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Effect of various origins conditioned media on the migration of neural cells in vitro

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An important direction in the development of the latest technologies for the restoration of damaged central nervous system is the use of stem/progenitor cells (SPCs), mainly neurogenic SPCs (NSPCs) and mesenchymal multipotent stromal cells (MMSCs). One of the main mechanisms of SPCs action is indirect paracrine effects due to the ability to produce a wide range of biologically active signaling molecules (secretome). The study of regenerative effects of conditioned media (CM) of NSPCs and MMSCs as a source of their secretome seems to be actual and potentially beneficial. The aim of the study is to compare the impact of CM from 24-h cultures of fetal neurogenic cells (NCs (E14), as a source of NSPCs) and adipose-derived mononuclear cells (AMCs as a source of MMSCs) on migration capacity of rat neural cells in vitro. AMCs-CM were obtained from 24-h cultures with prevalence of $CD105^+$ cells and ability upon further cultivation to form "spheroids" and potency to differentiate into different cell types. NCs-CM were obtained from 24-h cultures with prevalence of $Nestin^+$ cells and ability upon further cultivation to form "neurospheres" and potency to differentiate into astrocytes ($GFAP^+$) and neurons (β-Tubulin III^+). Rat fetal neural cells (E14) were cultured to achieve a confluent monolayer with basic cellular elements of nervous tissue (5-7th day), which was dissected with forming a transection site and DMEM with 10% fetal calf serum (control) or 0.1-0.3 mg/ml (by total protein amount) of NCs-CM or AMCs-CM were added. In control cultures of rat neural cells partial overgrowth of the dissected area of the monolayer was observed due to the migration of cells, formation of a network of processes and intercellular contacts; reaching 13.2% (4th day) – 23.2% (8th day) of its full length. The overgrown area increased after addition of CM: NCs-CM – 3 times (0.1-0.2 mg/ml) and 3-4 times (0.3 mg/ml, 4th-8th day), reaching 70.5% of full length of the transection site; AMCs-CM – 1.5 times (0.1-0.2 mg/ml) and 4-7 times (0.3 mg/ml, 4th-8th day), reaching 97.4-100% of full length of the transection site. The addition of NCs CM and AMCs CM resulted in β-catenin translocation into nucleus of cells in rat neural cell cultures, which correlated with the overgrowth of the transection zone. NCs-CM as well as AMCs-CM in dose-dependent manner stimulate migration processes in culture of rat neural cells, obviously, involving β-catenin signaling pathway, contributing to overgrowing of the dissected area (reparation of a mechanical defect). NCs-CM and AMCs-CM are a source of signaling molecules that modulate the microenvironment and activate endogenous repair mechanisms in culture (in vitro model of nerve tissue regeneration).

Key words: fetal neurogenic cells; adipose-derived mononuclear cells; conditioned media; secretome; cell migration; differentiation.

INTRODUCTION

An important direction in the development of the latest technologies for the restoration of damaged central nervous system (CNS) is the use of stem/progenitor cells (SPCs). Given the limited possibilities of endogenous neurogenesis due to neurogenic SPCs (NSPCs), located in the corresponding "niches" of the CNS of adult mammals [1] and providing natural compensation for nerve tissue cells that are lost in the life course or in pathological conditions [2], the ways to enhance endogenous neurogenesis through the appointment of exogenous trophic factors or transplantation of different types of SPCs as potentially effective therapeutic agents

are considered. To date, more than 460 clinical randomized controlled trials on the safety and efficacy of SPCs application in CNS diseases have been registered [3], of which 41 involve the use of NSPCs and 170 – mesenchymal multipotent stromal cells (MMSCs). Among available sources for MMSCs (bone marrow, adipose tissue (AT), umbilical cord, cord blood), AT is considered a rich source of MMSCs [4, 5].

For the efficient use of SPCs transplantation, it is necessary to understand the mechanisms of realization of their potential. It is believed that NSPCs may exhibit therapeutic effects such as cell replacement, neurotrophic support (neuroprotection) or immunomodulation [6, 7]. Cell replacement can occur due to proliferation, migration, differentiation of transplanted cells, their functional integration and reconstruction of neural connections in the recipient tissue, but only very small amount of transplanted NSPCs survive and differentiate into mature cells of nervous tissue [8, 9]. The neuroprotective effect is provided by the ability of transplanted NSPCs to significantly enhance the survival and functional activity of endogenous glial and neuronal SPCs after CNS damage, due to tropism and migration to the sites of the lesion and release into the microenvironment of molecules that prevent death and enhance the regeneration of target cell populations (NGF, BDNF, CNTF, GDNF). Immunomodulation occurs due to the impact of transplanted NSPCs through paracrine signaling mechanisms on the effector functions of the immune system components. Improvement of neurological functions after MMSCs transplantation in CNS lesions is also explained mainly by immunomodulatory and trophic effects (because only 0.01-0.001% of transplanted MMSCs are integrated into the ischemic cortex [10, 11]); the therapeutic impact of MMSC is directed at providing neuroprotection, neuroregeneration, stimulation of angiogenesis, prevention of apoptosis, activation of NSPCs migration, proliferation and differentiation, neurite outgrowth, synaptogenesis, restoration of the blood-brain barrier [12, 13].

Obviously, one of the main mechanisms of transplanted cells action is indirect paracrine effects due to the ability to produce a wide range of biologically active signaling molecules (secretome). It was demonstrated that NSPCs [14-16] as well as MMSCs, particularly AT-MMSCs [17-20], are able to produce cytokines, growth and neurotrophic factors, miRNA, etc, which play pleiotropic roles in key cell signaling processes.

At the same time, despite the dominant perception of low immunogenicity ("immune privilege") of SPCs, in particular NSPCs and MMSCs (low expression levels of major histocompatibility complex (MHC) class I (MHC-I) proteins, lacking MHC-II and costimulatory molecules expression) [21, 22], there is data evidencing dramatic increase in the MHC-I expression level in these cells in response to inflammatory stimuli (IFN-γ, TNF-α, IL-1β) in vitro and activation of native and adaptive immune response upon allogeneic transplantation in vivo [23-27]. That's why the entry of MHC-mismatched allogeneic SPCs into the lesion focus may contribute to the induction of MHC-I, -II expression by transplanted cells due to the inflammatory stimuli, and as a result – to recognition by immune cells, progressive immune cytolysis, rejection and development of immunocompromised complications, which in turn will dictate the need for immunosuppressive medication. It is considered that repeated clinical use of MMSCs should be limited to autologous or cross-matched allogeneic MMSCs, and upon single application of non-cross-matched allogeneic MMSCs pre-sensitization against donor MHC should be assessed [26]. Therefore, given the known paracrine effects of SPCs and their key role in mechanisms of regenerative impact, the use of their conditioned media (CM) as a source of secretome and a key component of the microenvironment ("niche") of these cells may be an attractive alternative to direct cell transplantation.

The development of this direction is at an early stage: to date only 2 clinical trials on the

early phase were announced (current status: recruiting and not yet recruiting) for using the CM from umbilical cord MMSCs in the treatment of cerebral palsy and acute ischemic stroke (Indonesian National Brain Center and Gatot Soebroto Hospital, Jakarta, Indonesia) [3]. That's why the study of regenerative effects of CM from SPCs, as a source of their secretome, particularly CM from NSPCs and MMSCs, seems to be actual and potentially beneficial.

The purpose of the study was to compare the impact of conditioned media from cultures of fetal neurogenic cells (NCs, as a source of NSPCs) and adipose-derived mononuclear cells (AMCs, as a source of MMSCs) on the migration capacity of rat neural cells in vitro.

METHODS

All procedures with experimental animals used in the work (outbred sexually mature Wistar rats, females (n = 14, aged 3 months, weight 230 ± 20 g), breeding vivarium of the State Institution "Romodanov Neurosurgery Institute, National Academy of Medical Sciences of Ukraine" (SI "INS NAMS") were carried out in compliance with legal norms and requirements of the Law of Ukraine N3447 IV "On protection of animals from cruel treatment", "European Convention for the protection of vertebrate animals used for research and other scientific purposes" (Strasbourg, 1986), principles of bioethics and biosafety standards. Animals were kept in standard conditions of the accredited vivarium, anesthesia and euthanasia were performed under ether anesthesia. The study was approved by the Ethics and Bioethics Commission of the SI "INS NAMS" (Minutes No. 26 of May 11, 2018).

Isolation of AMCs and obtaining the AMCs-CM. AT was removed from the groin area of rats (n = 14) anesthetized by diethyl ether ("Sigma", Germany), washed several times in fresh portions of phosphate buffer saline (PBS) without Ca^{2+} and Mg^{2+} ("Sigma", Germany), crushed to fragments of 1-2 mm (total volume 1-2 cm^3), incubated in 0.075% solution of collagenase (type I, "Sigma", Germany) in PBS ("Sigma", Germany) 60 min at 37°C with stirring. The enzyme was inactivated by adding (1:1) DMEM ("Sigma", Germany) with 10% fetal calf serum (FCS, "BioWest", France), the cells were pelleted by centrifugation for 10 min at 1000 rpm. The liquid phase containing the mature adipocytes and debris was removed with a Pasteur pipette ("Greiner Bio-One GMBH", Germany), the pellet containing the stromal-vascular fraction of cells was resuspended in DMEM ("Sigma", Germany) with 10% FCS ("BioWest", France), filtered through a nylon filter, mononuclear cells were isolated by centrifugation for 30 min at 1000 rpm in a density gradient of Histopaque ("Sigma", Germany). The resulting cell suspension was washed twice in DMEM ("Sigma", Germany) for 10 min at 1000 rpm. The number and viability of the obtained cells were determined in a standard cytological test with 0.2% aqueous solution of trypan blue ("Merch", Germany). The volume of the obtained AMCs suspensions at a concentration of $6.0 \cdot 10^6$ cells was adjusted to 6 ml by adding serum-free DMEM ("Sigma", Germany) with concanavalin A (0.10 mg/ml, "Sigma", Germany), the cells were kept for 2 h in a CO_2 incubator ("Nuve", Turkey). Next, the cells were pelleted by centrifugation for 5 min at 1500 rpm, resuspended in DMEM ("Sigma", Germany), and seeded $2 \cdot 10^6$ on the bottom of plastic Petri dishes (d = 35 mm, "Greenpia Technology", Korea). Cultures were kept in a CO_2 incubator (95% humidity, 37°C, 5% CO_2) for 24 h, then AMCs-CM were collected with a Pasteur pipette ("Greiner Bio-One GMBH", Germany), cell debris were removed by centrifugation for 5 min at 1500 rpm and finally, supernatant was filtered through a filter with a pore diameter 0.2 mm ("TRP", Switzerland). Cell cultures were fixed in 10% neutral formalin ("Bio-Optica", Italy) and stained on CD105 (endoglin, membrane glycoprotein type I, MMSCs marker) using murine monoclonal antibody ENG (CD105) ("OriGene", USA, 1:200).

Formation of 3D spheroids from AMCs. AMCs were cultured in serum-free DMEM ("Sigma", Germany) supplemented with EGF (20 ng/ml, "Sigma", Germany) and FGF (10 ng/ml, "Sigma", Germany) to study the ability to form "spheroids" as well as in adhesive plastic Petri dishes (d = 35 mm, "Greenpia Technology", Korea) in DMEM supplemented with 20% FCS ("BioWest", France), 8 mg/ml glucose, 100 IU/ml penicillin, 100 mg/ml streptomycin (all – "Sigma-Aldrich", USA) to study the differentiation capacity. The nutrient medium was replaced in 3 days. Cultures were kept in a CO_2 incubator (95% humidity, 37°C, 5% CO_2). Lifetime observation of the culture growth dynamics was performed using an inverted microscope TS100 ("Nikon", Japan).

Isolation of NCs and obtaining the NCs-CM. The cerebral cortex and hippocampal area removed from fetuses (E14, n = 128) of female rats (n = 14) anesthetized by diethyl ether ("Sigma", Germany), were washed in DMEM ("Sigma", Germany), transferred to fresh DMEM, and mechanically dissociated by repeated pipetting. The resulting cell suspensions were precipitated by centrifugation for 5 min at 1500 rpm, washed in DMEM ("Sigma", Germany), fresh DMEM was added to the cell pellet and resuspended. The number and viability of the obtained cells were determined in the test with 0.2% aqueous solution of trypan blue ("Merch", Germany). The volume of the obtained NCs suspensions at a concentration of $6 \cdot 10^6$ cells was adjusted to 6 ml by adding serum-free DMEM ("Sigma", Germany) with concanavalin A (0.10 mg/ml, ("Sigma", Germany), the cells were incubated for 2 h in a CO_2 incubator ("Nuve", Turkey). Next, the cells were pelleted by centrifugation for 5 min at 1500 rpm, resuspended in DMEM, and seeded $2 \cdot 10^6$ on the bottom of plastic Petri dishes (d = 35 mm, "Greenpia Technology", Korea). Cultures were kept in a CO_2 incubator (95% humidity, 37°C, 5% CO_2). After 24 h of incubation, the NCs-CM were collected with a Pasteur pipette ("Greiner Bio-One GMBH", Germany), cell debris were removed by centrifugation for 5 min at 1500 rpm and finally, supernatant was filtered through a filter with a pore diameter 0.2 mm ("TRP", Switzerland). Cell cultures were fixed in 10% neutral formalin ("Bio-Optica", Italy) and stained on Nestin (intermediate filament protein, NSPCs marker) using rabbit affinity antibodies Anti-Nestin ("Sigma-Aldrich", USA, 1:100).

In CM obtained from 24-h cultures of AMCs and NCs the total protein concentration was determined by Lowry and by photometric test according to Bradford method using automated bioanalyzer Respons^{920} DiaSys ("Diagnostic Systems International", Germany). CM were concentrated at 30°C using concentrator Eppendorf (Germany) to final concentration of 5.0 mg/ml, aliquoted and stored at –20°C.

Formation of 3D spheroids from NCs. NCs were cultured in serum-free DMEM ("Sigma", Germany) supplemented with EGF (20 ng/ml, ("Sigma", Germany) and FGF (10 ng/ml, ("Sigma", Germany) to study the ability to form "neurospheres" as well as in DMEM supplemented with 20% FCS ("BioWest", France), 8 mg/ml glucose, 100 IU/ml penicillin, 100 mg/ml streptomycin (all – "Sigma-Aldrich", USA) to study the differentiation capacity. The nutrient medium was replaced in 3 days. Cultures were kept in a CO_2 incubator (95% humidity, 37°C, 5% CO_2). Lifetime observation of the culture growth dynamics was performed using an inverted microscope TS100 ("Nikon", Japan).

Scratch assay. After reaching by NCs cultures the confluent layer with main cellular types of nervous tissue a mechanical model of neurotrauma (transection of the cell growth zone with a metal blade) was performed. Cells detached from the surface were removed by washing with DMEM ("Sigma", Germany). Immediately after transection of the growth zone the DMEM ("Sigma", Germany) with 10% FCS ("BioWest", France) – standard culture conditions (control), or the AMCs-CM or NCs-CM in concentrations 0.1, 0.2 or 0.3 mg/ml (according to the total protein amount) were

added. Cultures were kept in a CO_2 incubator (95% humidity, 37°C, 5% CO_2), the DMEM with 10% FCS was replaced in 3 days.

Neural cell staining. On the 4th and 8th day after transection the cultures were fixed in 10% neutral formalin and stained with hematoxylin-eosin (USA) according to Karachi, thionine ("Janssen Chimica", Belgium) according to Nissl. Immunocytochemical staining was performed using rabbit affinity antibodies Anti-Nestin ("Sigma-Aldrich", USA, 1:100), mouse monoclonal antibody Anti-Tubulin III (neuronal) ("Sigma-Aldrich", USA, 1:1000), rabbit polyclonal antibodies Anti-GFAP ("Millipore", USA, 1:1000), Anti-Ki-67 ("Millipore", USA, 1:300) and Beta-catenin ("Thermo Scientific", USA, 1:100). Visualization of the immunocytochemical reaction was performed using the "Immunoperoxidase secondary Detection System" kit ("Millipore", USA).

Microscopic examination and photo registration of cytological preparations of cultures were performed on a light optical microscope NIKON Eclipse E200 (Japan). Quantitative studies were performed in 10 representative fields of view with a standard measuring scale (object - micrometer) using ImageView software (2020, China). The number of migrated cells and immunopositive cells were determined; the length of the overgrown transection area was calculated by summing up the size of distinct overgrown regions throughout its length.

Statistical analysis. Obtained data were analyzed using the software package Statistica 8.0 (StatSoft, Inc. 2007). The non-parametric methods of variation statistics (rank discriminant analysis Kruskal-Wallis ANOVA test for multiple comparison and Mann-Whitney U-test for pairwise comparisons of independent groups, Wilcoxon test for pairwise comparison of dependent groups (in the dynamics of observation), Spearman Rank Order Correlations analysis) were used. The normality of data distribution was determined by the Shapiro-Wilk test. Data are presented as M ± m, where M – the average value; m – the standard deviation from the mean value. Differences were considered statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

Characterization of AMCs. After 24-h of AMCs cultivation two types of $CD105^+$ cells (immunopositive for endoglin, one of the MMSCs markers) were identified – small rounded cells with a narrow cytoplasm (average cell diameter 6.1 ± 0.5 μm, average nucleus diameter 3.6 ± 0.1 μm, Fig. 1A) and larger in size rounded cells with a broad cytoplasm (average cell diameter 21.3 ± 3.9 μm, average nucleus diameter 6.7 ± 0.8 μm, Fig. 1B). The average number of $CD105^+$ cells in 24-h AMCs cultures was 82.8 ± 0.7%. Upon the further cultivation in serum-free DMEM supplemented with EGF and FGF the AMCs formed cellular clusters of round form ("spheroids", Fig. 1C). The formation of spheroidal derivations in culture growth dynamics is believed to be the fundamental property of SPCs, in particular, MMSCs. After attachment of spheroidal cell aggregates to the surface and stratification, the cells migrated from them and several long processes protruded out from the cell bodies and a growth zone was formed.

During cultivation on an adhesive surface in DMEM supplemented with 20% FCS, 8 mg/ml glucose, AMCs formed monolayer of cells with morphological characteristics similar to fibroblasts. From the 5th day the spontaneous differentiation of fibroblast-like cells occurred with the acquisition of cells of several phenotypes: 1) cells of a star shape and long branched processes forming a network (Fig. 1D); 2) monolayer growths of cells of spindle-like and polygonal shape with round nucleus and numerous liposomal granules in the bulk cytoplasm (Fig. 1E) – obviously adipocytes (in addition, round drops of fat were observed, released by cells into the environment); 3) cells of polygonal shape and thick shoots which were connected with each other (Fig. 1F). Hence upon cultivation in DMEM supplemented with 20 %

FCS, 8 mg/ml glucose, AMCs differentiated into at least three cell types, which evidence their multipotency.

Thus, characteristics of the AMCs in 24-h cultures from which the CM were prepared (prevalence of $CD105^+$ cells, the ability to form "spheroids", the potency upon cultivation in adhesive surface to differentiate into at least three cell types), i.e. morphological, adhesive properties, immunophenotype and differentiating potential confirm that obtained AMCs contain the predominant ATSCs.

Characterization of NCs. After 24-h of NCs (E14) cultivation, the number of $Nestin^+$ cells (immunopositive for type VI intermediate filament protein, one of the NSPCs markers) were 66.9 ± 0.9%, the part of them formed spheroidal multi-cellular complexes (so-called "neurospheres", Fig. 2A). Such complexes were observed also upon NCs cultivation in serum-free DMEM supplemented with EGF and FGF. This is a characteristic feature of NSPCs [28], and is believed to be the fundamental property of SPCs.

Upon further NCs cultivation on an adhesive surface with polyethyleneimine in DMEM supplemented with 10% FCS, on the 5th-7th day the confluent monolayer of cells of astrocytic phenotype was formed creating the feeder layer for the survival of bi- and multipolar neurocytes with elongated processes (Fig. 2B, C). Immunocytochemical staining confirmed the affiliation of the cells in cultures to main cell phenotypes of nervous tissue – neurons (immunopositive for β-tubulin III, microtubule element of the tubulin family, a neuronal marker, Fig. 2D) and $GFAP^+$ astrocytes, star-shaped cells with numerous long processes (immunopositive for glial fibrillar acidic protein (GFAP), type III intermediate filament protein, Fig. 2E). At the

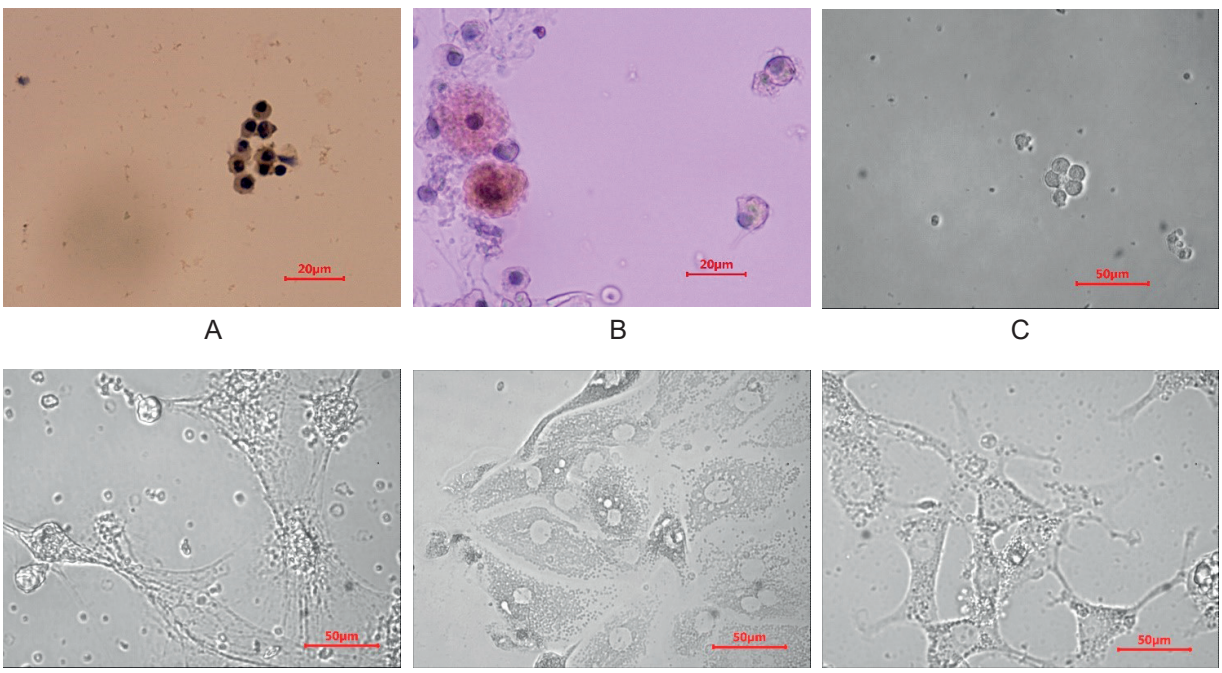

Fig. 1. Microphotographs of AMCs cultures cytopreparations. Light microscopy: A, B – immunocytochemical staining for CD105 (endoglin, MMSCs marker); C-F – unstained culture. A, B – 24 h of cultivation, $CD105^+$ cells of II types: small rounded cells with a narrow cytoplasm (A) and larger rounded cells with a broad cytoplasm (B); C – 24 h of cultivation, forming of initial spheroid cellular cluster; D – 5th day of cultivation, star-shaped cells with long branched processes; E – 13th day of cultivation, monolayer growths of cells of spindle-like and polygonal shape with round nucleus and numerous liposomal granules in the bulk cytoplasm; F – 8th day of cultivation, cells of polygonal shape and thick shoots

same time, cells in culture retain their mitotic activity – in the field of view 1-2 cells were in a state of mitosis and immunopositive for Ki-67 (nuclear protein, marker of proliferation, Fig. 2F).

Thus, characteristics of the NCs in 24-h cultures from which the CM were obtained (prevalence of Nestin$^+$ cells, the ability to form "spheroids", the potency upon cultivation to differentiate into neurons and astrocytes), confirm that obtained NCs contain the predominant NSPCs fraction.

Impact of NCs-CM and AMCs-CM on fetal neural cells in the mechanical model of neurotrauma. On the 5-7th day of rat neural cells cultivation after the formation of confluent monolayer with the histotypic signs of neuroglial growth and basic cellular elements of nervous tissue (Fig. 2B-E), in particular β-tubulin III$^+$ neurons and GFAP$^+$ astrocytes, the growth zone was dissected, a region of transection was created with an average width of ~0.45 mm, length 13 mm. Next, after adding DMEM with 10 % FCS (control cultures) or obtained CM (0.1 mg/ml), the signs of endogenous regeneration processes were observed in both control and experimental cultures, more intensive and numerous after NCs-CM or AMCs-CM influence: germination of long branched processes of cells from the edges of the dissected monolayer in the transection site along its entire length and perpendicularly in the direction of the opposite edge, eviction of individual cells and cell complexes, migration of cells to the dissection site, germinating of cell processes and forming a network, contributing to overgrowth of distinct regions of transection area.

In control cultures after mechanical transection on 4th day of cultivation the average length of the overgrown area of dissection was

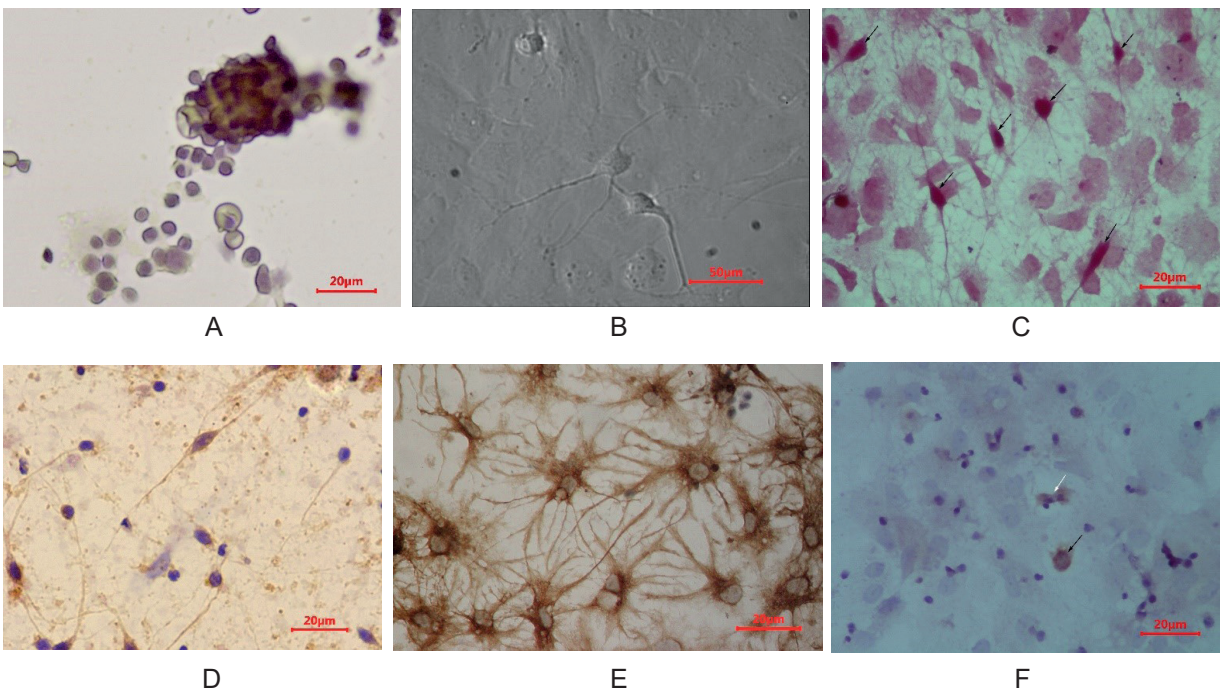

Fig. 2. Microphotographs of NCs (E14) cultures cytopreparations. Light microscopy: A – 24 h of cultivation, multi-cellular "neurospheres" with Nestin$^+$ cells (immunocytochemical staining, hematoxylin co-staining); B, C – 8th day of cultivation, confluent monolayer of glial cells, and bi- and multipolar neurocytes with elongated processes (arrows) located on the top, unstained culture (B) and hematoxylin staining (C); D – 7th day of cultivation, β-tubulin III$^+$ neurons (immunocytochemical staining); E – 7th day of cultivation, star-shaped astrocytes with GFAP$^+$ filaments in cytoplasm and processes (immunocytochemical staining) and rounded nucleus (hematoxylin co-staining); F – 7th day of cultivation, Ki67$^+$ cells (arrows, immunocytochemical staining) among cell monolayer (hematoxylin co-staining), one of them – on the terminal stage of mitosis (white arrow)

1.72 ± 0.19 mm, i.e. 13.2% of its full length (Fig. 3A, D). To the 8th day the average length of the overgrown area enlarged 1.75 times (P = 0.89, Mann-Whitney U-test), reaching 23.2% of the "trench" full length (Fig. 3D).

The addition of NCs-CM (0.1 mg/ml) to the standard culture medium significantly increased the overgrown area on 4th day of cultivation (in 3.3 times, P = 0.002, Mann-Whitney U-test; Fig. 3D); the same degree of overgrowth persisted for up to 8th day of observation. There were no differences between the NCs CM effects in concentration of 0.1 or 0.2 mg/ml, but addition of 0.3 mg/ml NCs-CM resulted in significant enhancement of "trench" filling with cellular elements: the overgrown area expanded in 3–3.8 times (respectively P = 0.015, P = 0.024, compared to control indices on 4th and 8th day, Mann-Whitney U-test), differing on the last term from indices of NCs-CM (0.1 mg/ml) (P = 0.029, Mann-Whitney U-test) and reaching 70.5% of full length of the "trench" (Fig. 3B, D).

The addition of AMCs-CM (0.1 mg/ml) increased the overgrown area on 4th day of cultivation (in 1.6 times, P = 0.53, Mann-Whitney U-test; Fig. 3D); the same level was registered on 8th day and after addition of 0.2 mg/ml AMCs-CM. Instead, the addition of 0.3 mg/ml AMCs-CM significantly increased the covering of damaged area by dense network of cells and their processes: the overgrown area expanded in 4.3–7.4 times (respectively P =

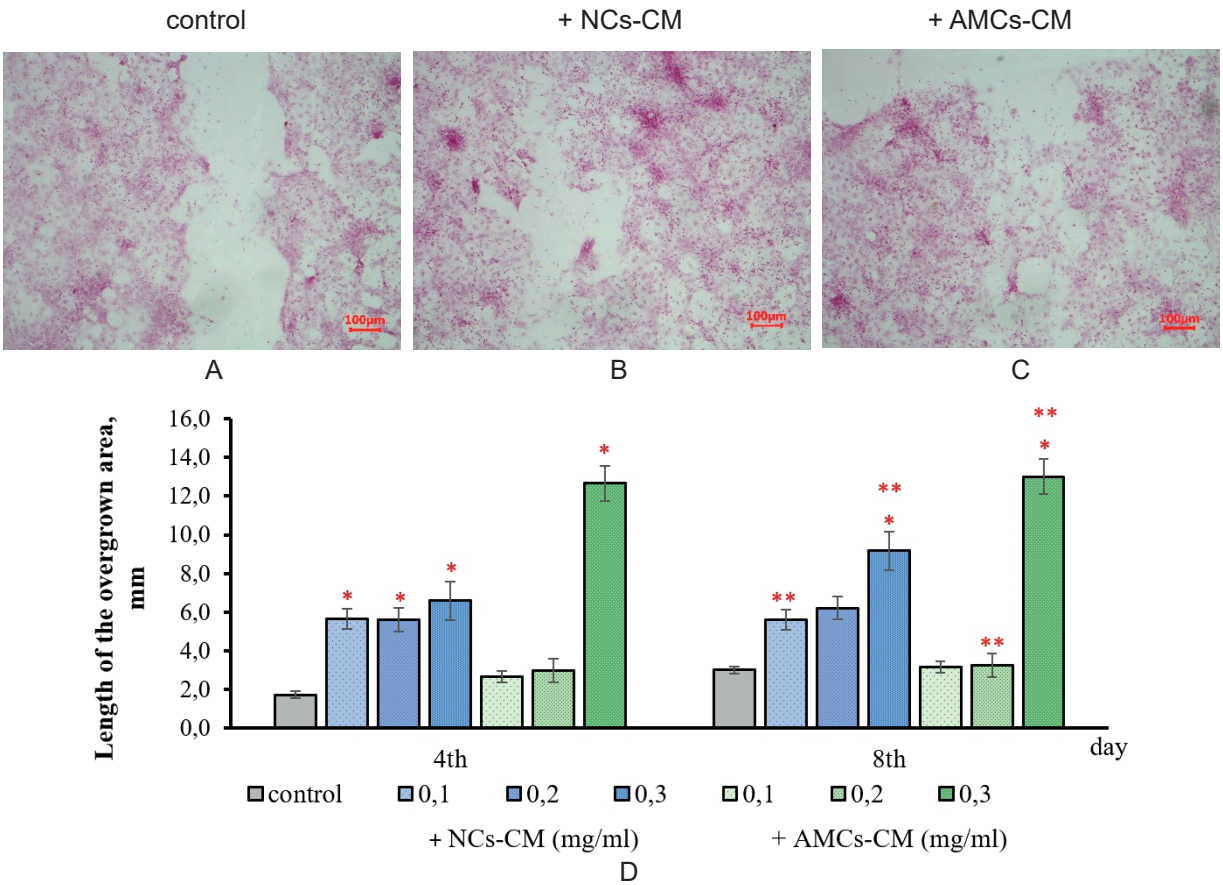

Fig. 3. Overgrowing of the transection site in rat neural cell (E14) cultures. A-C: Microphotographs of rat neural cell (E14) cultures cytopreparations, transection zone, 11th day of cultivation, 4th day after transection and addition of DMEM with 10% FCS (A) or NCs-CM (B) or AMCs-CM (C). Light microscopy, hematoxylin-eosin staining. D - enlargement of the transection site overgrowth under the influence of AMCs-CM and NCs-CM; differences are statistically significant: * – in comparison with control (p <0.05); ** – between indicators within the group at different CM concentration impact (p <0.05).

0.015, P = 0.049, compared to control indices on 4th and 8th day, Mann-Whitney U-test, Fig. 3C), exceeding the indices of NCs-CM (0.3 mg/ml) and reaching 97.4-100% of full length of the "trench" (Fig. 3D).

As follows NCs-CM as well as AMCs-CM stimulated the overgrowth of transection zone of neural cell culture in a dose-dependent manner contributing respectively to 70.5% and almost complete overgrowing of the dissected area. We consider that filling of the "trench" was fulfilled to a greater extent due to cell migration and differentiation, and to a lesser extent due to cell proliferation. So, immunocytochemical staining did not reveal significant differences between the growth zone of control and experimental rat neural cell cultures, but there were differences between transection zone. There were similar trends of increasing in amount of Nestin$^+$, β-tubulin III$^+$ and Ki-67$^+$ cells under the influence of NCs-CM and AMCs-CM, unlike the average amount of GFAP$^+$ cells in "trench" (Fig. 4). We can assume a relationship in increased amount of Nestin$^+$ and Ki-67$^+$ cells under the influence of NCs-CM and AMCs-CM (Spearman Rank Order Correlations 0.155, P > 0.05) and state the negative correlation between GFAP and Ki-67 expression as well as β-tubulin III expression (Spearman Rank Order Correlations -0.494, -0.542, P < 0.05).

The obtained data can be partly explained by known co-expression of Nestin and GFAP in astrocytes of the cerebral cortex (in E14 mice astrocytes expressed Nestin having the phenotype of a mature cell, besides most cells in the culture expressed Nestin and were not fully mature) [29]. Thus, the cells in the overgrowth zone of "trench" belong to different phenotypes (Nestin$^+$ non-differentiated cells, Nestin$^+$ postmitotic neurons, Nestin$^+$ early neuroblasts, Nestin$^+$GFAP$^+$ astrocytes, β-tubulin III$^+$ neurons), their fraction can be changed under the influence of CM from NCs or AMCs. The NCs-CM impact slightly amplified the cell proliferation but intensified the migration and cell differentiation into Nestin$^+$GFAP$^+$ astrocytes and β-tubulin III$^+$ neurons (through the stages of Nestin$^+$ postmitotic neurons and early neuroblasts), stimulating branching of processes (including dendrites and axonal branching) and forming a network. As compared to NCs-CM, the AMCs-CM impact more significantly increased cell proliferation, intensified the migration and cell differentiation into β-tubulin III$^+$ neurons (through the stages of Nestin$^+$ postmitotic neurons and early neuroblasts), and to a lesser extent – into GFAP$^+$ astrocytes, stimulating branching of processes (including dendrites and axonal branching) and forming a network.

To disclose the possible mechanisms involved in enhancing cell migration in neural cell culture under exposure of AMCs-CM and NCs-CM, we focused on the expression of β-catenin, the key element of Wnt/ β-catenin signaling pathway, which is critical in basic biological functions, including cell fate determination, migration, polarity, organogenesis and neural patterning during embryogenesis [30, 31]. Wnt proteins in the SCs niche control the behavior of various types of SCs maintaining them in a self-renewing state [32]. β-catenin is ubiquitously expressed cytoplasmic protein associated with E-cadherin at cellular junctions; when activating of canonical Wnt/β-catenin pathway the β-catenin stabilizes, accumulates in the cytoplasm and is free to translocate into the nucleus [33], where β-catenin acts as coactivator of transcriptional factors of the TCF/LEF family. The genes regulated by Wnt/β-catenin signaling include those of transcription factors, ECM components, cell adhesion proteins, enzymes and hormones (upregulation of C-myc, Tcf-1, LEF-1, PPAR-delta, c-jun, MMP-7, Axin-2, Nr-CAM, Claudin-1, VEGF and downregulation of osteocalcin, E-cadherin) [33, 34]. Wnt regulates the stability and organization of microtubules that influence alignment of mitotic spindles and the segregation of chromosomes during cell division [35]; these in turn influence cell migration and polarization. Wnt induces changes in morphology and behavior of axons via

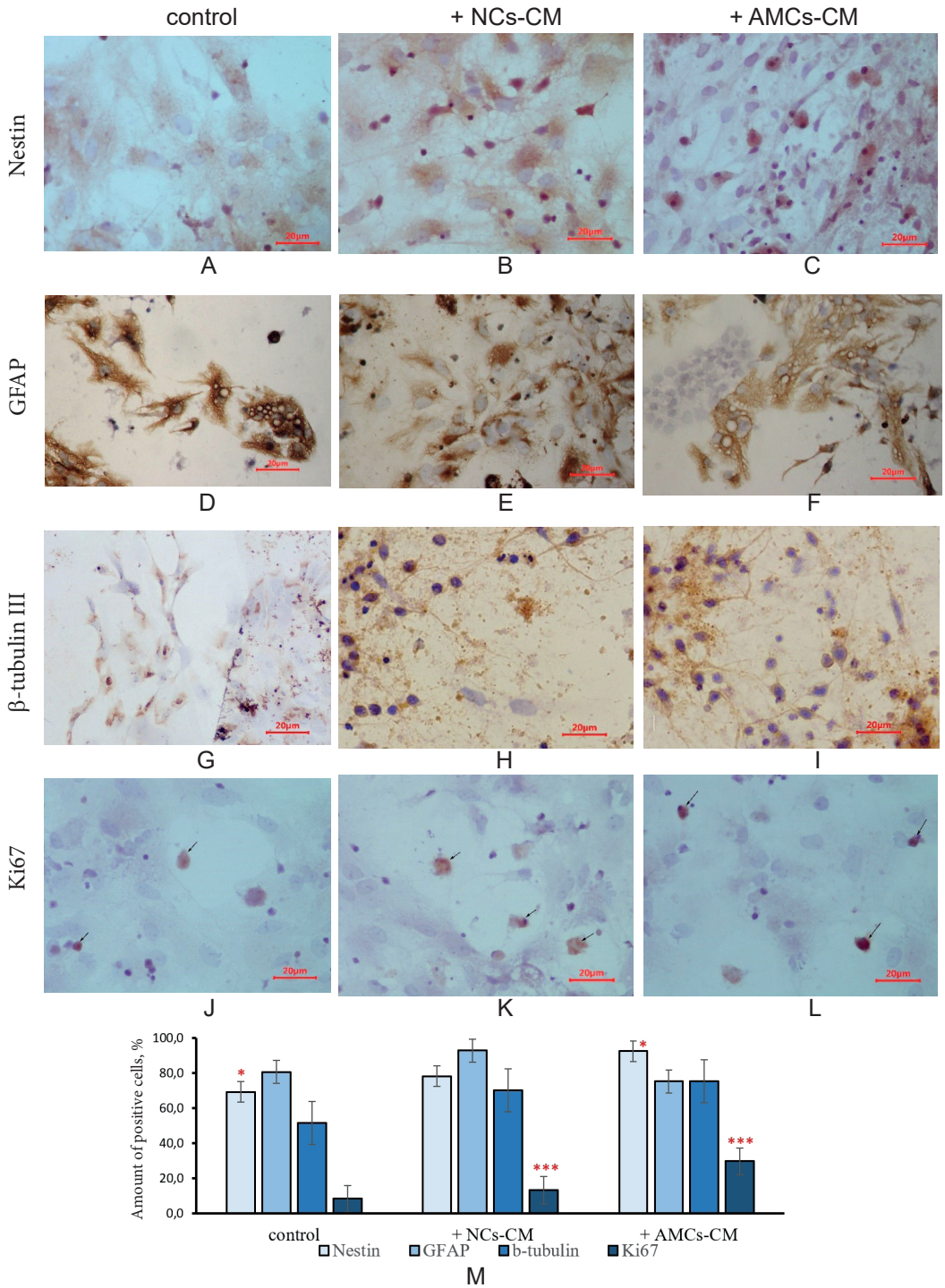

Fig. 4 . Distribution of Nestin+, GFAP+, β-tubulin III+, Ki-67+ cells in the transection zone under different cultivation conditions. A-L: microphotographs of rat neural cell (E14) cultures cytopreparations, transection zone, 11th day of cultivation, 4th day after transection and addition of DMEM with 10% FCS (control cultures, A, D, G, J) or NCs-CM (B, E, H, K) or AMCs-CM (C, F, I, L). Light microscopy, immunocytochemical staining on Nestin (A-C), GFAP (D-F), β-tubulin III+ (G-I), Ki67 (J-L, Ki67+ cells marked with arrows), hematoxylin co-staining. M - diagram with quantitative distribution of Nestin, GFAP, β-tubulin III, Ki-67 cell expression under the influence of AMCs-CM and NCs-CM; differences are statistically significant: * – in comparison with control (p =0.001); *** – between indicators under the influence of AMCs-CM and NCs-CM (p =0.042).

tethering of microtubules, which decrease the axon extension and increase the axon branching [36].

The addition of NCs-CM and AMCs-CM resulted in β-catenin translocation in nucleus of cells in rat neural cell cultures: the distribution of cells with β-catenin$^+$ nucleus both in the growth zone and transection site significantly increased in a dose-dependent manner from 4th to 8th day of cultivation (Fig. 5) and positively correlated with the overgrowth of the transection zone (Spearman Rank Order Correlations 0.308, $P < 0.05$) as well as with the distribution of Nestin$^+$ cells in transection site (Spearman Rank Order Correlations 0.332, $P < 0.05$). There was the absence of correlation between β-catenin translocation in the cell nucleus and β-tubulin and Ki-67 expression and negative correlation with GFAP expression (Spearman Rank Order Correlations -0.598, $P < 0.05$).

Taking into account the mechanism of Wnt/β-catenin signaling, it can be argued that components of NCs-CM and AMCs-CM activate the signal cascade of canonical Wnt/β-catenin pathway, stabilizes the β-catenin and initiates its translocation into the nucleus of cells with predominant Nestin expression, which in turn, activate transcriptional factors downregulating E-cadherin and stimulating cell migration and differentiation (into Nestin$^+$GFAP$^+$ astrocytes, β-tubulin III$^+$ neurons (through the stages of Nestin$^+$ postmitotic neurons and early neuroblasts), and increasing the axon branching, contributing to the overgrowth of the transection zone in the rat brain neural cells cultures. These findings accord with known data that NCs and MMSCs secretome promote axonal growth [4, 5, 19, 20].

Thus, the studies showed a dose-dependent stimulating effect of CM from rat NCs (E14) and AMCs on the overgrowth of the transection zone in the rat brain neural cells cultures due to migration of cells and their further differentiation (in particular, into Nestin$^+$GFAP$^+$ astrocytes, β-tubulin III$^+$ neurons). The stated endogenous regeneration stimulating effect of CM from NCs or AMCs, obviously, involve the target gene transcription regulation via canonical Wnt/β-catenin pathway and, apparently, is based on the effects of biologically active agents (neurotrophic and growth factors, cytokines, extracellular vesicles) which are a part of CM of the studied cells.

It is known a wide range of secreted molecules by NSCs/NPCs (IL-1α, -1β, -2, -4, -6, -10, -17, IFN-γ, TNF-α, TGF-β1, -β2, VEGF, NGF, BDNF, NT-3) [14-16]; as well as AT-MMSCs (IL-1α, -1β, -2, -4, -6, -7, -8, -9, -10, -12, -13, -15, -17, IFN-γ, TNF-α, TGF-β1, -β2, NGF, VEGF, IGF-1, GM-CSF, G-CSF, PDGF, FGF, EGF, HGF, BDNF, GDNF, CNTF) [17-20]. Among them, TGF-β attracted special interest because of suggested role for TGF-β signaling in synapse formation and neuronal plasticity and known relationship between Wnt/β-catenin and TGF-β signaling pathways [37]. In different developmental contexts, Wnts and TGF-β signaling can interact either antagonistically or synergistically and could thus provide a flexible way of controlling the formation, growth and maintenance of synapses. In cultured neurons, Wnt-7a has been shown to regulate axonal microtubule organization and the clustering of presynaptic proteins [38]. The TGF-β family has also been implicated in dendritic arborization and neuronal plasticity in cultured neurons [39]. It is possible that TGF-β, given its known properties, is one of the affecting factors of recorded NCs or AMCs neuroregenerative effects in neural cell culture, which via paracrine signaling, in particular, Wnt/β-catenin pathway, stimulate the processes of cell migration and differentiation and ultimately promotes the overgrowth of the transection area.

The presented data are obtained as a result of the morphological and morphometric study, which imposes certain restrictions, but opens the way for in-depth research in several directions: clear identification of molecules in NCs or AMCs CM composition; study of the key components of signaling pathways machinery involved in neural cell after NCs or AMCs CM

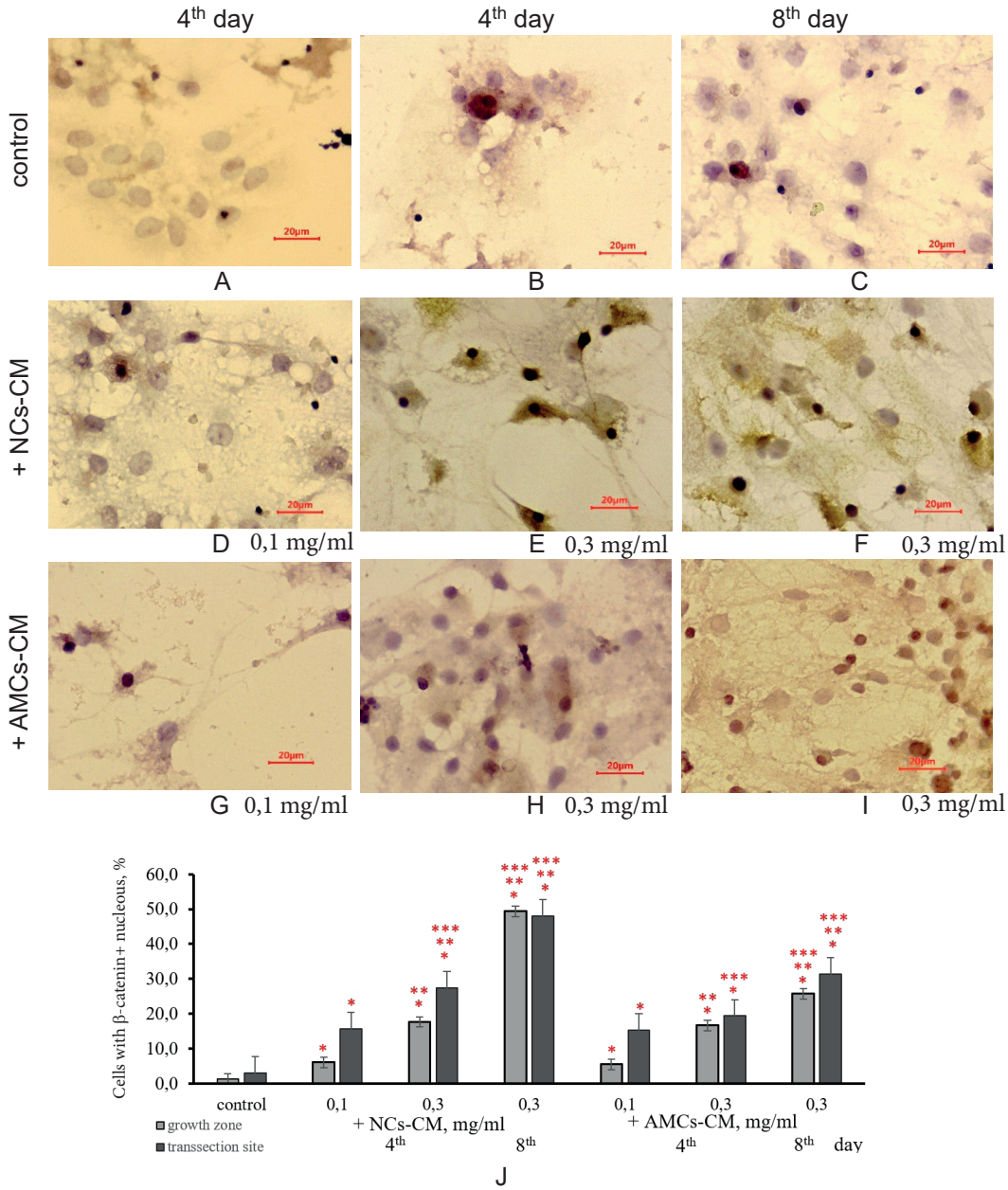

Figure 5. Distribution of β-catenin in cells in transection site of rat neural cell (E14) cultures under different cultivation conditions. A-I: microphotographs of rat neural cell (E14) cultures cytopreparations, transection zone, 4th, 8th day after transection and addition of DMEM with 10 % FCS (control cultures, A, B, C) or NCs-CM (0,1 mg/ml (D), 0,3 mg/ml (E, F) or AMCs-CM (0,1 mg/ml (G), 0,3 mg/ml (H, I). Light microscopy, immunocytochemical staining on β-catenin, hematoxylin co-staining. J - diagram with quantitative distribution of cells with β-catenin translocation in nucleus in transection site and growth zone in rat neural cell culture under the influence of AMCs-CM and NCs-CM; differences are statistically significant: * – in comparison with control ($p < 0.005$); ** – between indicators within the group at different CM concentration impact ($p < 0.02$); *** – between indicators under the influence of AMCs-CM and NCs-CM ($p < 0.02$).

impact; study of NCs or AMCs CM regenerative effects in model systems in vivo. Thus, our data may become the basis for in-depth developments of using a CM in the treatment of CNS traumatic injuries and degenerative diseases to restore and regenerate damaged nerve tissue when introduced into the body as a possible alternative to direct SPCs transplantation as well as an additional component to SPCs transplantation or conventional treatment regimens for CNS pathology.

CONCLUSIONS

CM from NCs (predominant NSPCs fraction) and AMCs (predominant ATSCs fraction) in a dose-dependent manner stimulate migration processes in the culture of rat neural cells, contributing to overgrowing of the dissected area (reparation of a mechanical defect). After addition of NCs-CM the overgrown area reached 70.5% of full length of the transection site; after addition of AMCs-CM – 97,4-100%. The addition of NCs-CM and AMCs-CM resulted in β-catenin translocation into the nucleus of cells in rat neural cell cultures, which correlated with the overgrowth of the transection zone, evidencing the involving of β-catenin signaling pathway in stimulating of rat neural cells migration. NCs-CM and AMCs-CM are a source of signaling molecules that modulate the microenvironment and activate endogenous repair mechanisms in culture (in vitro model of nerve tissue regeneration).

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ВПЛИВ КОНДИЦІЙОВАНИХ СЕРЕДОВИЩ РІЗНОГО ПОХОДЖЕННЯ НА МІГРАЦІЮ НЕРВОВИХ КЛІТИН IN VITRO

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Важливим напрямком розвитку новітніх технологій відновлення пошкодженої ЦНС є використання стовбурових/прогеніторних клітин (СПК), переважно нейральних СПК (НСПК) та мезенхімальних мультипотентних стромальних клітин (ММСК). Одним з основних механізмів дії СПК є непрямий паракринний вплив, зумовлений здатністю продукувати широкий спектр біологічно активних сигнальних молекул (секретом). Вивчення регенеративних ефектів кондиційованих середовищ (КС) НСПК і ММСК як джерела їх секретому є актуальним і потенційно перспективним. Мета нашого дослідження – порівняти вплив КС культур фетальних нейрогенних клітин (НК (Е14) як джерела НСПК) та мононуклеарних клітин жирової тканини (МКЖТ, як джерела ММСК) на міграційну здатність нервових клітин щура in vitro. КС МКЖТ отримували після 24 год культивування з переважанням у культурах $CD105^+$-клітин і здатністю утворювати «сфероїди» та диференціюватися в різні типи клітин. КС НК отримували після 24 год культивування з переважанням у культурах $Nestin^+$ клітин і здатністю утворювати «нейросфери» та диференціюватися в астроцити (GFAP+) і нейрони (β-тубулін III^+). Нейральні клітини плода (Е14) щура культивували для отримання конфлуентного моношару з основними клітинними елементами нервової тканини (5–7-й день), який розсікали з формуванням ділянки перерізу та додавали середовище DMEM з 10% фетальної телячої сироватки (контроль) або 0,1–0,3 мг/мл (за загальною кількістю протеїнів) КС НК або КС МКЖТ. У контрольних культурах нейральних клітин щурів спостерігали часткове зарощення розсіченої ділянки моношару внаслідок міграції клітин, утворення мережі відростків і міжклітинних контактів, що сягало 13,2% (4-а доба) – 23,2% (8-а доба) її повної довжини. Площа заростання збільшувалася після додавання КС: КС НК – утричі (0,1-0,2 мг/мл) та у 3-4 рази (0,3 мг/мл, 4–8-ма доба), сягаючи 70,5% повної довжини ділянки перерізу; КС МКЖТ – у 1,5 раза (0,1-0,2 мг/мл) та у 4–7 разів (0,3 мг/мл, 4–8-ма доба), сягаючи 97,4–100% повної довжини ділянки перерізу. Додавання КС НК і КС МКЖТ призводило до транслокації β-катеніну в ядра клітин у культурах нейральних клітин щура, що корелювало із зарощенням ділянки перерізу. Таким чином, КС НК та КС МКЖТ дозозалежно стимулюють міграційні процеси в культурі нейральних клітин щура, очевидно, із залучен-

ням β-катенін-сигнального шляху, сприяючи заростанню розсіченої ділянки (репарація механічного дефекту). КС НК і КС МКЖТ є джерелом сигнальних молекул, що модулюють мікросередовище та активують ендогенні механізми відновлення в культурі (модель регенерації нервової тканини in vitro).

Ключові слова: фетальні нейрогенні клітини; мононуклеарні клітини жирової тканини; кондиційовані середовища; секретом; клітинна міграція; диференціація.

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