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3D CULTIVATION OF NEURAL CREST-DERIVED MULTIPOTENT STEM CELLS IN COLLAGEN AND FIBRIN HYDROGELS: EFFECTS ON CELL VIABILITY AND PROLIFERATION

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The possibility of culture of neural crest-derived multipotent stem cells in 3D collagen and fibrin hydrogels has been shown. The effects on cell viability and growth have been explored. It is shown that both hidrogels contribute to cell adhesion and spreading. During culture it maintains high cell viability in both types of hydrogels as well as network formation of the interconnected cells. When cultured for two weeks in a collagen hydrogel cell number increased by 1.4 fold, while the fibrin hydrogel shows almost a threefold increase in cell number. The results obtained can be used for development of neural crest-derived multipotent stem cell-based tissue-engineered constructs.

Key words: neural crest-derived multipotent stem cells, 3D culture, hydrogel, fibrin, collagen.

Neural crest-derived multipotent stem cells (NC-MSCs) possess a considerable interest for the regenerative medicine. NC-MSCs are capable of directed differentiation into adipocytes, osteoblasts, keratocytes, neurons, Schwann cells and other cell types [1, 2]. Due to these properties, NC-MSCs can be used to restore the cranial bone defects, peripheral nervous system and corneal stroma lesions. However, cell suspension transplantation has several restrictions as a similar approach can be applied not for all tissue defects. It was noted also a low efficiency of cell transplantation associated with a weak rate of cell engraftment in the target tissue after their administration by injection due to a "leakage" and/or loss of substrate-dependent cells via apoptosis mechanism (anoikis) [3]. Efficacy of transplantation may be enhanced by creation of three-dimensional (3D) cell agglomerates (spheroids) [4] or cell- and extracellular matrix-based tissue equivalents [5]. A promising direction is the development of biocompatible hydrogels. Their advantages include: homogeneous cell distribution within a construct, high cell viability, the ability to receive constructs of a given shape using various natural and synthetic polymers and their combinations thereof, the ability to adjust physical characteristics (elasticity, stiffness, porosity, etc.) [6]. The aim of a study was to explore the effects of NC-MSC culture within 3D collagen and fibrin hydrogels on their viability and growth.

Materials and Methods

The experiments were performed in compliance with the principles of bioethics and biosafety standards, as confirmed by the Committee on Bioethics of State Institute of Genetic and Regenerative Medicine of NAMS. Experiments were carried out with use of 5 cell cultures derived from FVB mice males of 4 to 6 months aged.

Isolation and culturing of NC-MSCs from a bulge region of whisker follicle

Briefly, NC-MSC culture was obtained from explants of the bulge region (BR) of the whisker follicle (WF) by Sieber-Blum method [7] in our modification [2]. By 3 BRs were explanted into the 35 mm Petri dish and cultured in DMEM:F12 medium supplemented with 10% fetal bovine serum (FBS), 5 ng/ml bFGF, 1% MEM vitamins, 1% Neuronal Stem Cell Supplement (PAA, Austria) and 2 mM

L-glutamine (all from Sigma-Aldrich, USA) in a multi-gas incubator CB210 (BINDER, Germany) at 37 °C and saturating humidity, in artificial atmosphere, consisting of 90% N_2, 5% O_2 and 5% CO_2. Cultures were passaged with use of 0.05% trypsin in 0.53 mM Na_2EDTA (Sigma-Aldrich, USA). Phenotype of NC-MSCs and their functional properties were determined as described previously [2]. In all experiments, third passage (P3) cells were used.

Collagen and fibrin hydrogel modeling

Collagen hydrogel was prepared according to E. Bell method [8]. For this purpose, a sterile solution of 0.34M NaOH was mixed with a ×10 medium MEM (Sigma, USA) in a ratio of 1:2. Then 100 mg of *L*-glutamine (Sigma, USA) and 9 ml of 7.5% sodium bicarbonate solution (Sigma, USA) were added per 100 ml of the resulting mixture. On ice, the resulting composition was mixed with a solution of collagen type I/III (3 mg/ml, Sigma, USA) in a ratio of 1:4. Required cell number in growth medium (cell suspension volume was 10% of gel volume), was thoroughly mixed with resulting composition and placed over 30 min in an incubator for hydrogel polymerization.

Fibrin hydrogel was prepared by mixing on ice 0.5 ml of fibrinogen (4 mg/ml, Sigma, USA) with 0.5 ml of cell-loaded growth medium, which also contain 1U of thrombin (Sigma, USA). Resulting composition was mixed thoroughly and placed for 30 min in an incubator for hydrogel polymerization.

After complete hydrogel polymerization, they were covered with 1 ml of growth medium. Hydrogels were prepared in 35 mm Petri dishes with 1 ml of hydrogel containing 0.5×10^6 cells. For each culture ($n = 5$) by two hydrogels of each type were fabricated (10 samples of each hydrogel type in total). Half of the samples of each hydrogel type have been used for cell isolation and calculation after 7 days of culturing, the second half — after 14 days of culturing.

Assessment cell viability in 3D collagen and fibrin hydrogels

To estimate cell viability cultured in 3D hydrogel, it was carried out combined staining with fluorescein diacetate (FDA, Sigma, USA) and propidium iodide (PI, Sigma, USA). For this purpose a culture medium was removed, the samples were washed three times with Hanks' Balanced Salt solution, HBSS (PAA, Austria) and then filled for 20 min with HBSS containing 1 mg/ml FDA and 2 mg/ml PI. After incubation, a dye solution was removed, cultures washed 3 times with HBSS and visualized. FDA stains live cells with bright green color. PI penetrates the dead cells and stains the nuclei with orange color. For each sample, five random visual fields were chosen, pictured, and calculated in each field a total cell number in one plane, a number of live and dead cells. Viability was determined as a live cell number / total cell number ×100%.

Cell isolation from hydrogels

For cell isolation from a collagen hydrogel it was treated with 0.1% solution of collagenase IA (Sigma, USA) for 30 min at 37 °C and permanent stirring. The resulted cell suspension was centrifuged (200 g, 5 min) and treated for 5 min with 0.05% trypsin solution (Sigma, USA) for dissociation of cell agglomerates.

For cell isolation from a fibrin hydrogel, the samples were treated with 0.1% solution of pronase (Sigma, USA) for 20 min at at 37 °C and permanent stirring. The resulted cell suspension was centrifuged (200 g, 5 min) and treated for 5 min with 0.05% trypsin solution (Sigma, USA) for dissociation of cell agglomerates.

Assessment cell viability in suspension

Assessment of a cell number and viability in suspension prior to seeding into the hydrogels and after hydrogel releasing was performed by staining with 0.4% solution of trypan blue (Sigma, USA) to count the total cell and dead cell number with Goryaev's chamber (MiniMed, Russia).

Microscopy

The inverted fluorescence microscope Axio Observer A1, equipped with a digital camera AxioCam ERc 5s and software ZEN 2012, have been used (Carl Zeiss, Germany).

Statistics

All numerical data are represented as mean values and their standard deviations ($M\pm s$). Significance of the differences was evaluated using Student's *t*-test.

Results and Discussion

Biological properties of NC-MSCs from a bulge region (BR) of whisker follicle (WF) were described earlier by us and other authors [1, 2, 7]. The identity of NC-MSCs used in the experiment has been confirmed with their phenotype (nestin+/CD44+/CD73+/CD90+/Sca-1+/cytokeratin−/CD45−) and the capacity to differentiate into the adipocytes and osteoblasts (data not show).

The article shows a principal possibility for NC-MSCs to be cultured in three-dimensional structures based on collagen and fibrin hydrogels. Both types of hydrogels are easy to handle, require a short time for preparation and polymerization, and allow producing a uniform cell distribution within them. Immediately after hydrogel fabrication, NC-MSCs in their composition have a rounded shape (Fig. 1, *A, B*). 24 h post-culture, the NC-MSCs changed their shape on fibroblast-like with several long processes formed (Fig. 1, *C, D*). To cell culture Day 7 in both types of hydrogels the cells formed an interconnected network. When cultured for 14 days, NC-MSCs maintained their fibroblast-like morphology and cellular network organization, also showing a significant viability index (Fig. 2; Table). At all stages of hydrogel fabrication and cell culturing within them, the NC-MSC viability was above 90%, as it was shown via two different methods for determination of cell viability. Lower NC-MSCs viability values obtained when assessing trypan blue exclusion compared to the FDA/PI stains may be due to additional traumatic procedure of cell release from 3D constructs using enzymatic treatment. It also may explain a statistically significant difference in cell viability prior hydrogel seeding and after cell release from them. At the same time, cell viability values, determined by different methods, had no significant differences between collagen and fibrin hydrogels. Cell morphology, network organization and viability of NC-MSCs within collagen and fibrin hydrogels were similar during culture, but cell growth values were significantly different (Table). So, NC-MSC number when cultured for 14 days in a collagen hydrogel increased by 1.45-fold, while NC-MSC number when cultured in a fibrin hydrogel increased by 2.8-fold. Thus, NC-MSC growth rate was twice as high in a fibrin hydrogel as in a collagen hydrogel.

Thereby, we have demonstrated a principle possibility for culture of the NC-MSCs in hydrogels of two different types, based on natural extracellular matrix components — collagen and fibrin. Wherein, NC-MSC proliferation rate was significantly higher for cells cultured in a fibrin than in collagen hydrogel. It may be due to the fact that fibrin is a key component of a provisional matrix during wound healing and it has to maintain an active cell migration and proliferation. Whereas collagen type I is a main component of the connective tissue extracellular matrix and it promotes to maintain a differentiated state of the cells and tissue homeostasis. Various types of hydrogels can be used for repair of damages of different tissues. Collagen hydrogel may be preferable for restoration of damages of slowly renewing tissues, for example for bone tissue. The use of fibrin hydrogel may be more effective in cases when a rapid tissue remodeling is required for repair process, e.g. for reparation of peripheral nerve injuries or for healing of skin wounds. We have previously shown that

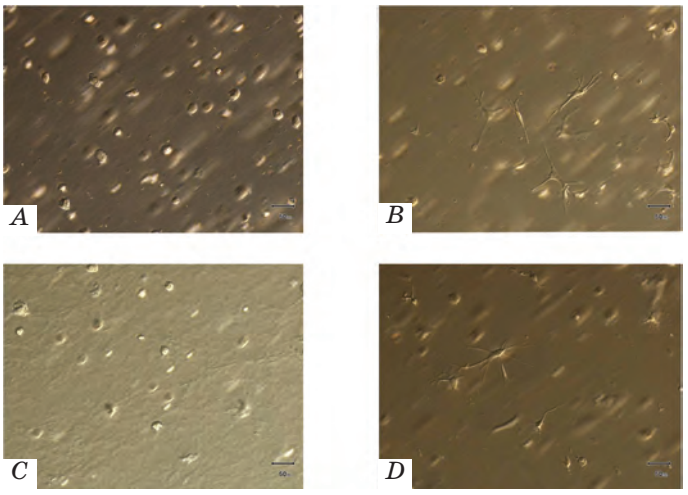

Fig. 1. **Morphological changes of NC-MSCs during initial stages of culturing in 3D collagen and fibrin hydrogels:**

A — collagen hydrogel, one hour after modeling;
B — collagen hydrogel, 24 h of culture;
C — fibrin hydrogel, one hour after modeling;
D — fibrin hydrogel, 24 h of culture;
relief contrast; 50 μm scale

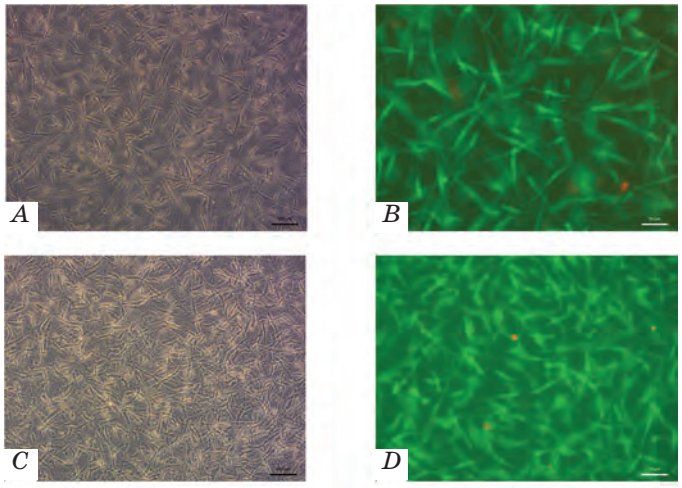

Fig. 2. **NC-MSC culture (14 days) in hydrogels:**
A — NC-MSCs morphology in a collagen hydrogel;
B — NC-MSCs viability in a collagen hydrogel;
C — NC-MSCs morphology in a fibrin hydrogel;
D — NC-MSCs viability in a fibrin hydrogel;
A, B — phase contrast, 50 μm scale;
C, D — fluorescence microscopy, FDA/PI combined stains; 100 μm scale.
B, D — live cells — green; dead cells — orange.

NC-MSC number and viability at various stages of a 3D hydrogel modeling and culturing (M±*s*)

	Prior hydrogel seeding (control)	Collagen hydrogel, Day 7	Fibrin hydrogel, Day 7	Collagen hydrogel, Day 14	Fibrin hydrogel, Day 14
Cell number, $\times 10^3$	508±13	654±62*,#	1090±136*,#	728±31*,#,&	1412±101*,#,&
Viability, FDA/PI,%	–	94.8±1.9	95.2±1.9	94.4±2.1	94.8±1.3
Viability, trypan blue,%	95.6±2.3	90.2±1.9*	90.8±2.6*	90.6±2.9*	91.2±1.5*

* $P < 0.05$ versus control;
# $P < 0.05$ between collagen and fibrin hydrogels at same time point;
& $P < 0.05$ between different time point into same group (collagen and fibrin hydrogels).

adipose-derived stem cells (ADSCs) within fibrin hydrogel composition contribute more effectively to the healing of experimental full-thickness burns in mice than ADSCs within a collagen hydrogel, which could be associated with a greater stimulation of neoangiogenesis [9].

Thereby, our results can be used for development and modeling of tissue-engineered constructs (live tissue equivalents) on a basis of neural crest-derived multipotent stem cells for restoration of defects of different tissues.

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3D-КУЛЬТИВУВАННЯ МУЛЬТИПОТЕНТНИХ СТОВБУРОВИХ КЛІТИН — ПОХІДНИХ НЕРВОВОГО ГРЕБЕНЯ У КОЛАГЕНОВОМУ І ФІБРИНОВОМУ ГІДРОГЕЛЯХ: ВПЛИВ НА ЖИТТЄЗДАТНІСТЬ ТА ПРОЛІФЕРАЦІЮ

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Виявлено можливість культивування мультипотентних стовбурових клітин — похідних нервового гребеня у тривимірних конструкціях на основі колагенового і фібринового гідрогелів. Досліджен вплив на життездатність та ріст клітин. Показано, що обидва гідрогелі сприяють адгезії та розпластуванню клітин. За культивування зберігається висока життєздатність клітин в гідрогелях обох типів та відбувається утворення мережі з'єднаних між собою клітин. За культивування протягом двох тижнів у колагеновому гідрогелі кількість клітин збільшується у 1,4 раза, тоді як у фібриновому гідрогелі — майже у 3 рази. Отримані результати можуть бути використані при розробленні тканинно-інженерних конструкцій на основі мультипотентних стовбурових клітин — похідних нервового гребеня.

Ключові слова: нервовий гребінь, мультипотентні стовбурові клітини, 3D-культури, гідрогелі, фібрин, колаген.

3D-КУЛЬТИВИРОВАНИЕ МУЛЬТИПОТЕНТНЫХ СТВОЛОВЫХ КЛЕТОК — ПРОИЗВОДНЫХ НЕРВНОГО ГРЕБНЯ В КОЛЛАГЕНОВОМ И ФИБРИНОВОМ ГИДРОГЕЛЯХ: ВЛИЯНИЕ НА ЖИЗНЕСПОСОБНОСТЬ И ПРОЛИФЕРАЦИЮ

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Установлена возможность культивирования мультипотентных стволовых клеток — производных нервного гребня в трехмерных конструкциях на основе коллагенового и фибринового гидрогелей. Изучено влияние на жизнеспособность и рост клеток. Показано, что оба гидрогеля способствуют адгезии и распластыванию клеток. При культивировании сохраняется высокая жизнеспособность клеток в гидрогелях обоих типов и происходит формирование сети соединенных между собой клеток. При культивировании в течение двух недель в коллагеновом гидрогеле количество клеток увеличивается в 1,4 раза, в то время как в фибриновом гидрогеле происходит почти трехкратное увеличение количества клеток. Полученные результаты могут быть использованы при разработке ткане-инженерных конструкций на основе мультипотентных стволовых клеток — производных нервного гребня.

Ключевые слова: нервный гребень, мультипотентные стволовые клетки, 3D-культуры, гидрогели, фибрин, коллаген.