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CYTOGENETIC ANALYSIS OF RAT PANCREATIC CELL CULTURES AT EARLY PASSAGES

ABSTRACT

Cell culture obtained from the pancreas can serve as a source of physiologically competent substitute for primary islets of Langerhans in the treatment of diabetes. However, it is possible to obtain the required number of cells only at long-term cultivation in vitro. Therefore, it is necessary to investigate the risks of neoplastic transformation of cells in vitro before transplantation.

MATERIALS AND METHODS. *Cell culture was obtained by explant method from pancreas of 12-day-old rats. Cell cultures of the first to sixth passages were used for the cytogenetic analysis. In this study the number of cells with altered karyotype, cells with micronuclei, binucleated cells and the cells in a state of apoptosis were considered, mitotic index was calculated.*

RESULTS. *Aneuploid cells were noted at all passages in an amount of 2.2 % (1st) to 16.6 % (4th). Polyploidy manifested in a population of cells from the second (1.1 %) to the sixth passage (4.4 %) with a maximum at passage four (7.8 %). A significant increase in their number was observed since the second passage (0.3 %). We have seen a significant increase in the number of binucleated cells from the first (0.1 %) to the sixth passage (0.8 %). During the study there was a decrease in mitotic index from the first (2.7 %), to the third passage (1.5 %) and its gradual increase in fourth (1.7 %) and sixth (2.0 %) passages. In addition, there was discovered a small percentage of cells in apoptosis, their number gradually increased to the 4th passage (0.5 %). The 5th-6th passages showed decrease in the number of apoptotic cells to 0.1 %.*

CONCLUSION. *There have been revealed changes in the rat pancreatic cells culture as aneuploidies, polyploidies and micronuclei, the intensity of which varied depending on the passage. However, karyotype variability of mentioned cell did not exceed the level of spontaneous mutations characteristic of mammalians.*

KEYWORDS: *pancreatic cells culture, cytogenetic analysis, micronucleus test*

Diabetes mellitus (DM) of the first type is a chronic disease that affects genetically predisposed individuals, whose insulin-producing pancreatic β-cells of Langerhans islets are selectively and irreversibly destroyed due to an autoimmune attack of the body [12]. For over 80 years the main therapeutic approach was limited to the treatment of the DM symptoms with substitution insulin therapy. Scientific sources prove that tight regulation of blood glucose at the intensive insulin therapy leads to a significant increase in the risk of severe hypoglycemic reactions, such as seizures and coma; and does not exclude the possibility of secondary destructive changes of DM (nephropathy, neuropathy and cardiovascular disease) [17, 19].

Modern studies on DM therapy are aimed at searching for drugs, which are approximated at most to physiological conditions of insulin secretion [1]. Quite a promising method for the treatment of DM is transplantation of cell material as a virtually unlimited source of physiologically competent substitute for primary Langerhans islets [9, 10].

The results of preclinical and clinical studies indicate a high efficiency of using stem cells as replacement therapy compared with other treatments. Clinical use requires a large number of cell transplants, which can be achieved through long-term cultivation of cells under conditions *in vitro*.

At the same time, an important condition for obtaining high-quality cellular material by culture method is to maintain genetic stability of the cell culture outside the body. Available literature revealed a number of conflicting data on the risks of neoplastic transformation of cells *in vitro* [2, 5, 13-16, 18], which has necessitated further researches on chromosomal stability of rats' pancreatic cell culture during cultivation as the main models for biological research.

OBJECTIVE. To investigate the changes in karyotype of rat pancreatic cells culture at early passages and assess stability of the resulting culture.

MATERIALS AND METHODS

All experimental procedures with animals were in compliance with article 230 of the Law of Ukraine «On protection of animals from cruelty» (# 3447-IV, 21.02.2006), international principles of the European Convention for the protection of vertebrate animals used for experimental and other

scientific purposes (European convention, Strasburg, 1986), and all norms of bioethics and biosafety. 9 nonlinear 12-day-old rats from vivarium of National University of Life and Environmental Sciences of Ukraine were used for studies.

Euthanasia of experimental animals was carried out by decapitation under ether anesthesia. The pancreas was removed from the abdominal cavity in a sterile Petri dish where it was washed with phosphate-buffered saline (*Sigma*, USA). Then glandular part was separated and crushed into pieces with scissors to 1-2 mm, which were transferred to Petri dishes (d = 3 cm) at the rate of 10-15 per cup. Cultivation was performed in a standard complete medium: 80 % DMEM (*Sigma*, USA); 20 % fetal bovine serum (*Sigma*, USA); 10 μL/mL antibiotic-antimycotic solution (*Sigma*, USA) at 37°C and 5 % CO_2 for 10 days prior to monolayer formation. The cells were passaged by the standard method (0.25 % trypsin/EDTA solution) [6]. Microscopic analysis and assessment of the culture was carried out using inverted microscope Axiovert 40 (*Carl Zeiss*, Germany).

Cytogenetic analysis was performed on 30 metaphase plates of cells from each passage. To obtain chromosomes preparations, a modification of standard cytogenetic methods was used [6]. Fixation of chromosomes was carried out 48 hours after cells seeding. Colchicine (*Sigma*, USA) was added to the culture medium in a final concentration of $1 \cdot 10^{-7}$ M and incubated for 4 hours at 37°C. The cells were removed from the dishes with standard solution of 0.25 % trypsin/EDTA solution. After centrifugation 9 mL 0.56 % hypotonic solution of KCl were added to 1 mL cell suspension. Fixation of chromosomes was performed in freshly prepared fixative (glacial acetic acid/methanol – 1:3) 3-4 times with 10-20 minutes exposure. The obtained preparations were stained with Leucodif 200 (*Erba Lachema*, Czech Republic) according to manufacturer's instructions. Analysis of metaphase plates was performed with a microscope Axiovert 40 (*Carl Zeiss*, Germany) at magnification ×400 and ×1000.

A quantitative abnormality of chromosomes (aneuploidy, polyploidy) and mitotic index (the percentage of cells in division of the total analyzed cells) were analyzed [3]. The percentage of binucleated cells, cells with micronuclei and apoptotic cells was calculated for 500 cells. Statistical analysis was performed by non-parametric Mann-Whitney test. The differences between the values at $p < 0.05$ were assumed as statistically significant.

RESULTS AND DISCUSSION

Karyotype analysis of rat pancreatic cells during their cultivation showed that they are characterized by quantitative disorders (aneuploidy and polyploidy). The research results of change in the number of chromosomal disorders are shown in **Table 1**.

Aneuploid cells were noted from the first to the sixth passage in an amount of 2.2 % to 16.6 %. Cytogenetic variability (aneuploidy) was mainly accounted in cells which karyotype had 39 or 40 chromosomes (**Fig. 1, c**). The difference between the average values on this basis in populations passages 3-6 was reliable ($p < 0.01$), compared with the first ones. The highest level of aneuploidy (16.6 %) was observed in the fourth passage, then there was a decrease in the number of aneuploid cells (passage 5) and increase in their number in the sixth passage.

These changes can be explained by the fact that programmed cell death *in vitro* is induced by accumulation of genetic errors, as was noted on fourth passage by increase of apoptotic cells percentage (Table 2) [8]. At the same time obtained data did not exceed spontaneous level of somatic mutagenesis, characteristic of mammals' lymphocytes [11].

Fold increase in the number of chromosomes (polyploidy) manifested itself in a population of cells from the second to the sixth passage (**Fig. 1, b**). Polyploidy can be caused by a deviation from the normal mitosis, and as a result of two cells fusion, a characteristic of the cell cultures [4]. Starting from the second passage there was observed a tendency to increase in this genomic mutation to 7.8 %. However, our result was lower than spontaneous chromosomal variability for mammals (6-15 %) [7, 11].

To evaluate the cytogenetic changes in rat pancreatic cells culture, micronucleus test was performed. Micronuclei are formed as a result of

PASSAGE NO.	CELLS WITH NORMAL KARYOTYPE, %	ANEUPLOIDY, %	POLYPLOIDY, %
I	97.8 ± 1.3	2.2 ± 1.3	0.0 ± 0.0
II	94.5 ± 1.3	4.4 ± 2	1.1 ± 1.3
III	85.5 ± 1.3**	8.9 ± 1.3**	5.6 ± 1.3*
IV	75.5 ± 1.2**	16.6 ± 2.0**	7.8 ± 1.3**
V	83.3 ± 2.0**	12.2 ± 1.3**	4.5 ± 2.6
VI	82.3 ± 1.3**	13.3 ± 0.0***	4.4 ± 1.3*

Table 1. The results of cytogenetic analysis of rats' pancreas cell culture 1-6th passages, M ± m, n = 3.

*Note: * – $p < 0.05$;*
*** – $p < 0.01$;*
**** – $p < 0.001$ compare with passage I*

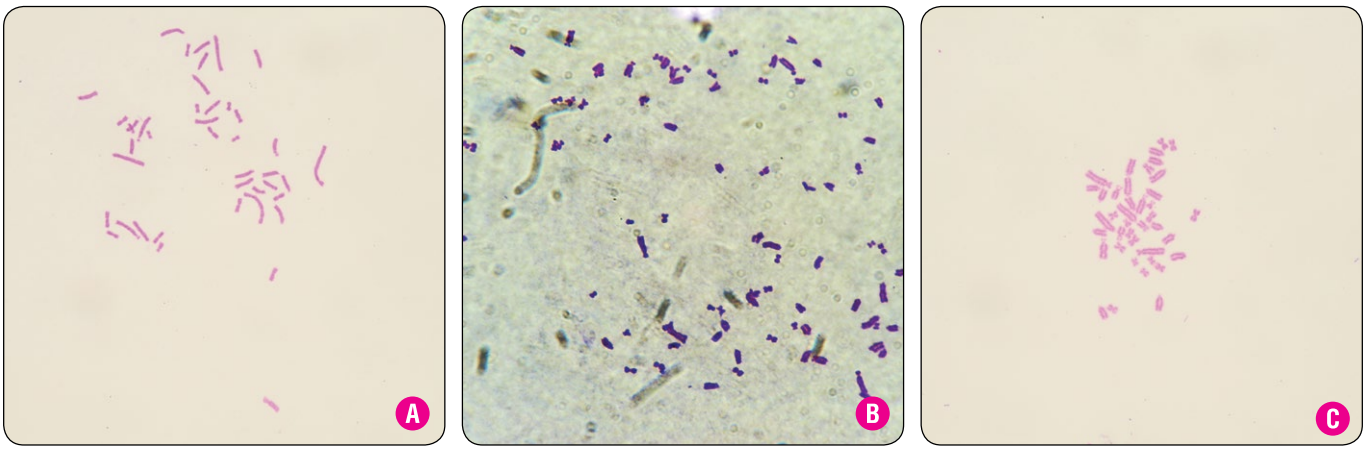

Fig. 1. Photomicrographs of metaphase plates of rat pancreatic cells culture (passage 4), Leucodif 200 staining; oc. ×10, ob. ×100: A) normal karyotype (42 chromosomes); B) polyploidy (84 chromosomes); C) aneuploidy (40 chromosomes).

the spindle abnormality, which leads to mitotic nondisjunction or delay in the chromosomes divergence to the poles of the cell. The micronuclei may include both separate complete chromosomes and their fragments (**Fig. 2, a**). They are pathological structures and their formation is associated with chromosomal instability [3, 4].

The results shown in **Table 2** indicate the presence of micronuclei in all passages. Moreover, a significant increase in their number was observed since the second passage. However, the percentage of cells with micronuclei was in the normal range for mammals (1.6-5.6 %) [7, 20].

At the same time we have seen a significant increase in the number of binucleated cells from the first to the sixth passage, which may be due to the extension of the cell cycle, including cytokinesis (**Fig. 2, b**). However, their number was less than spontaneous mutations characteristic of mammals (5.4%) [7, 20].

During the study there was a decrease in mitotic index from the first (2.7 %), to the third passage (1.5 %) and its gradual increase in fourth (1.7 %) and sixth (2.0 %) passages. A normal rate for mammals is 2.9–4.1 % [7, 11].

In addition, there was discovered a small percentage of cells in apoptosis (**Fig. 2, c**), their number gradually increased to the 4th passage (0.5 %). The 5th-6th passages showed decrease in the number of apoptotic cells to 0.1 %. The level of apoptotic cells was normal.

Recently, the question of genetic stability of cell culture was raised by many scientists. The majority of publications describe properties of only mesenchymal stem cells [2, 5, 14-16, 18]. Part of the studies on mesenchymal stem cells highlights the issue of the effect of genome instability on spontaneous immortalization and malignant transformation of cell culture [16]. At the same time some researchers point to a lack of genetic errors in cell culture *in vitro*, at their cultivation up to 2 months [15, 18]. Studies of other authors indicate the absence of tumors in immunodeficient mice after injection of cell culture with changes in karyotype at the early stages of cultivation [14].

However, the majority of researchers believe that malignant transformation is possible with long-term expansion of cell culture [2, 13, 14, 16, 18]. These data prove that the number of cells with altered karyotype increases with the time of cultivation. However, they point out that a source of cell culture, cultivation conditions (index of proliferation), enzyme treatment play an important role in maintaining of the culture stability [2, 5, 13].

Our data regarding the chromosomal stability of the rat pancreatic cells culture at early passages confirm that an increase in time of cultivation increases the number of cells with karyotype abnormality. Consequently, study on the safety of cell culture is an important condition for their further application.

PASSAGENO.	CELLS WITH NORMAL NUCLEUS, %	THE CELLS WITH MICRONUCLEI, %	BINUCLEATED CELLS, %	APOPTOTIC CELLS, %	MITOTIC INDEX, %
I	99.7 ± 0.1	0.1 ± 0.1	0.1 ±0.1	0.1 ± 0.1	2.7 ± 0.1
II	99.4 ± 0.2	0.3 ± 0.1	0.3 ±0.1	0.1 ± 0.1	2.1 ± 0.1**
III	99.0 ± 0.0***	0.5 ± 0.1*	0.5 ±0.1*	0.1 ± 0.1	1.5 ± 0.1***
IV	98.0 ± 0.1***	0.7 ± 0.2*	0.8 ±0.1**	0.5 ± 0.1*	1.7 ± 0.1***
V	98.5 ± 0.1***	0.5 ± 0.1*	0.8±0.0***	0.2 ± 0.1	1.4 ± 0.0***
VI	98.4 ± 0.2**	0.7 ± 0.1**	0.8±0.1***	0.1 ± 0.1	2.0 ± 0.1**

Table 2. The results of nucleus analysis of rat pancreatic cells culture, M ± m, n = 3.

Note:
** – p < 0.05;*
*** – p < 0.01;*
**** – p < 0.001*
compare with passage I.

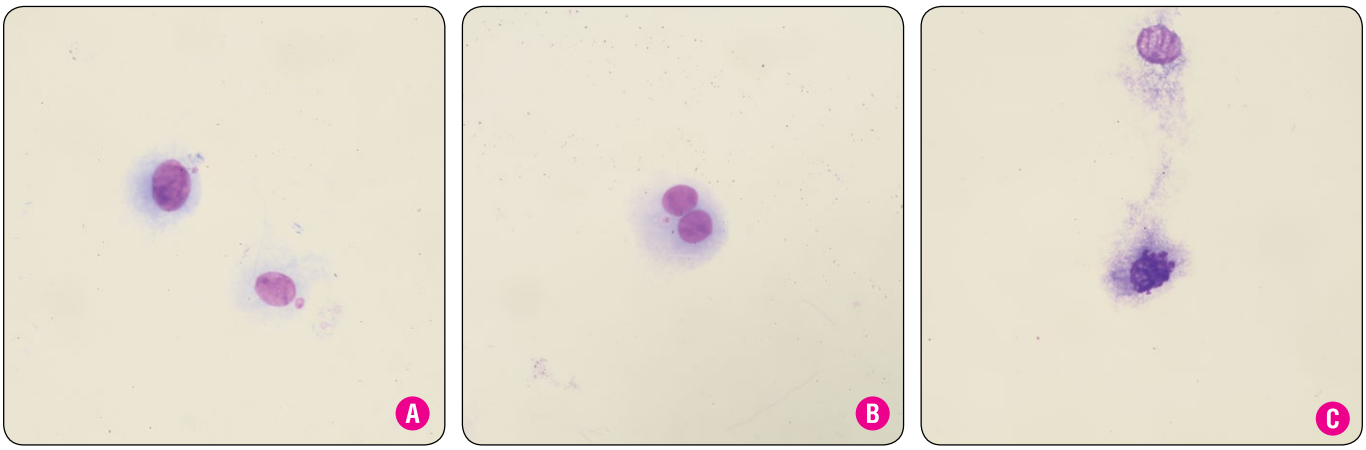

Fig. 2. Photomicrographs of cytopreparations of rat pancreatic cells culture (passage 4) with changes in the nucleus, Leucodif 200 staining; oc. 10, ob. 100: A) cells with micronuclei; B) binucleated cells; C) apoptotic cells.

CONCLUSION

1. ***Analysis of karyotype of the rat pancreatic cells culture above showed that the amount of aneuploidies and polyploidies changes with each passage, but does not extend beyond the spontaneous mutagenesis characteristic of mammals.***
2. ***Cytogenetic study of the culture revealed that the number of cells with micronuclei, binucleated cells and cells in the apoptosis state is in the normal range from the first to the sixth passage.***

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