood, milled, and mechanically disaggregated into single cells using vibration [7]. The resulting cell suspension was filtered through a nylon filter with a pore diameter of 200 μm, after which the viability and the number of cells were determined. When isolated by the enzymatic-mechanical method, the brain tissue was washed from blood, then transferred into a trypsin-versene solution and incubated at 37 °C for 2 minutes. After that, the tissue was transferred into DMEM/F12 supplemented with 10 % adult rat blood serum (ARBS) [8] and mechanically disaggregated into single cells using vibration [7]. The resulting suspension was filtered through a nylon filter and then washed from trypsin by centrifugation at 100 g for 2 min. The resulting cell pellet was suspended in DMEM/F12 containing 10 % ARBS.

Cell viability was assessed by the trypan blue (TB) exclusion test and by staining with propidium iodide (PI) [9]; the number of cells was counted in a Goryaev chamber. The number of cells was also recalculated per fetus.

Cells were cultured in 24-well plates (Corning, USA) at concentrations of **0.2|4×10^6** cells/ml in DMEM/F12 supplemented with 0.6 % glucose and 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, in the presence of or without 10 % ARBS [8]. The cells were cultivated in a CO_2 incubator at 37 °C in an atmosphere of 5 % CO_2, 95 % air. The cell culture medium was replaced with a fresh one every 3–4 days.

Formation of NC aggregates. To form aggregates, the NC that settled on the bottom of

the well were suspended with a pipette in 15 and 30 min after seeding. The formed aggregates were carefully collected in a minimal volume of medium, subcultured, and further cultured in DMEM/F12 supplemented with 10 % ARBS.

To assess the viability and concentration of NC in aggregates/spheroids, all aggregates and spheroids, formed in one well, were carefully collected, disaggregated into single cells by gentle pipetting, and their concentration and viability were assessed as described above.

Immunocytochemical studies of cell cultures were carried out after their fixation in a solution of 4 % paraformaldehyde. The fixed cells were permeabilized with absolute alcohol, blocked with 5 % goat serum (Sigma, USA), and incubated with primary antibodies overnight at 4 °C. The cells were further washed and cultured with secondary antibodies for 40 minutes. β-tubulin 3 (mouse, Sigma, USA) was used as the primary antibody for neuron identification. IgG conjugated to FITC (Sigma, USA) was used as a secondary antibody. Cell nuclei were stained with Hoechst 33342 dyes (Sigma, USA).

Analysis of the proliferative activity of NC was performed using bromodeoxyuridine (BrDU) (Sigma, USA). The formed NC aggregates were transferred into 24-well plates with coverslips. BrDU was added to them to a final concentration of 10 μM and cultured for 2 days at 37 °C. Then the coverslips with NC were washed with phosphate buffer (PB) and fixed in 4 % paraformaldehyde. The fixed cells were permeabilized with 1 % Triton X-100, DNA was denatured by incubation with 1 and then 2 N HCl, the cells were further incubated for 10 min in 0.1 M Tris-borate buffer at room temperature.

The cells were then washed and incubated with 5 % normal goat serum in PB containing 1 % Triton X-100 and 1M glycine at 37 °C for 4 hours. The solution was further removed, the primary antibodies against BrDU (Sigma, USA) were added to the cells and incubated overnight at room temperature. The cells were then washed in PB and incubated at room temperature in the dark with the secondary antibodies conjugated with FITC (Sigma, USA). Cell proliferative activity was assessed using fluorescence microscopy.

Microphotography of cell cultures was performed using Observer Z1 (Carl Zeiss, Germany) and AmScope (USA) microscopes.

Morphometric analysis of NC and aggregates/spheroids was carried out using AxioVision Rel. 4.8 and Zeiss Image Examiner (Carl Zeiss, Germany), and AmScope software (USA).

The results obtained were statistically processed by the Student's t-test using Microsoft Excel.

The work with animals was carried out in accordance with the "General Principles of Experiments on Animals" approved by the V National Congress on Bioethics (Kiev, 2013), consistent with the provisions of the "IV European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes" (ETS 123, Strasbourg, 1986) and permission of the Bioethics Committee of the Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine.

Results and Discussion

Both mechanical and enzymatic-mechanical methods allowed obtaining viable cells from

the brain tissue of rat fetuses. The viable cells are characterized by a rounded shape and a clearly delineated plasma membrane.

The viability of freshly isolated NC did not depend on the method of isolation and the gestation period of rat fetuses (Table 1). At the same time, the viability of NC, determined by staining with propidium iodide, did not differ significantly from the viability determined by the trypan blue test (Table 1), and averaged 40.3 % in the cells obtained from the fetuses ED16 and 43.2 % — from the E15. However, when using the enzymatic-mechanical method, 1.9 and 1.7 times more cells were obtained from the brain tissue of the fetuses ED15-16 in comparison with the mechanical method (Table 1). Also, the use of the enzymatic-mechanical isolation method allowed the obtaining of 1.5 times more cells from the fetuses ED16 compared to the number of cells obtained from th fetuses ED15 (Table 1).

During cultivation in the absence of serum, most of the cells are quickly settled on the substrate, some of them attached and spread. The spread cells had glial morphology. The number of spread cells was 2-5 % of the settled ones. During cultivation, the settled cells did not form a monolayer and eventually died.

The NC cultivation in the presence of 10 % serum (Table 2) results in spontaneous formation of aggregates within a few hours after cell seeding.

Table 1. **Viability and number of freshly isolated rat fetuses NC depend on the embryonic day (ED) of development and the isolation method (M±m)**

Isolation Method	Gestation period, days	Fetuses, n	Viability, %		Cell Concentration (1×10^6 cells/fetus)
			By Trypan Blue staining	By Propidium Iodide staining	
Enzymatic-mechanical	16	13	44.2±15.8	39.8±12.5	13.4±4.2*#
Mechanical	16	12	41.5±12.1	35.6±10.0	7.0±3.1
Enzymatic-mechanical	15	10	44.7±10.2	41.3±9.6	8.5±3.0
Mechanical	15	12	46.3±12.3	40.6±10.4	5.0±2.5

Note: * — the differences are statistically significant compared to the mechanical isolation method of the same gestation period ($p < 0.01$); # — the differences are statistically significant compared to NC isolated from fetuses ED15 ($p < 0.01$)

Table 2. **Influence of rat fetuses NC viability and concentration on aggregate formation efficiency during cultivation in the presence of 10 % ARBS (n=40).**

Concentration (1×10^6 cells/ml)	Viability, %	Characterization of cell aggregates			
		The number of cells combined into aggregates, %	Size, μm	Density	Attachment and migration, %
0.1÷0.5	< 20	No aggregate formation			
1.0÷3.0	< 20	20÷30	20÷50	Loose	20-50
1.0÷2.0	> 20	30÷60	30÷120	Compact	70-90
>3.0	> 20	Dense suspension of merging aggregates			

Note: The cultivation was carried out in a 24-well plate.

The number, size, and compactness of aggregates depended on the concentration and viability of the seeded cells.

The cells with viability of less than 20 %, when cultured at concentrations lower than 0.5×10^6 cells/ml, formed few small aggregates (10–20 μm) with a loose packing of cells in them. Such aggregates did not attach to the substrate (Table 2).

Cultivation of NC with viability of less than 20 % at concentrations of $(1–3)\times10^6$ cells/ml was characterized by the formation of small and medium-sized (20–50 μm) aggregates, the number of which depended on the cell seeding density (Table 2, Fig. 1 A).

The packing of cells in these aggregates was mostly loose. After subculturing, 20–30 % of these aggregates attached to the substrate, and their cells then migrated and differentiated into neurons and glial cells.

Active spontaneous formation of aggregates occurred during the cultivation of NC with cell viability higher than 25 % and the cell density in the seeding culture $(1–2)\times10^6$ cells/ml (Table 2, Fig. 1 B, C). The packing of cells in these aggregates was generally compact; the size of the aggregates was 30-120 μm. During cultivation, the number of aggregates could increase, some of the aggregates merged, some aggregates turned into spheroids, which, if not attached, increased in size (Fig. 1, D). This indicates the presence of stem/progenitor cells capable of proliferation in the aggregates. Cultivation of NC, seeded at concentrations above 3×10^6 cells/ml, was characterized by the formation of a dense suspension of merging aggregates

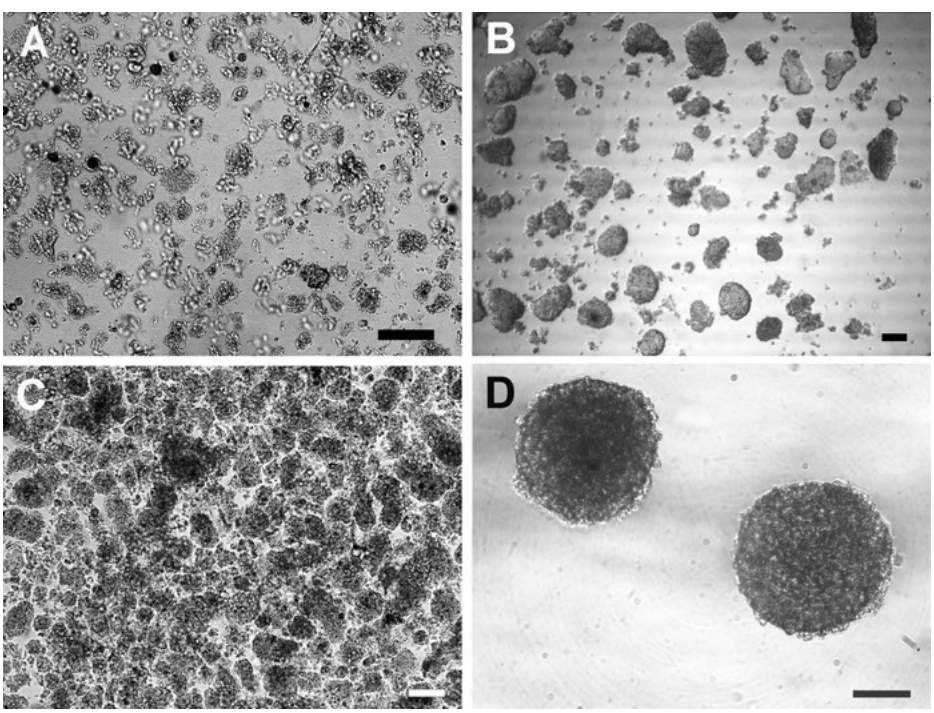

Fig. 1. Formation of aggregates by freshly isolated NC of rat fetuses ED15 after 2 hours of cultivation in the presence of 10 % ARBS (*A* — viability 10 %, 1×10^6 cells/ml; *B* — viability 30 %, 1×10^6 cells/ml; *C* — viability 30 %, 4×10^6 cells/ml), *D* — formation of spheroids from aggregates (1 day of cultivation). Scale: *A, B, C* — 100 μm; *D* — 50 μm.

(Table 2, Fig. 1 C). The merging activity of the aggregates was directly proportional to their number, which, in turn, was determined by the concentration of the seeded cells.

A decrease in serum concentration to 5 % was accompanied by a decline in the efficiency of aggregates formation (at the concentration (1–2)×10^6 cells/ml and viability higher than 20 % about 30 % of cells formed aggregates) and a decrease in the size of aggregates to 30–50 μm. An increase in serum concentration up to 20 % did not significantly affect the efficiency of aggregates formation.

Erythrocytes, which sometimes contaminated the freshly isolated NC suspension, were not included in aggregates.

The viability of NC that formed aggregates, determined after 1 day of cultivation, was 85.4±6.5 %, which is more than 2 times higher than the viability of freshly isolated cells (44.5±9.5 %).

The majority of aggregates formed by NCs with a viability higher than 25 % and a size (diameter) greater than 30 μm, as well as all spheroids, were attached to the substrate after 1 day of cultivation (Fig. 2A). At the same time, the attachment of aggregates/spheroids was stimulated by subculturing or replacement of the medium with a fresh one. Subculturing of aggregates that did not attach to the substrate during 1–2 days of cultivation also led to their attachment to the substrate. After attachment, the cells of the aggregates/spheroids actively migrated and spread out (Fig. 2A). Also, the cells of attached aggregates/spheroids formed the long processes (Fig. 2), along which the undifferentiated cells migrated (Fig. 2C). Individual cell processes are generally connected, forming the structures similar to nerve fibers (Fig. 2B). Through the processes, the attached aggregates/spheroids and migrating cells were coalesced into a network (Fig. 2A, B). Immunocytochemical staining showed that all processes were β-tubulin 3-positive (Fig. 2D).

During further cultivation, the cells of aggregates/spheroids formed a glial monolayer (Fig. 3A), on the surface of which β-tubulin 3-positive cells with neuronal morphology were identified (Fig. 3B).

Large aggregates (300–400 μm), formed as a result of the fusion of the smaller ones, also attached to the substrate, their cells formed processes, migrated and differentiated. At the same time the cells and fragments of the inner layer of such aggregates detached from the main part of the latter, transferred into a floating state and lost the ability to attach to the substrate with further cultivation, which suggests their nonviability. This fact indicates that in the inner layers of large aggregates the unfavorable conditions are created for NC functioning (lack of nutrients and oxygen, excess of metabolic products), which leads to their death.

The experiments have shown that the rate of monolayer formation depends on the initial viability of NC and their seeding concentration. At the same time, the monolayer is formed not only due to the cell migration from aggregates, but also through the cell proliferation, shown by BrDU incorporation into the cells of monolayer (Fig. 4), which indicates the presence of stem/progenitor cells.

After the formation of a monolayer on 70 % of the surface area of the well (4–6 days of cultivation), the neuroblast-like cells were the first to appear on it (Fig. 5A, black arrows), and then (8–10 days of cultivation) the colonies of undifferentiated cells (Fig. 5A, light arrows).

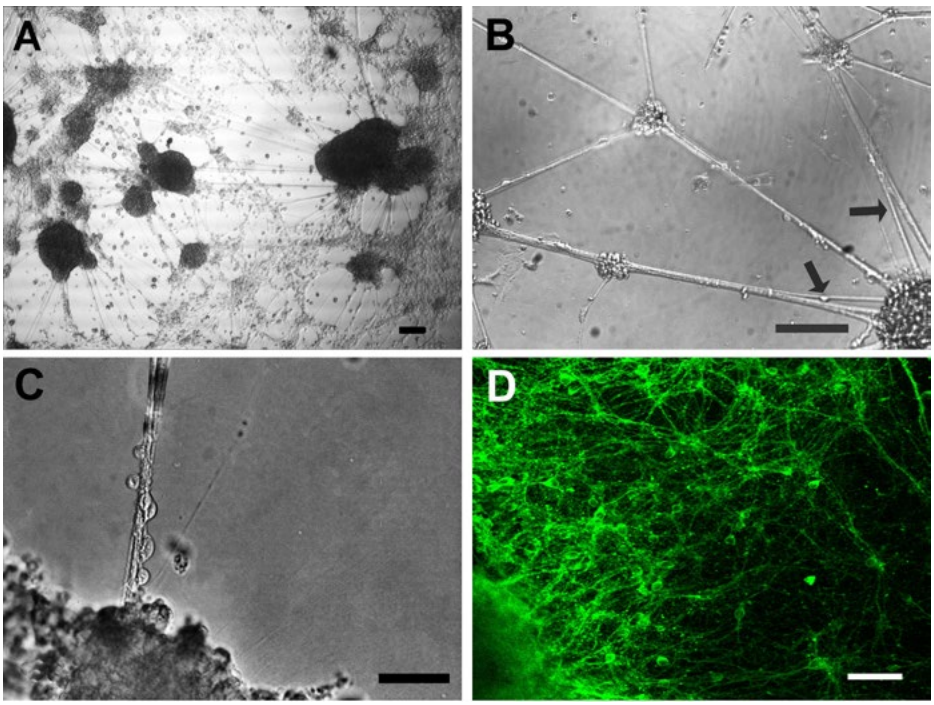

Fig. 2. Attachment of NC aggregates/spheroids after subculturing (*A*). Formation of long processes by cells of aggregates and their connection (arrows) (*B*). Migration of undifferentiated cells along the processes (*C*). Immunohistochemical staining of the culture of attached aggregates with antibodies to β-tubulin 3 (*D*). Scale: *A, B* — 100 μm, C, *D* — 50 μm.

During cultivation, the number of colonies and their size increased. The colony cells formed the processes connecting them into a network (Fig. 5A). The neuroblast-like cells also formed **the processes that increased in size** and formed a cellular network (Fig. 5B).

Immunohistochemical staining showed that both the colonies and neuroblast-like cells were β-tubulin 3-positive (Fig. 6).

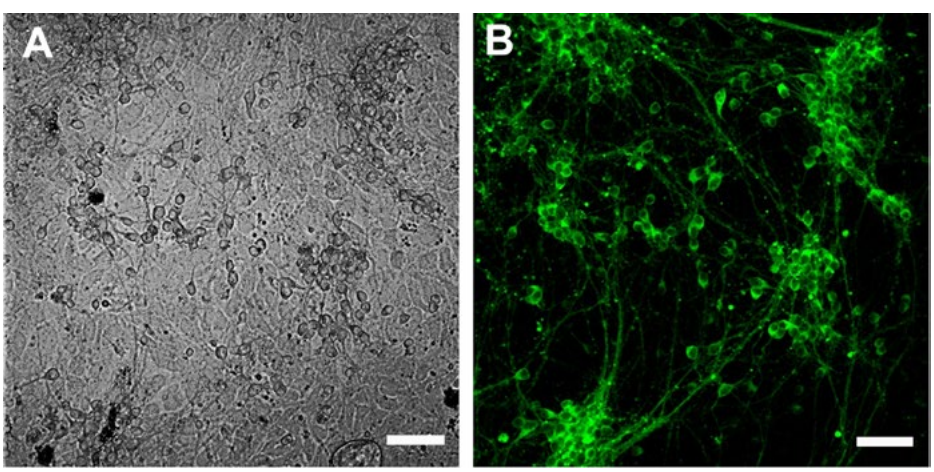

Fig. 3. Monolayer formation by cells of aggregates/spheroids (during 3 days of cultivation) (*A*), on the surface of which there were β-tubulin-3-positive cells (*B*). Scale: 50 μm

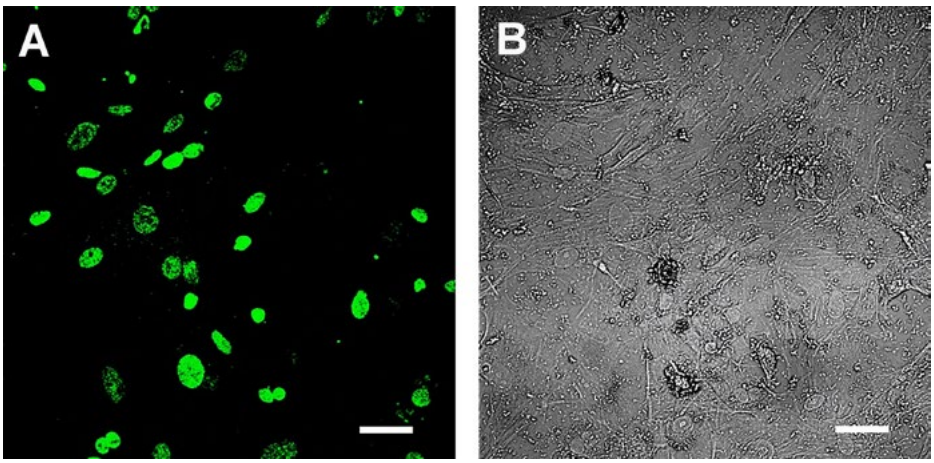

Fig. 4. BrDU incorporation (*A*) into cells of a monolayer (*B*) formed during two-day cultivation of attached aggregates of NC. Scale: 50 μm.

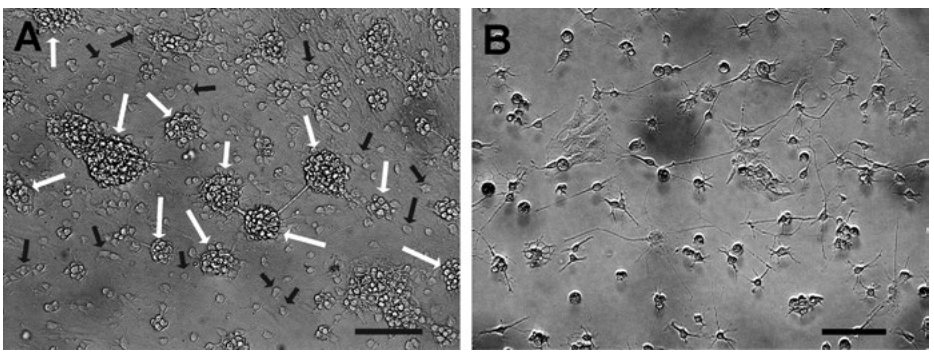

Fig. 5. *A* — formation of neuroblast-like cells (black arrows) and colonies of undifferentiated cells (light arrows) on the glial monolayer (10 days of cultivation of rat fetuses NC aggregates). *B* — formation of a network of neuroblast-like cells. Scale: 100 μm.

Thus, the studies have shown that both mechanical and enzymatic-mechanical methods allow the obtaining of viable cells from the fetal rat brain tissue. The resulting cell suspensions are heterogeneous and contain both differentiated (living and dead) and viable stem/progenitor neural cells.

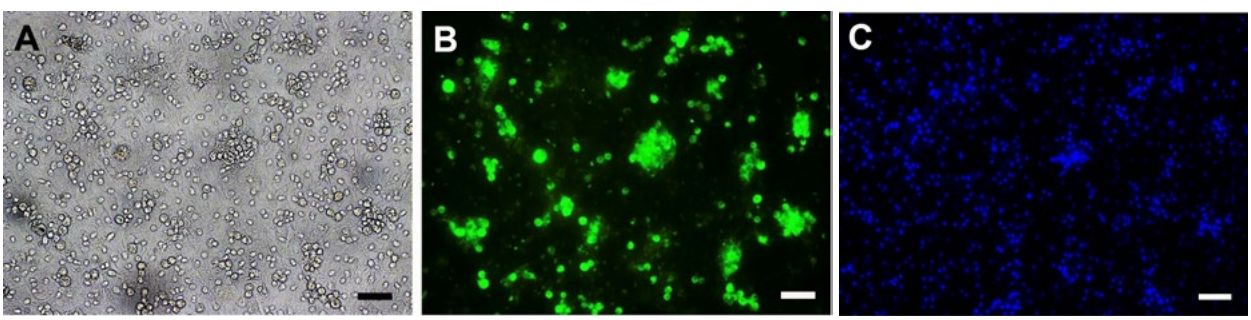

Fig. 6. Immunohistochemical staining of neuroblast-like cells and colonies of undifferentiated cells formed on the monolayer (*A*) with antibodies to β-tubulin 3 (*B*). *C* — [the] staining of cell nuclei with Hoechst 33342 dyes. Scale: 50 μm.

During cultivation in a medium containing 10 % serum without mitogens, the freshly isolated rat fetal NC spontaneously aggregate, forming multicellular aggregates. At the same time, the viability of cells in aggregates is 2–2.5 times higher than the viability of the initial cell suspension and averages 85 % (Table 2). This indicates that only viable cells are combined into aggregates.

The experiments showed that the optimal conditions for the formation of aggregates are the presence of 10 % serum in the culture medium, high seeding density (1-4×10^6 cells/ml) and cell viability higher than 20 %. This can be explained by the fact that in the process of NC isolation, cell microdamages can occur, with the subsequent loss of adhesion molecules (integrins and e-cadherin) [10], which restrains the attachment of cells to the substrate. When cultured at high concentrations, under conditions that prevent rapid cell settling, the remaining adhesion molecules are probably sufficient for the formation of intercellular bonds and the formation of multicellular, three-dimensional aggregates. Further cultivation is probably accompanied by the synthesis of lost adhesion molecules [10], which leads to the compaction of aggregates and their transformation into spheroids, and also promotes their attachment to the substrate with further migration, differentiation, and spreading of their cells.

A number of aggregates in the process of cultivation turn into spheroids, which are morphologically and functionally practically indistinguishable from neurospheres — thus, they are characterized by both the ability to increase in size and the ability of their cells to incorporate BrdU, which indicates the presence of proliferating stem/progenitor cells in their composition; after the attachment of spheroids to the substrate, their cells migrate and differentiate into neurons and glial cells, similar to the cells of neurospheres. The presence of stem/progenitor cells in aggregates is also indicated by the formation of neuroblasts and colonies of undifferentiated cells during cultivation. These data point to a similar cellular composition of aggregates and neurospheres, including both committed and stem/progenitor cells, and similar mechanisms of their functioning. However, unlike neurospheres, the aggregates are not the clones of a single stem cell, but are a result of the aggregation of a heterogeneous suspension of NC.

Conclusions

Isolated rat fetuses NC, when cultured in the presence of 10 % serum and concentrations above 1×10^6 cells/ml, spontaneously form multicellular aggregates.

The structure of aggregates formed during short-term cultivation depends on the initial cell viability, and the size of aggregates depends on the initial viability and seeding densities of NC.

Rat fetuses NC aggregates/spheroids contain stem/progenitor cells that can proliferate and differentiate.

NC aggregates/spheroids are three-dimensional structures, in which the favorable conditions are formed for the survival and adequate functioning of the committed and stem/progenitor NC.

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Отримання і характеристика агрегатів/сфероїдів нейральних клітин плодів щурів

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Мета. Пошук оптимальних умов формування агрегатів/сфероїдів нейральними клітинами (НК), отриманими з тканини головного мозку плодів щурів. Вивчення особливостей поведінки в культурі НК в складі тривимірних агрегатів/сфероїдів. **Методи.** Виділення НК з тканини головного мозку плодів щурів; визначення життєздатності клітин; культивування; імуноцитохімічне фарбування; аналіз проліферативної активності. **Результати.** Встановлено, що умовами формування агрегатів є: 1) присутність в середовищі культивування 10 % сироватки крові дорослих щурів, 2) посівна концентрація у межах 1–4×10^6 клітин/мл та 3) вихідна життєздатність клітин вища за 20 %. **Висновки.** Ізольовані НК плодів щурів при культивуванні в присутності 10 % сироватки і концентраціях вище 1×10^6 клітин/мл спонтанно формують багатоклітинні агрегати; структура агрегатів, що утворюються в процесі короткочасного культивування, залежить від вихідної життєздатності, а розмір — від вихідної життєздатності і концентрації посіяних НК; агрегати/сфероїди НК плодів щурів містять в своєму складі стовбурові/прогеніторні клітини, здатні проліферувати і диференціюватися; агрегати/сфероїди НК є тривимірними структурами, в яких створюються сприятливі умови для виживання і адекватного функціонування комітованих і стовбурових/прогеніторних НК.

Ключові слова: плоди щурів, нейральні клітини, агрегати, сфероїди, культивування.

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