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O.M. Sukach, O.R. Mayorova, O.V. Ochenashko, S.O. Vsevolodska
Institute for Problems of Cryobiology and Cryomedicine, NAS of Ukraine
23, Pereyaslavska Str., Kharkiv, Ukraine, 61016
alexsukach587@gmail.com

ONTOGENETIC STAGE AFFECTS RAT NEURAL CELL SPHEROIDS FORMATION AND PROPERTIES

Aim. *To compare the formation efficiency and morphometric, mechanical, and morphofunctional properties of neural spheroids derived from brain cells of rat embryos (embryonic day 15, E15) and neonatal rats (postnatal day 0, P0).* ***Methods.*** *Neural cells were isolated by mechanical-enzymatic dissociation, and spheroids were formed by the hanging drop method. Spheroid formation efficiency, diameter, sphericity, mechanical stability, and cell behavior after spheroid transfer to an adhesive substrate were evaluated.* ***Results.*** *Embryonic brain-derived cells demonstrated higher spheroid formation efficiency, producing larger, more spherical structures with less size variability and greater mechanical stability than neonatal cells. After attachment, E15 spheroids rapidly disintegrated, accompanied by intense radial cell migration and the formation of a confluent layer exhibiting signs of neuronal and glial differentiation. In contrast, neonatal P0 spheroids maintained a compact 3D structure and formed stable neuronal networks.* ***Conclusions.*** *The ontogenetic stage of neural cells significantly affects spheroid self-organisation, mechanical stability, and morphofunctional properties, highlighting its importance in selecting cell sources for 3D neural tissue models.*

Keywords: *neural cells, spheroids, 3D cultivation, ontogenesis, hanging drop, morphofunctional properties.*

Introduction

Three-dimensional (3D) cell models are increasingly used in biology, neurobiology, regenerative medicine, and pharmacology, as they more adequately reproduce cell-cell interactions, tissue architecture, and microenvironmental features than traditional two-dimensional (2D) cultures [1, 2]. By enabling spatial organisation, dense intercellular contacts, and gradients of metabolites and growth factors, 3D systems more closely mimic *in vivo* conditions [3, 4].

Spheroids formed by cell self-assembly represent one of the simplest and most informative

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forms of 3D cellular organisation. They are widely used to model development, differentiation and cell interactions in various tissues, particularly in the nervous system [5]. For neural cells (NCs), spheroid models are especially valuable because they reproduce the heterogeneous cellular composition of nervous tissue, spatial interactions between neuronal, glial and progenitor populations, and the formation of primitive neuronal networks [6]. However, cell self-organisation is a complex, multifactorial process determined by cell viability, metabolic and mechanical properties of the initial population, and the level of intercellular adhesion [7].

The hanging drop method is one of the most common approaches for obtaining spheroids, as it enables the study of cell self-assembly under controlled conditions without solid matrices or artificial scaffolds [8, 9]. This approach provides opportunities to analyse intercellular interactions, geometric organisation of aggregates, and their mechanical properties. Nevertheless, comparative studies of neural spheroids formed by cells at different stages of brain development remain limited. In particular, the differences in morphometric characteristics, mechanical stability and subsequent cellular behaviour between spheroids derived from embryonic and neonatal brain NCs are insufficiently explored.

An important criterion for the functional integrity of 3D structures is their ability to attach, migrate and differentiate after transfer to an adhesive surface [10], as these processes reflect integrative cellular properties and determine the suitability of spheroids for modelling *in vitro* neurogenesis and applied neurobiological research. These properties may vary depending on the developmental stage of cells, reflecting differences in their proliferative capacity, differentiation status and intercellular interactions. Embryonic day 15 (E15) corresponds to a stage of active neurogenesis with a high proportion of neural progenitors [11], while postnatal day 0 (P0) reflects a more differentiated cellular composition [12]. Such ontogenetic contrast provides a relevant model for analysing how developmental stage influences neural cell self-assembly and spheroid characteristics.

Therefore, this study aimed to investigate neural spheroid formation in hanging drop cultures using brain cells from embryonic day 15 (E15) and postnatal day 0 (P0) rats, and to perform a comparative assessment of self-assembly efficiency, morphometric parameters, mechanical stability, and morphofunctional cellular behaviour following the transition from 3D culture to adhesive growth conditions.

Materials and Methods

Obtaining neural cells

Primary neural cell (NC) suspensions were isolated by mechanical-enzymatic dissociation of whole brains from neonatal rats (P0; n = 6) and embryonic rats (E15). For the embryonic group, whole brains from all embryos of each pregnant dam (n = 6) were pooled to obtain the cell suspension for each individual experiment. Brain tissue was removed, washed with sterile saline, incubated for 2 min at 37 °C in 0.25% trypsin (Biowest, France), transferred to DMEM/F12 medium (Biowest, France) supplemented with 10% rat serum [13] and antibiotics (50 U/mL penicillin and 50 μg/mL streptomycin) and mechanically dissociated into single cells by vibration [14]. The suspension was filtered through a 70 μm cell strainer (nylon mesh; Bioswisstec, Switzerland) and centrifuged for 3 min at 100 g to remove trypsin. The cell pellet was resuspended in DMEM/F12 with 10% rat serum. Cell viability was determined using 0.4% trypan blue (Sigma, USA) and expressed as a percentage. Cell number was counted in a Goryaev chamber.

Cultivation of neural cells

Freshly isolated NCs were seeded in 24-well plates (TPP, Switzerland) at 2×10^6 cells/well in DMEM/F12 medium supplemented with 10% rat serum, 2 mM L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin. No additional

coating with adhesive substrates was applied. The cells were cultured at 37 °C in 5% CO_2 and 95% air. Half of the culture medium was replaced every 3—4 days.

Upon reaching subconfluence, the cells were detached using a 1:4 mixture of 0.25% trypsin (Biowest, France) and versene solution (Simesta, Ukraine). The reaction was stopped by adding a 5-fold volume of DMEM/F12 with 10% serum, followed by centrifugation for 4 min at 100 g. The pellet was resuspended in culture medium, and cell number and viability were assessed. These precultured NCs (passage 0) were used for spheroid formation.

Preparation of NC spheroids

Spheroids were generated using the hanging drop method [15]. Drops (20 µl) of precultured NC suspension containing 4×10^3 cells were placed on the inner surface of 100 mm Petri dish lids (SPL Life Sciences, Korea). The lids were inverted over the dish bottoms containing sterile saline with antibiotics and cultured in a CO_2 incubator at 37 °C in an atmosphere of 5% CO_2 and 95% air for 24—72 hours. The viability of cells within the formed spheroids was assessed using fluorescein diacetate/propidium iodide (FDA/PI) staining [16]. For subsequent analysis, 5 spheroids were randomly selected from each independent experiment (i.e., from spheroids derived from neural cells of all embryos of a single pregnant female or from a single neonatal rat).

Morphometric parameters

Microscopic analysis and microphotography of cell cultures were performed using a laser scanning confocal microscope LSM 510 META (Carl Zeiss, Germany) and an inverted light microscope (AmScope MT3000, USA). For each spheroid, diameter, projection area, and sphericity (circularity index) were measured. Spheroid formation efficiency was calculated as the percentage of drops in which morphologically stable spheroids with clearly defined boundaries were formed.

The circularity index of spheroids was calculated using the formula:

$$\text{Circularity} = 4\pi \times A/P^2$$

where: $\pi \approx 3{,}1416$, A — projection area of spheroid, P — the perimeter of spheroid.

Evaluation criteria: Circularity = 1.0 — perfect circle (sphere); 0.8—0.95 — high sphericity (typical of well-formed spheroids); <0.7 — deformed, asymmetric or loose aggregates.

Mechanical stability of spheroids was evaluated by their ability to maintain integrity after 4 cycles of aspiration and expulsion through the tip of a 200 µL automatic pipette. Spheroids were then classified into three categories: stable — retained a regular spherical shape and clear boundaries; partially deformed — individual cells or small conglomerates detached from the spheroid while the core remained intact; destructured — the spheroid completely disintegrated into single cells or shapeless aggregates.

The degree of spheroid fragmentation after the mechanical stability test was calculated as the difference between 100% and the proportion of intact spheroids after mechanical stress, according to the formula:

Degree of fragmentation (%) = 100% — proportion of intact spheroids (%).

Data analysis

The experiments were performed in at least five independent replicates. Statistical analysis was conducted using Microsoft Excel 2021 and GraphPad Prism 9.0. The data are presented as mean ± standard error of the mean (SEM). Normality was assessed using the Shapiro-Wilk test. As the data showed normal distribution, comparisons between two independent groups (E15 and P0) were performed using Student's t-test. Differences were considered statistically significant at $p < 0.05$.

Bioethical norms

Animal experiments were conducted in accordance with the "General Principles of Animal Experiments" (V National Congress on Bioethics,

Kyiv, 2013), the "IV European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes" (ETS 123, Strasbourg, 1986), and with the approval of the Bioethics Committee of the Institute for Problems of Cryobiology and Cryomedicine of the NAS of Ukraine (Protocol No. 2, 23/02/2021).

Results

In our previous studies [17], freshly isolated NCs demonstrated a limited ability to self-aggregate into spheroids. This limitation may be associated with methodological factors, particularly the cell isolation procedure, which may result in partial

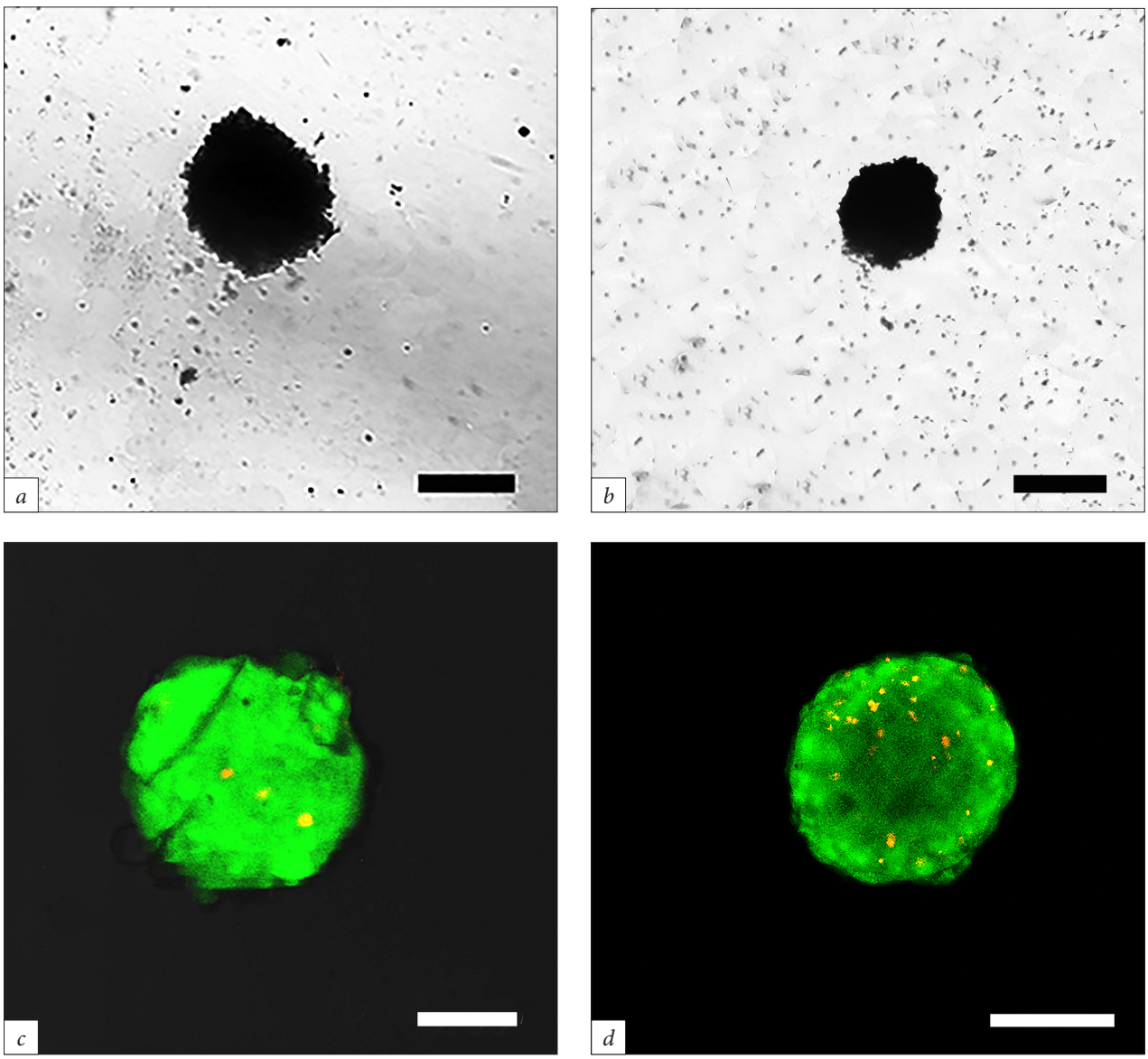

Fig. 1. Spheroids formed after 24 h hanging drop culture from embryonic (E15, *a*, *c*) and neonatal P0 (*b*, *d*) rat brain cells. Cells within spheroids (*c*, *d*) were stained with fluorescein diacetate/propidium iodide. Scale: *a*, *b* — 100 μm, *c*, *d* — 50 μm

loss of adhesion molecules. Precultivation improves the cell suspension quality by reducing the proportion of damaged cells and enriching the population with viable cells (~90% viability). Therefore, in the present study, spheroids were generated using the hanging drop method from precultured NCs. All key stages of 3D self-assembly of precultured NCs occurred within the first 24 hours of culture. If spheroid formation did not initiate during this period, further cultivation in hanging drops did not result in their formation. Cultivation of formed spheroids for 7 days caused no significant changes in morphometric parameters, except for moderate compaction accompanied by a slight reduction in size.

Typical spheroids are shown in Fig. 1, and their morphometric and physico-mechanical characteristics are summarised in Tables 1 and 2. During the first day of cultivation, significant differences were observed between cells of embryonic and neonatal origin. Embryonic NCs formed spheroids with a larger mean diameter (123.5 ± 9.6 μm) compared to neonatal cells (111.0 ± 12.9 μm). The coefficient of variation was lower in the embryonic group (17.4% vs 26.1%), indicating more homogeneous aggregation. The sphericity index was also higher in embryonic spheroids (0.87 ± 0.04) than in neonatal ones (0.75 ± 0.05; $p < 0.05$).

Shape distribution analysis (Table 2) showed that 60% of embryonic spheroids exhibited high sphericity (≥0.80), compared to 40% in the P0 group. No deformed aggregates were detected in the E15 group, whereas 20% of neonatal spheroids had a sphericity (circularity index) <0.70.

Additionally, the spheroids formed by neonatal rat NCs exhibited pronounced geometric deterioration with increasing size: at diameters > 110 μm, the circularity index markedly decreased, often resulting in deformed or loose aggregates. In contrast, the embryonic spheroids maintained a compact, nearly spherical morphology even at larger diameters (up to ~150 μm).

Table 1. **Comparative characteristics of neural spheroids derived from embryonic (E15) and neonatal (P0) rat brain cells (m±SEM)**

Parameter	Embryonic E15 (n = 30)	Neonatal P0 (n = 30)
Mean spheroid diameter, μm	123.50 ± 9.60*	111.00 ± 12.90
Percentage of formed spheroids per drop, %	82.00 ± 4.34**	47.90 ± 6.43
Mean sphericity	0.87 ± 0.04*	0.75 ± 0.05
Percentage of loose structures, %	22.50 ± 3.35**	52.40 ± 4.14
Spheroid resistance to mechanical test, %	90.00 ± 5.16**	51.50 ± 5.86
Degree of spheroid fragmentation, %	10.00 ± 5.16**	48.50 ± 5.86
Coefficient of variation (CV) of spheroid diameter, %	17.40	26.10

Notes: E15 — embryonic day 15; P0 — postnatal day 0; differences are significant compared to neonatal rat neural cells: * — $p < 0.05$; ** — $p < 0.001$.

Table 2. **Distribution of circularity index-based shape categories of spheroids derived from embryonic (E15) and neonatal (P0) rats after 24 h of culture**

Form category (sphericity)	Embryonic E15 (n = 30), %	Neonatal P0 (n = 30), %
High (≥0.80)	60	40
Moderate (0.70—0.79)	40	40
Deformed (<0.70)	0	20

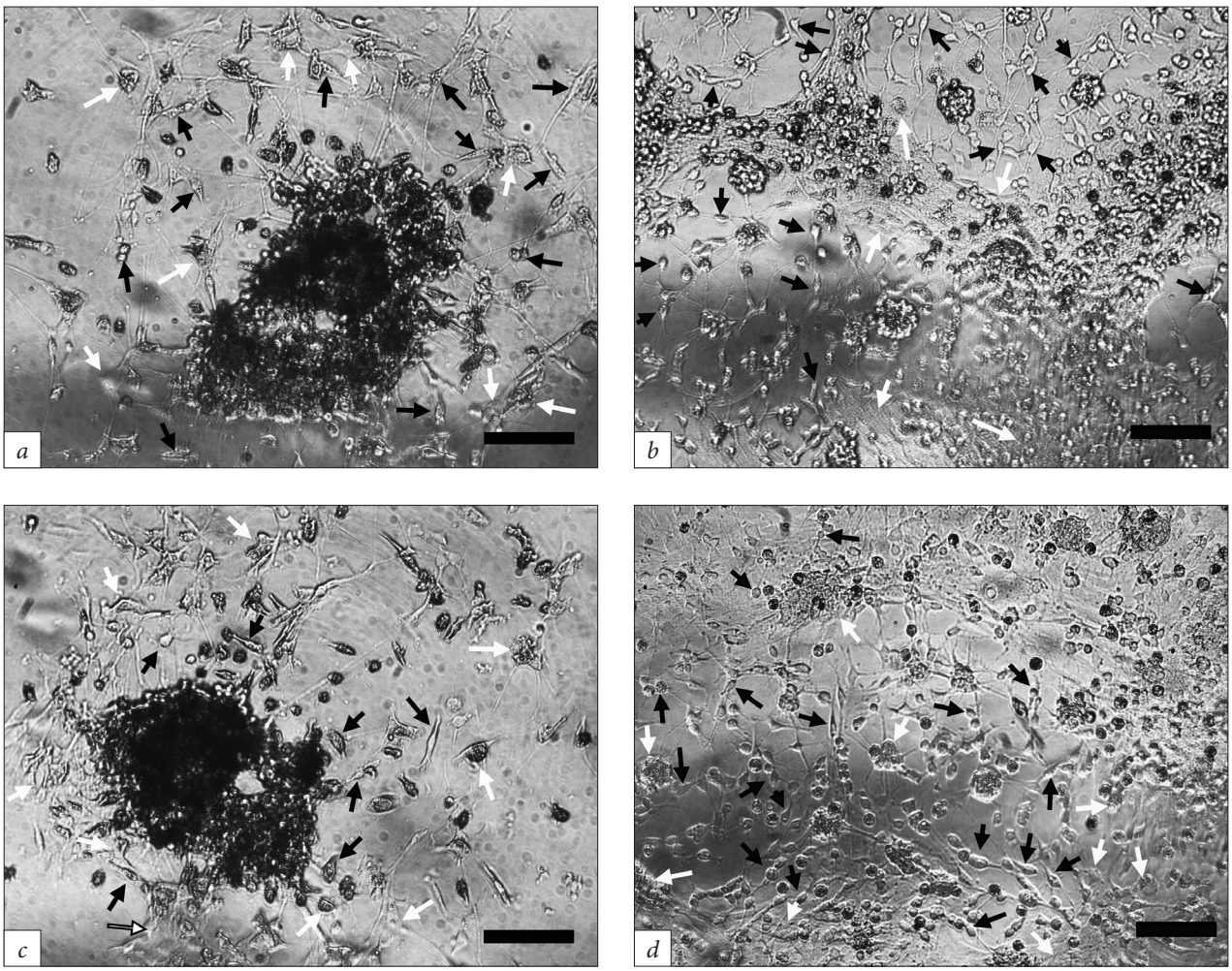

Fig. 2. Migration and differentiation dynamics of spheroid-derived cells after transfer to an adhesive surface. Spheroids formed by neural cells from E15 rat embryos on days 2 (*a*) and 7 (*b*) of culture. Spheroids formed by neural cells from P0 neonatal rats on days 2 (*c*) and 7 (*d*) of culture. Black arrows indicate neuroblast-like and neuron-like cells; white arrows indicate glia-like cells. Scale: 50 μm

Mechanical stability assessment (Table 1) supported these findings. The proportion of intact spheroids after mechanical loading was significantly higher in the E15 group (90.0 ± 5.16%) than in the P0 group (51.5 ± 5.86%; $p < 0.001$). Accordingly, fragmentation was nearly fivefold higher in neonatal spheroids (48.5% vs 10.0%; $p < 0.001$), indicating weaker intercellular adhesion and reduced mechanical resistance. Overall, embryonic NCs not only formed spheroids with better geometry but also provided greater structural integrity and resistance to mechanical stress.

To evaluate functional viability, the mechanically stable spheroids were transferred to adhesive 24-well plates and cultured for 7 days. On day 1, both embryonic and neonatal spheroids exhibited 100% attachment**.**

By day 2, the embryonic spheroids demonstrated active radial cell migration and spreading, forming a wide migration zone (Fig. 2a). Numerous

elongated spindle-shaped cells with thin cytoplasmic processes migrated outward from the aggregate. In parallel, flattened polygonal cells with larger spreading areas were observed, likely corresponding to immature glial elements. Migrating cells established early intercellular contacts through extending processes, forming an initial cellular network around the spheroid. The spheroid core remained compact but showed signs of partial loosening at the periphery.

By day 7, the embryonic spheroids had completely dispersed due to extensive outward migration, resulting in a nearly confluent monolayer on the culture surface (Fig. 2*b*). The culture exhibited marked cellular heterogeneity and a highly developed network architecture. A large number of elongated neuroblast-like and neuron-like cells with long, thin neurite-like processes formed dense interconnected structures resembling the neuropil. Stellate and flattened polygonal cells, morphologically consistent with astroglial or radial glial-like elements, were widely distributed among the neuronal cells and likely contributed to substrate organization and cell adhesion. The culture exhibited high cell density and significant overlap of migration zones, indicating strong proliferative and migratory activity of embryonic neural stem/progenitor and differentiated cells.

The neonatal spheroids at day 2 also showed active radial migration and substrate colonization (Fig. 2*c*). Migrating cells were predominantly bipolar or spindle-shaped with clearly distinguishable contours and short cytoplasmic protrusions. Compared with embryonic cultures, the migration zone appeared more compact and spatially ordered. Individual cells migrated radially around the spheroid, while only early stages of network formation were observed. The spheroid core retained a dense rounded morphology with moderate peripheral dispersal.

By day 7, the neonatal spheroids likewise underwent almost complete dispersal with the formation of a broad, nearly confluent cellular monolayer (Fig. 2*d*). Numerous small elongated cells with long branching processes formed an extensive interconnected cellular network throughout the culture area. Flattened polygonal cells were interspersed among neuron-like cells, likely representing astroglial elements contributing to substrate support and intercellular interactions. Compared with day 2, the density of cellular processes and degree of intercellular connectivity markedly increased, indicating progressive maturation of the culture.

Discussion

The results indicate that neural cells from rat embryos (E15) exhibit a significantly higher potential for spheroid formation compared to neonatal cells. This is reflected in a greater proportion of formed spheroids, larger average diameters, higher sphericity, lower size variability, and markedly improved mechanical stability. These differences align with the notion that embryonic neural cells possess greater plasticity, proliferative capacity, and self-organisation potential than postnatal brain cells, which are at more advanced stages of differentiation [18, 19]. The data also show that all key stages of spheroid self-assembly occur within the first 24 hours of culture, and prolonged residence in hanging drops does not lead to the formation of additional aggregates. This observation is consistent with the literature reports indicating that the early phases of 3D aggregation are governed by rapid activation of intercellular adhesion, actin cytoskeleton reorganisation, and the balance of mechanical stresses within the cell aggregate [20, 21]. The recent reviews on 3D neural models further emphasise that the first 24—48 hours of culture are critical for establishing stable spheroid architecture and enabling subsequent functional differentiation [22].

Higher sphericity values and lower diameter coefficient of variation in embryonic spheroids indicate a more homogeneous and predictable pattern of self-organisation. As reported in studies on neural spheroids and brain organoids, the geometric regularity of aggregates is closely associated with the consistent cell proliferation, uniform distribution of cell types, and stable intercellular

contacts [23]. In contrast, the decline in sphericity observed in neonatal spheroids with increasing diameter may reflect the limited proliferative potential and greater heterogeneity of the cell population, which are characteristic of more mature neural cells [24].

The mechanical testing results confirmed the morphometric observations: embryonic spheroids exhibited significantly higher resistance to mechanical stress and a markedly lower level of fragmentation. The mechanical stability of 3D aggregates is known to depend on the density of cell-cell contacts, the expression of adhesion molecules, and the production of extracellular matrix, which further reinforces spheroid structure [19]. These mechanisms likely underlie the observed differences between embryonic and neonatal spheroids. These differences may also be explained by underlying molecular mechanisms regulating cell-cell interactions. In particular, enhanced expression of adhesion molecules (e.g., N-cadherin, NCAM) in embryonic cells could promote stronger intercellular cohesion and higher mechanical stability, consistent with the established role of cadherin-mediated adhesion in neural tissue organisation [19, 20, 25]. In parallel, the increased cytoskeletal plasticity and active actin remodelling may facilitate efficient aggregation and spheroid integrity [20, 21]. Differences in the formation of intercellular junctions and integrin-mediated interactions with extracellular matrix components may further influence spheroid architecture and post-adhesive behaviour, as reported for 3D neural models [21—23, 26]. It should be noted that these mechanisms were not directly investigated in this study and remain hypothetical. Therefore, the targeted analysis of adhesion molecules, cytoskeletal organisation, and associated signalling pathways represents an important direction for future research.

The functional viability of mechanically stable spheroids from both groups was confirmed by 100% attachment to the adhesive surface; however, their subsequent spatio-temporal behaviour and morphological organization differed depending on their origin.

By day 2, the embryonic spheroids exhibited rapid attachment and robust radial migration, forming an extensive migration zone (Fig. 2*a*). This process culminated by day 7 in the nearly complete dispersal of the 3D aggregate and the formation of a dense, highly heterogeneous monolayer (Fig. 2*b*). This behaviour aligns with the current models of neurospheres and organoids, in which embryonic NCs exhibit a high capacity to transition from 3D organisation to active migration and differentiation under adhesive growth conditions [23]. The presence of long, neurite-like processes forming neuropile-like structures, alongside large flattened glia-like elements, suggests advanced structural maturation of the embryonic culture.

In contrast, the neonatal spheroids initially demonstrated a more compact and spatially ordered radial migration (Day 2, Fig. 2*c*). While they also underwent nearly complete dispersal by day 7, forming a confluent monolayer (Fig. 2*d*), their organization remained more uniform compared to the embryonic group. Neonatal cultures were characterized by a denser population of smaller neuron-like cells and a more regular arrangement of cellular elements, which is consistent with the formation of stable neuronal networks in postnatal 3D cultures [24]. This suggests that while both cell types effectively transition to 2D growth, the neonatal cells maintain a more organized architecture, potentially supporting more structured synaptic connectivity.

It should be noted that the identification of cell types in this study is based on morphological criteria and should be considered preliminary. Further studies will include detailed phenotyping using specific molecular markers

Conclusion

The results indicate that the ontogenetic stage of NCs is a key determinant of their self-organisation efficiency under three-dimensional culture conditions. It influences the morphofunctional and mechanical properties of the resulting spheroids, as well as the patterns of cell migration

and differentiation following transition to adhesive growth. The embryonic brain NCs exhibit higher plasticity and self-organisation potential, whereas the neonatal brain NCs tend to form more stable and structurally ordered neuronal networks. These differences highlight the importance of considering the ontogenetic origin of cellular material when selecting a cell source. Such consideration is critical for developing physiologically relevant 3D models of neural tissue tailored to specific experimental goals, including studies of neurogenesis, cell migration and differentiation, neuroregeneration, or pharmacological screening.

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О.М. Сукач, О.Р. Майорова, О.В. Оченашко, С.О. Всеволодська
Інститут проблем кріобіології і кріомедицини НАН України
вул. Переяславська, 23, Харків, Україна, 61016
alexsukach587@gmail.com

ВПЛИВ СТАДІЇ ОНТОГЕНЕТИЧНОГО РОЗВИТКУ
НА ФОРМУВАННЯ ТА ВЛАСТИВОСТІ СФЕРОЇДІВ НЕРВОВИХ КЛІТИН ЩУРІВ

Мета. Порівняти ефективність формування та морфометричні, механічні й морфофункціональні властивості нейральних сфероїдів, отриманих із клітин головного мозку ембріонів щурів 15-ї доби гестації (E15) та неонатальних щурів (P0). ***Методи.*** Нейральні клітини ізолювали методом механіко-ферментативної дисоціації та формували сфероїди методом висячої краплі. Оцінювали ефективність сфероїдоутворення, діаметр, сферичність, механічну стійкість і поведінку клітин після перенесення сфероїдів на адгезивний субстрат. ***Результати.*** Клітини ембріонального мозку демонстрували вищу ефективність сфероїдоутворення та формували сфероїди з більшим діаметром, вищою сферичністю, меншою варіабельністю розмірів і кращою механічною стійкістю порівняно з неонатальними. Після прикріплення сфероїди E15 швидко дезінтегрували з інтенсивною радіальною міграцією клітин і формуванням конфлюентного шару з ознаками нейрональної та гліальної диференціації, тоді як неонатальні сфероїди зберігали компактну 3D-структуру та формували стабільні нейрональні мережі. ***Висновки.*** Онтогенетична стадія нейральних клітин суттєво впливає на самоорганізацію, механічну стабільність і морфофункціональну поведінку нейральних сфероїдів, що є критичним для вибору клітинного джерела у 3D-моделях нервової тканини.

***Ключові слова*:** нейральні клітини, сфероїди, 3D-культивування, онтогенез, висяча крапля, морфофункціональні властивості.