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INVESTIGATION OF ENZYMATIC ACTIVITY IN HUMAN DERMAL FIBROBLASTS DURING VARIOUS CULTIVATION PERIODS

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The cultivation of human dermal fibroblasts plays a leading role in the field of biomedical research and regenerative medicine, thanks to their ability to produce extracellular matrix and thereby maintain the integrity of connective tissue, wound closure (1). In the cultures, there is an exhaustion of the cell population and an increase in the number of cells that enter the state of senescence (2). Despite numerous studies of these cells and their features of maintaining stability during the proliferation of fibroblasts for the correction of involutional-dystrophic changes, it remains an unresolved issue to this day.

Aim. Research into the change in enzymatic indicators of cell activity during the aging of human dermal fibroblasts in culture from 3 to 15 passages to determine the most optimal terms for cell transplantation to patients for further cell therapy.

Methods. Fibroblasts were obtained from the dermis of donors A, B, and C aged 40 to 60 years and cultured in MEM Alpha culture medium containing 5% FBS, 1% antibiotic/antimycotic, 5ng bFGF until they reached 3, 6, 9, 12, and 15 passages (s1p3-s1p15).

When fibroblasts that had reached the required passage were removed, the cells were washed with saline solution and precipitated on a centrifuge. Fixation was carried out with a 0.1% solution of formalin. At the end of the fixation time, the cells were centrifuged and left in 70% ethanol at −20 °C. At least after 3 hours, the cells were washed twice with saline and centrifuged. To determine number of cells at stages of the cell cycle by the quantitative content of DNA, fibroblasts were stained with propidium iodide/RNase buffer. At the end of the sample preparation, the sample was analyzed on a flow cytometer (Beckman Coulter, China).

To study the enzymatic activity of mitochondria, fibroblasts were seeded in a sterile 96-well microplate and cultured for 72 hours. As a control, wells without fibroblasts were subjected to the same manipulations as the experimental wells. After that 0.3 mg/ml MTT was added to the wells. In four hours' time DMSO and 0.015 mg/ml glycine were added to all wells. The contents of the wells were homogenized and measured with a photometer (BioSan, Latvia) at 490 nm.

To determine the activity of lysosomal enzymes of fibroblasts cultured in Petri dishes for 72 hours, cells were washed with saline and fixed with formalin. After the end of fixation, fibroblasts were again washed several times with saline solution and stained with the addition of synthetic dye (1.25% reagent C, 1.25% reagent B, 2.5% X-Gal and 95% X-Gal buffer) for at least 2 hours at 37 °C. Cells were washed with saline from dye residues and photographed the results.

The results of the groups were compared using the ANOVA test. Differences were observed at a significance level of $P < 0.05$.

Results and Discussion. It is well known that aging cells can be characterized by cell cycle arrest, affecting the phenomenon of cell proliferation (3). Dermal fibroblasts maintain their mitotic activity even up to the 15th passage (Fig. 1). In addition, the average percentage of fibroblasts that were at the stage of mitosis 58.88±4.49% was higher than the average value of indicators at the stages of interphase (41.17±2.72%).

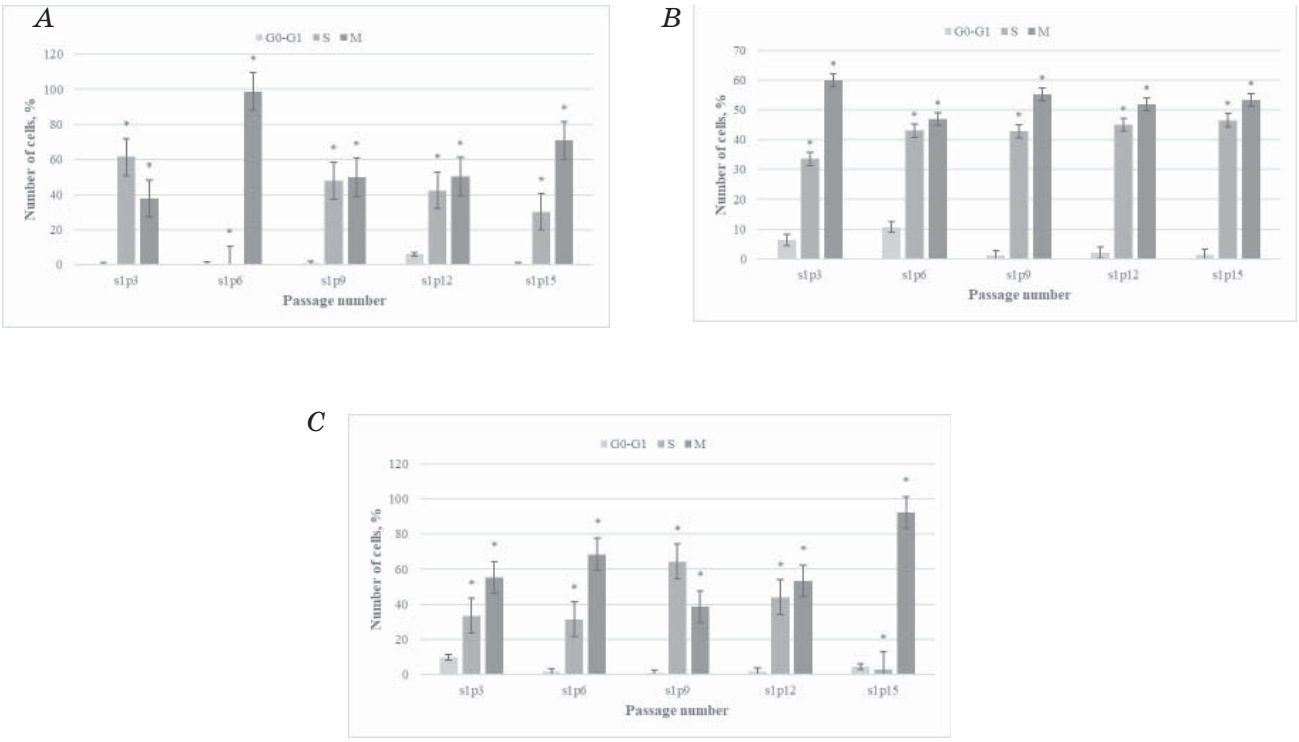

Fig. 1. **Percentage distribution of human dermal fibroblasts placed at different stages of the cell cycle with a change in the cultivation period for cells of donor A (*A*),** donor B (*B*), and donor C (*C*)
* — $P < 0.05$ compared to the studied group G0-G1

Disruption of stable enzymatic activity is characterized by an increase in the mass of organelles in aging cells. The population of mitochondria can increase by 90–94 times compared to young cells (4). The optical density, which depends on the concentration of formazan in the wells of the plate, did not show significant changes with the change in the term of cultivation of dermal fibroblasts from early to late passages (Fig. 2).

There is an increase in the activity of formazan formation by donor cells at different passages, which does not show a linear dependence and corresponds to a high percentage of cells in mitosis. This can be explained by the enhancement of the activity of oxidoreductase of mitochondria with an increase in their mitotic activity. Between these methods was found a moderate positive correlation, confirming the possibility of using the MTT test additionally for measuring the proliferative ability of cells.

An increase in lysosomal mass and enhanced release of lysosomal enzymes can also be present in aging cells (5). When studying microphotographs of early passages (Fig. 3), to detect cells with enhanced release of β-galactosidase, signs of aging of dermal fibroblasts of donors were not noticed, spindle-shaped cells with processes, were all uniformly stained with dye without changing its color. At late passages, cells did not show signs of aging culture: the change in the color of the cytochemical reaction did not occur, the number of large, flat, vacuolized cells did not increase.

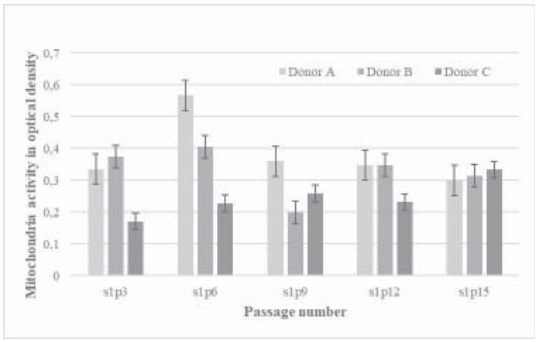

Fig. 2. **Optical density indicators at different passages of human dermal fibroblast cultivation, obtained by the colorimetric method**

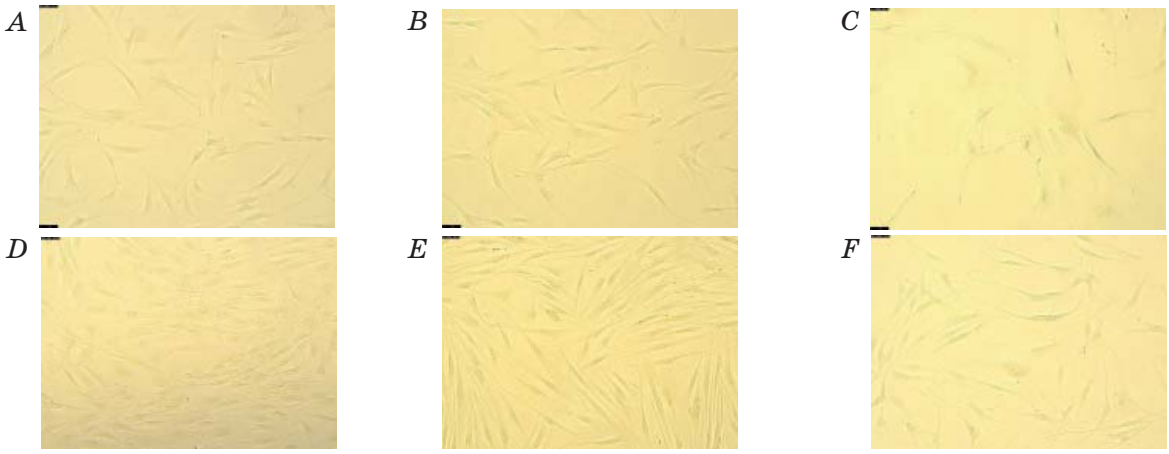

Fig. 3. **Microphotographs of early (*A*-*C*) and late (*D*-*F*) passages of cells for the detection of cells positive for β-galactosidase**
X-gal staining; Mag. ×150

Conclusions. Thus, using various cytochemical methods, it has been proven that the culture of human dermal fibroblasts from donors of the age group from 40 to 60 years maintains stability during their cultivation from 3 to 15 passages. The cells of the culture do not show signs of aging and do not show significant changes in indicators during passaging: a consistently high percentage of mitotically active cells and the absence of a significant increase in the indicators of enzymatic activity of mitochondria and lysosomes.

Key words: human dermal fibroblasts, senescence, cell proliferation, metabolic activity.

Authors` Contribution. AAT worked with mastering and refining methodologies, obtaining preliminary results and analysis of data, OKV editing the manuscript, YMK and DMP made the conception of the work, interpreted the results.

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