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Подано експериментальні дані про особливості будови, розвитку та функціонування рослинних і тваринних організмів, флору та фауну України, одержані на основі досліджень, що проводяться науковцями ННЦ "Інститут біології" в галузях фізіології рослин і тварин, генетики, ботаніки, зоології, мікробіології, вірусології. Викладено також нові дані стосовно біохімічних і біофізичних основ регуляції у клітинах і органах у нормі й після впливу різноманітних фізико-хімічних факторів, наведено результати нових методичних розробок.

Для викладачів, наукових співробітників, аспірантів та студентів.

Подано экспериментальные данные об особенностях строения, развития и функционирования растительных и животных организмов, флору и фауну Украина, полученные на основе исследований, проводимых учеными ОНЦ "Институт биологии" в области физиологии растений и животных, генетики, ботаники, зоологии, микробиологии, вирусологии. Изложены также новые данные обиохимических и биофизических основ регуляции в клетках и органах в норме и после воздействия различных физико-химических факторов, приведены результаты новых методических разработок.

Для преподавателей, научных сотрудников, аспирантов и студентов.

Collection of articles written by the scientists of ESC "Institute of Biology" contains data on research in molecular biology, physiology, genetics, microbiology, virology, botanics, zoology concerning the structure, development and function of the plant and animal organisms, flora and fauna of Ukraine. Results of newly developed biophysical methods of biological research, biochemical data regarding metabolic regulation under the influence of different factors are presented.

For scientists, professors, aspirants and students.

ЗМІСТ

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EXPRESSION OF *REG1A* GENE IN RAT DUODENAL UPON LONG-TERM HYPOACIDITY AND WITH ADMINISTRTION OF MULTIPROBIOTIC "SYMBITER® ACIDOPHILIC" CONCENTRATED

The appearance of Reg1a gene's expression in rat's duodenal villus epithelial cells and the increasing of mRNA level of Reg1a in crypt epithelial cells upon hypoacidic conditions were shown. The level of Reg1α expression in villus epitheliocytes was decreased (in 1,6 times), while the level of Reg1α gene mRNA was similar to the control value in crypt epitheliocytes upon treatment of hypoacidic rats with multiprobiotic "Symbiter® acidophilic" concentrated.

Key words: hypoacidity, duodenal, gene expression, multiprobiotics.

Introduction. The *Reg1α* gene is an interleukin (IL)-6-inducible gene which encodes regenerative protein (regenerating islet-derived protein 1*a*) [11, 12]. REG Ia protein plays important roles in the process of wound healing in non-neoplastic gastric mucosa [11, 12]. Also it provides formation of endocrine islands and regeneration of pancreatic tissue upon pathological conditions and is also involved in the differentiation of pancreatic cells upon regeneration [10, 13].

In the small intestine REG I a protein is localized in Paneth cells and nonmature columnar cells of the human intestinal crypts, but never in functional villous epithelium [2]. Therefore, such topographical distribution is associated with growth and also with differentiation process in proliferative cells of intestinal epithelium [2].

The *Reg1α* gene does not express in healthy high differentiated tissues of many organs: liver, spleen, brain and so on [7, 15], while the appearance of this gene expression in above mentioned tissues (not in the active regeneration upon pathological conditions in some organs [15]) may be related with tumorigenesis [7, 11, 12, 16]. According to a scientific literature, besides these, increased expression of *Reg1α* gene encoding eponymous protein is associated with regeneration of pancreatic islet cells and diabetogenesis upon damage of the gland [10], with inflammation (ulcerative colitis, Crohn's disease and so on) and carcinogenesis in the gastrointestinal tract (GIT) [9, 11, 12, 16]. REG Ia overexpression is linked to STAT3 (signal transducer and activator of transcription 3 signaling) activation by activating the Akt/Bad/Bcl-xL pathway in human gastric cancer tissues. These data suggest that REG Ia protein plays a critical anti-apoptotic role in gastric tumorigenesis under STAT3 hyperactivation [11, 12].

Therefore, different alterations in *Reg1α* gene expression may lead to the development of various morbid conditions in GIT [2, 9, 11, 12].

In recent decades, proton-pump inhibitors (PPI) of gastric parietal cells, such as omeprazole, remain the most effective therapeutic agents against acid-related disorders [5, 13]. Development of dysbiosis is one of the key consequences of long-term hypoacidity. Colonization of GIT by opportunistic microbiota forms stable sources of endogenous infection and additionally promotes gastric carcinogenesis [13]. It is proved in clinical trials that probiotics are able not only to cure dysbiotic states, but also to immediately reduce damage ratio of GIT [4, 5].

Multiprobiotics of "Symbiter®" group (hereinafter referred to as Symbiter) are characterized by complexity, wide array of bioactivity and composition that is maximally close to nature microbial populations of human and animals [5].

Analysis of scientific literature showed lack of data on pattern of above mentioned gene expression in duodenal upon experimental or natural hypoacidity. Data on effect of probiotics on gene expression in duodenal upon these conditions are also absent.

Therefore, the aim of current investigation was to determine the expression of *Reg1a* gene in rat duodenal upon long-term injection of omeprazole and with administration of Symbiter.

Materials and methods. The international recommendations on performance of medical and biological investigations with the use of animals according to European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes were followed. Experiments were carried out on white non-strain male rats with initial weight around 180-200 g.

All animals were divided into four groups. The rats injected abdominally with 0,2 ml of physiological solution and 0,5 ml of water for injections orally were used as a control (first group). Animals of second group were treated with Symbiter (manufactured by LLC "O.D. Prolisok") orally (0,14 ml/kg) during 28 days. Hypoacidity (third group) was modeled by everyday intraperitoneal injection of omeprazole (14 mg/kg) during 28 days [14]. Fourth experimental group simultaneously with omeprazole obtained Symbiter in the same dose. Number of animals in each experimental group was 6.

Crypts and villi of duodenal epithelial cells were extracted by means of low-temperature method [5]. RNA was isolated following *Chomczynski* [3]; cDNA was synthesized in 20 μl of reaction mix containing 2 μg of RNA, 1 mM dNTP, 50 U of reverse transcriptase "MultiScribe™ Reverse Transcriptase", corresponding buffer, 20 U of ribonuclease inhibitor "RNase Inhibitor" ("Applied Biosystems", США), 20 pmol of reverse primer. Synthesis was carried out in the following conditions: 37° C – 2 hour. Polymerase chain reaction was performed in 30 μl of reaction mix containing 10 μl of cDNA, PCR buffer, 200 μM of each dNTP, 30 pmol (1,0 μM) of each primer, 2,5 mM of $MgCl_2$ and 1,5 U of Taq DNA polymerase ("iTaq™", "Bio-Rad", США).

PCR amplifications consisted of an initial denaturing step of 94ºC for 4 min, followed by 35 (30 for *β-actin* – gene used as internal control of reaction due to its constitutive expression) cycles of 94ºC for 45 s, the annealing step (with optimal annealing temperature): *Reg1a* (608 b. p., 48º C – 45 s) and β-actin (521 b. p., 49º C – 40 s); the extending step at 72º C for 1 min 15 s (for *Reg1a*) or 1 min (for *β-actin*). Final extension step was performed upon 72º C for 5 min.

Such primer sequences were used in reactions: for *Reg1α* – forward – AGCCTGCAGAGATTGTTGAC and reverse – CCATAGGGCAGTGAGGCAAG; for *β-actin* – forward – TGGGACGATATGGAGAAGAT and reverse – ATTGCCGATAGTGATGAXCT. Separation of PCR products was performed electrophoretically in 1,6 % agarose gel with 0,5 x TBE buffer. For semi-quantitative analysis of amplicons expression based on densitometry the ImageJ 1.45s program was used. Indices of mRNA expression were calculated for each sample following *Konturek et al.* [8].

Mathematical and statistical processing of experimental data was performed using GraphPad Prism 4.03 ("GraphPad Software Inc.", USA). The normal Gaussian distribu-

tion of the data was verified by the Shapiro-Wilk normality test. Two-way analysis of variance (two-way ANOVA) and Bonferroni post tests were performed on obtained data. Statistical significance was set at $p \leq 0,05$. The data are expressed as means and standard deviations. For graphical data means and 95 % confidence intervals are used.

Results and discussion. PCR analysis of cDNA samples generated in the rat's duodenal crypt epithelial cells indicated the presence of a specific signal with the expected length (608 b. p.) for *Reg1a* gene both in the control and second groups of investigated animals. While mRNA of this gene wasn't detected in villous epithelium both of the control group and animals treated only with Symbiter (Fig. 1.).

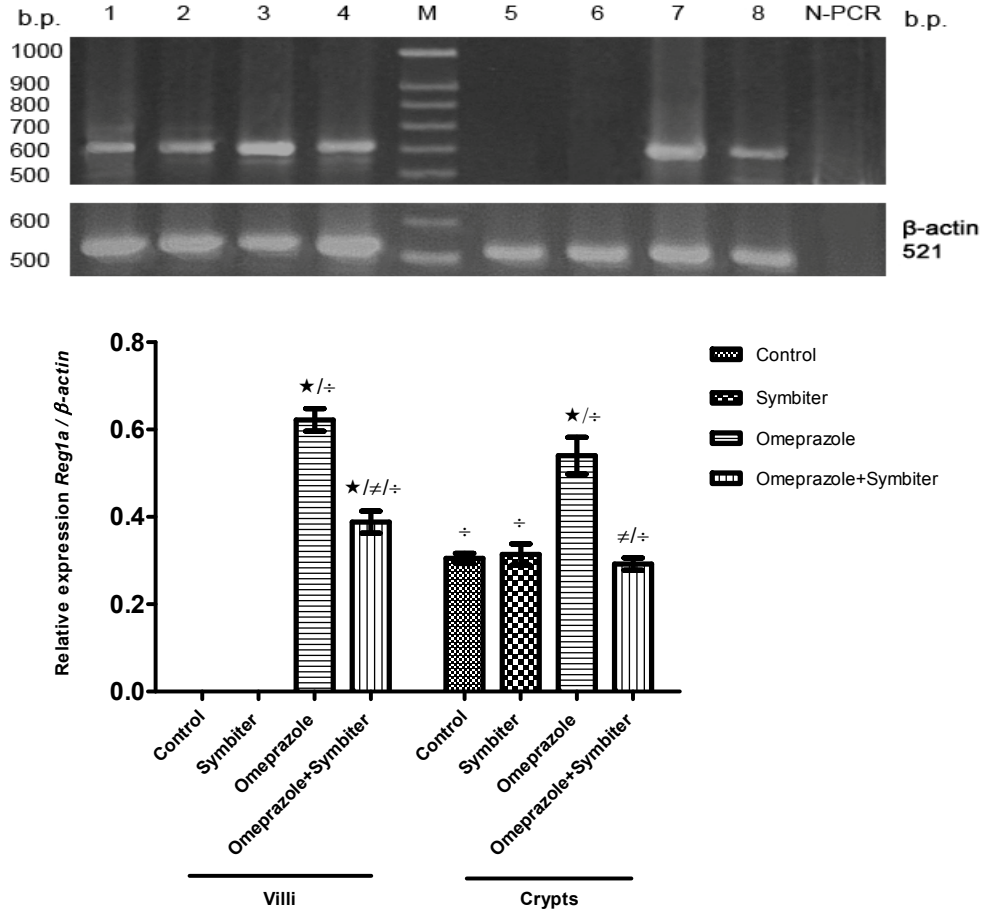

Fig. 1. Level of *Reg1a* gene mRNA in rat duodenal upon long-term hypoacidity and with administration of multiprobiotic "Symbiter® acidophilic" concentrated.

M – molecular mass marker; villus epithelial cells: 1 – control; 2 – Symbiter; 3 – omeprazole; 4 – omeprazole + Symbiter; crypts epithelial cells: 5 – control; 6 – Symbiter; 7 – omeprazole; 8 – omeprazole + Symbiter; N-PCR – negative PCR control; * – $p \leq 0,0001$ in relation to control; # – $p \leq 0,0001$ in comparison with animals treated with omeprazole; ÷ – $p \leq 0,0001$ villi in comparison with crypts. The levels of above mention gene's expression did not significantly differ *in the* crypt epithelial cells of the control and second groups from one another. On the other hand, the levels of *Reg1α* mRNA in duodenal samples of animals treated with omeprazole during 28 days and rats upon simultaneous administration of omeprazole with multiprobiotic Symbiter as significantly differentiate both in villous epithelium and crypts as between analyzed groups of animals ($F = 348,1$, $DFn = 3$, $DFd = 40$, $p \leq 0,0001$) (Fig. 1., Table. 1.).

Table 1. **Level of *Reg1a* gene mRNA in rat duodenal upon long-term hypoacidity and with administration of multiprobiotic "Symbiter® acidophilic" concentrated, (m ± SD, n = 6)**

Groups of animals	Epithelial cells	Relative expression *Reg1a / β-actin*
Control	villi	0
	crypts	$0,305 \pm 0,0110^{\div}$
Symbiter	villi	0
	crypts	$0,314 \pm 0,0231^{\div}$
Omeprazole	villi	$0,622 \pm 0,0247^{*\div}$
	crypts	$0,540 \pm 0,0403^{*\div}$
Omeprazole + Symbiter	villi	$0,388 \pm 0,0242^{\neq\div}$
	crypts	$0,292 \pm 0,0135^{\neq\div}$

Notes: SD – standard deviation;
** – $p \leq 0,0001$ in relation to control;*
≠ – $p \leq 0,0001$ in comparison with animals treated with omeprazole;
÷ – $p \leq 0,0001$ villi in comparison with crypts.

In functional villous epithelium of duodenal we found mRNA of *Reg1α* gene upon long-term hypoacidity. At the same time in fourth group of rats the level of this gene was in 1,6 times lower than in animals injected with omeprazole ($p \leq 0,0001$).

In crypts epithelium of animals treated only with omeprazole for 28 days the level of *Reg1α* mRNA was in 1,8 times higher than control values ($p \leq 0,0001$). While upon simultaneous administration of multiprobiotic Symbiter this parameter was approximately in 1,9 times lower than in animals of third group ($p \leq 0,0001$). In animals treated only with Symbiter this parameter was similar to the control value (Fig. 1., Table. 1.).

It is well known, that the main function of crypts is reproduction of cell, which are able to regenerate intestinal epithelial tissue. It is well known, that the crypt base columnar cells are only proliferative cells and that the mid-crypt columnar cells proliferate and also differentiate, whereas the crypt top columnar cells never divide, but continue to differentiate and then move along the lines of villi [2, 13].

As it was demonstrated, *Reg1a* gene mRNA expression was observed in Paneth cells, in the glands of Lieberkühn and nonmature columnar cells of the human intestinal crypts, but never in the mature functional villous epithelium [2]. Therefore, REG protein may be associated with growth and early differentiating of intestinal epithelium, as has been proposed for pancreas [2].

In our experiment we demonstrated the elevation of *Reg1a* mRNA level in crypt epithelial cells upon hypoacidic conditions (Fig. 1., Table. 1.). It can be assumed about intensification of inflammatory processes in duodenal [2, 9].

The appearance of *Reg1a* gene's expression in duodenal villus epithelial cells in rats treated only with omeprazole (Fig. 1., Table. 1.) may be associated with possible neoplasia in epithelial cells on later stages of pathology process development [2, 9, 11, 12, 16].

Thus, the obtained changes in expression of *Reg1a* gene in rat duodenal villus and crypts epithelial cells upon hypoacidic conditions should point out the development of teratoid displacements in duodenal tissue. Multidirectionality in alterations of *Reg1a* gene's expression in villus and crypts epithelial cells is determined by their structural and functional features.

Among probable mechanisms of Symbiter's action on gene expression in rat pancreas, firstly, it should be pointed out its ability to liquidate dysbiosis and bacterial colonization of GIT – it was observed in a number of investigations [1]. As a consequence, the burden of pathogenic microbiota is removed from GIT and associated organs [4, 5]. Furthermore, multicomponent probiotics such as Symbiter are able to increase *de novo* synthesis of the main low-molecular cellular antioxidant – reduced glutathione and, thus, to raise its content both in GIT and duodenal [1]. So, preliminary treatment with probiotics can ameliorate the rate of oxidative stress, inflammatory processes and damage of the duodenal [1].

Conclusion. In summary, we have shown, that long-term experimental hypoacidity is accompanied by changes in expression of *Reg1a* gene in rat duodenal. While upon simultaneous administration of multiprobiotic "Symbiter® acidophilic" the expression pattern of this gene is similar to control. The level of *Reg1α* expression in villus epitheliocytes was decreased, while the level of *Reg1α* gene mRNA was similar to the control value in crypt epitheliocytes upon treatment of hypoacidic rats with multiprobiotic "Symbiter®" acidophilic" concentrated. Based on the obtained data, it can be assumed, that there is some potential risk of duodenal carcinogenesis upon long-term use of omeprazole (and probably other PPIs). The final explanation of molecular mechanisms underlying the changes in expression of *Reg1a* gene in rat duodenal upon long-term hypoacidity and with administration of multiprobiotic Symbiter requires further experiments.

References

1. An H., Zhai Z., Yin S. et al. Coexpression of the superoxide dismutase and the catalase provides remarkable oxidative stress resistance in Lactobacillus rhamnosus // J. Agric. Food Chem. – 2011. – Vol. 59(8). – P. 3851-3856.

2. Bernard–Perrone F., Renaud W., Guy–Crotte O. Expression of REG Protein During Cell Growth and Differentiation of Two Human Colon Carcinoma Cell Lines // The Journal of Histochemistry & Cytochemistry. – 1990. – 47 (7). – P. 863–870.

3. Chomczynski P., Sacchi N. Single-step method of RNA isolation by acid guanidiniumthiocyanate-phenol-chloroform extraction // Anal. Biochem. – 1987. – Vol. 162. – P. 156-159.

4. Culligan E., Hill C., Sleator R. Probiotics and gastrointestinal disease: successes, problems and future prospects // Gut Pathogens. – 2009. – Vol.1, №19. – P. 38-49.

5. Falaleeva T.M., Virchenko O.V., Beregova T.V. et al. Correction of stress-induced changes in hypothalamic-pituitary-adrenal system by multiprobiotic "Symbiter" // World of medicine and biology. – № 1. – 2012. – P. 173-178.

6. Flint N., Cove F.L., Evans G.S.H. A low-temperature method for the isolation of small-intestinal epithelium along the crypt-vinlus axis // Biochem J. – 1991. – Vol. 280 (Pt 2). – P. 331–334.

7. Kobayashi S., Akiyama T., Nata K. Identification of a Receptor for Reg (Regenerating Gene) Protein, a Pancreaticb-Cell Regeneration Factor // The journal of biological chemistry. – 2000. – 275 (15). – P. 10723–10726.

8. Konturek P.C., Brzozowski T., Pierzchalski P. et. al. Activation of genes for spasmolytic peptide, transforming growth factor alpha and for cyclooxygenase (COX)-1 and COX-2 during gastric adaptation to aspirin damage in rats. // Aliment Pharmacol. Ther. – 1998. – 12. – P. 767–777.

9. Planell N., Lozano J., Mora-Buch R. Transcriptional analysis of the intestinal mucosa of patients with ulcerative colitis in remission reveals lasting epithelial cell alterations // Gut. – 2012. – 11. – P. 1–10.

10. Sanchez D., Mueller C., Zenilman M. Pancreatic Reg I and acinar cell differentiation: influence on cellular lineage // Pancreas. – 2009. – Vol.38 (5). – P. 572-577.

11. Sekikawa A., Fukui H., Fujii S. et al. Possible role of REG Ia protein in ulcerative colitis and colitic cancer // Gut. – 2005. – 54. – P. 1437–1444.

12. Sekikawa A., Fukui H., Fujii S. et al. REG Ia protein mediates an anti-apoptotic effect of STAT3 signaling in gastric cancer cells // Carcinogenesisvol. – 2008. – 29 (1). – P.76–83.

13. Textbook of gastroenterology / T. Yamada, D. Alpers, A. Kalloo et al. – Wiley-Blackwell; 5th edition, 2008. – 3712 p.

14. Tsyriuk O.I., Beregova T.V. Effect of omeprazole-induced hypergastrinemia on the basal gastric secretion in rats // Bulletin of biological and medical issues. – 2007. – № 3. – P. 38-43.

15. Wang J., Koyota S., Zhou X. et al. Expression and localization of regenerating gene I in a rat liver regeneration model // Biochem Biophys Res Commun. – 2009. – 380 (3). – P. 472-477.

16. Yuan R., Jeng Y., Chen H. et al. Opposite roles of human pancreatitis-associated protein and REG1A expression in hepatocellular carcinoma: association of pancreatitis associated protein expression with low-stage hepatocellular carcinoma, B-catenin mutation and favorable prognosis // Clin Cancer Res. – 2005. – 11. – 2568-2575.

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ЕКСПРЕСІЯ ГЕНА *REG1A* В ЕПІТЕЛІОЦИТАХ ДВАНАДЦЯТИПАЛОЇ КИШКИ ЩУРІВ В УМОВАХ ДОВГОТРИВАЛОЇ ГІПОАЦИДНОСТІ ТА ПРИ ВВЕДЕННІ МУЛЬТИПРОБІОТИКА "СИМБІТЕР® КОНЦЕНТРОВАНИЙ АЦИДОФІЛЬНИЙ"

Виявлено експресію гена Reg1a в епітеліоцитах ворсинок та зростання рівня мРНК цього гена в епітеліоцитах крипт дванадцятипалої кишки за гіпоацидних умов. При введенні мультипробіотика "Симбітер® ацидофільний" концентрований за тих же умов рівень експресії Reg1a в епітеліоцитах ворсинок зменшувався в 1,6 рази, у той час як вміст мРНК Reg1a в криптах був на рівні контрольних значень.

Ключові слова: гіпоацидність, дванадцятипала кишка, експресія генів, мультипробіотики.

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ЭКСПРЕССИЯ ГЕНА *REG1A* В ЭПИТЕЛИОЦИТАХ ДВЕНАДЦАТИПЕРСТНОЙ КИШКИ КРЫС В УСЛОВИЯХ ДЛИТЕЛЬНОЙ ГИПОАЦИДНОСТИ И ПРИ ВВЕДЕНИИ МУЛЬТИПРОБИОТИКА "СИМБИТЕР® АЦИДОФИЛЬНЫЙ"

Выявлено экспрессию гена Reg1a в эпителиоцитах ворсинок и показано увеличение уровня мРНК этого гена в эпителиоцитах крипт двенадцатиперстной кишки в гипоацидных условиях. При введении мультипробиотика "Симбитер® ацидофильный" концентрированный в тех же условиях уровень экспресии Reg1a в эпителиоцитах ворсинок уменьшился в 1,6 раза, в то время как содержание мРНК Reg1a в криптах было на уровне контрольных значений.

Ключевые слова: гипоацидность, двенадцатиперстная кишка, экспрессия генов, мультипробиотики.

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PROMOTER DNA METHYLATION OF E-CADHERIN GENE IN MOLECULAR PATHOGENESIS OF ADHESIVE COMPLEX AND ACTIVATION OF EMT MARKERS *SNAIL* AND *TWIST1* IN MYELOPROLIFERATIVE LEUKEMIA

EMT – the epithelial-to-mesenchimal transduction is the basis platform of the tumor microenvironment. EMT causatively binding with the tumor progression, by which are modulated the migrational, invasive and metastatic potentials of the tumor cells. Therefore, EMT is the crucial microenvironment metastatic niche for the final cancer cell aggressiveness. Moreover, EMT underlie cancer stem cell induction and also controls tumor drug sensitivity modulating the response of cancer cells to chemotherapy. The earlier event of EMT metastatic cascade is the epigenetic deregulation of the E-cadherin-beta-catenin adhesive signaling. The promoter DNA hypermethylation of E-cadherine gene as the key adhesive molecules results in the switch the E-cadherin-beta-catenin adhesive signaling on the canonic beta-catenin-Wnt signaling associated with epithelial-mesenchimal transduction. Thus, the epigenetic regulation of EMT might be considered as the target for a new cancer epigenetic therapy strategies.

Key words: epithelial–mesenchymal transition, E – cadherin, aberrant DNA methylation.

Introduction. The epithelial-mesenchymal transition (EMT) is the key phenomenon in conception of tumor microenvironment [1]. EMT is the tissue-specific mechanism of morphogenesis and organogenesis, which takes place during embryonic development and associated with transformation of the epithelial cells into mesenchymal stem cells [2; 3]. In carcinogenesis EMT is chimerical organogenesis manifested as metastatic tumors. Thus, it is abnormal program leads to migrational, invasive and metastatic properties of tumor cells [4]. These processes implement at different stages of cell differentiation [5; 6]. The violation of epigenetic control in carcinogenesis is associated with aberrant promoter DNA methylation of E – cadherin gene – the key molecule of adhesive complex. This event leads to abnormal activation of EMT in tumor progression [7]. Thus, epigenetic deregulation of E-cadherin-beta-catenin adhesive signaling plays the key role in violation of epigenetic control in myeloproliferative leukemia.

The aim of our study was to investigate the epigenetic regulation of EMT as a result of aberrant DNA methylation of E – cadherin gene and its association with EMT modulating transcription factors *Snail* and *Twist1* in progression of myeloproliferative leukemia, such as chronic and acute leukemia (CML and AML).

Tumor microenvironment is the central factor of carcinogenesis, especially in tumor progression and metastasis [8]. The mesenchimal stem cells (MSCs) are progenitors of tumor stem cells (CSCs) in cancer progression. The morphological heterogeneity is crucial criterion in classification of tumors. Chromosomal aberrations find in different types of tumor cells [9]. Scientists suggested, that aneuploidy might explain genetic instability in tumors with genetic mutations (aneuploidy theory of carcinogenesis, Theodore Bovary, 1914). These changes in chromosomes may occur millions of nucleotides [10]. Even the most advanced program of sequencing of the human genome does not provide definitive answers about the mechanisms of carcinogenesis [11].

The epithelial – mesenchymal transduction activates during carcinogenesis and leads to invasion and metastasis [12]. The EMT is activated by inhibition of the expression of E – cadherin protein [13, 14] and E – cadherin/β – katenin adhesive complex [15, 16]. Thus, mesenchymal phenotype is acquired by epithelial cells [17]. Epigenetic silencing of promoter DNA-methylation of E-cadherin is crucial event on primary stages in cancer [18]. This step results into the switch of the E – cadherine/β – catenin adhesive signaling [14] on the canonic β – catenin/Wnt signaling [19], which associates with epithelial – mesenchimal transduction.

The regulation of E-cadherin promoter occurs in two ways: transcription regulation through inhibition of factors *Snail, Slug, Twist1, Zeb1, Zeb2* in *E-box* region [20; 21] and epigenetic regulation through aberrant promoter methylation of E-cadherin gene [22; 23]. Unrestrained Wnt signaling and/or loss of the cell adhesion occur during progression of cancer [14, 22]. The key event of carcinogenesis is lack of the control over level of β – catenin, which may lead to the destabilization of adhesive complex [19, 24].

Materials and methods. The object of our study was samples of patient's peripheral blood from the National Institute of Cancer. In our work we used such methods as extraction of total DNA and genomic RNA, bisulfate modification of DNA using EpiTect Bisulfite Kit (QIAGEN), methyl – specific PCR of bisulfate modificated DNA and PCR with reverse transcription. In these methods primers to the promoter region of E–cadherine were used, where cytosine was replaced to thymine, which complementary to uracil (5'-TGGTTGTAGTTATGTATTTATTTTTAGTGGTGTT-3'-forward and 5'-ACACCAATACAACAAATC AAACCAAA-3'-reverse); primers to transcription factors *Snail* (560 bp) 5′-CAGACCCACTCAGATGTCAA-3′ (forward) та 5′-CATAGTTAGTCACACCTCGT-3′ (reverse), *Twist1* (130 bp) 5′-CGGGAGTCCGCAGTCTTA-3′ (forward) та 5′- TGAATCTTGCTCAGCTTGTC -3′ (reverse). We used program *TotalLab v. 2.01* for quantity estimation of amplification product.

Results and discussion:

In our work we used four samples of patent peripheral blood.

- Patient with CML (chronic myeloprolipherative lekemia).
- Patient with AML (acute myeloprolipherative lekemia).
- Patient with ET (essential trombocytosis).
- Negative control HD (healthy donor).

Total DNA was extracted from patient peripheral blood leucocytes with myeloproliferative leukemia. These DNA samples were exposed to bisulfate modification. Then methyl – specific PCR (MS-PCR) was conducted. Electrophoregramme of products of methyl – specific PCR for identification of promoter DNA – methylation of E – cadherin gene is demonstrating on Figure 1. The lack of amplification product was observed in patients with CML and AML, in compared to healthy donor. This indicates the presence of the aberrant promoter DNA – methylation of E – cadherin gene in patients with CML and AML.

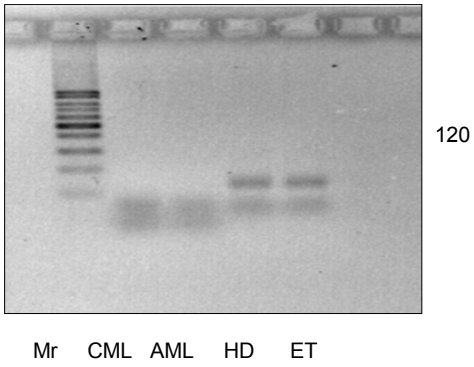

Fig. 1. Electrophoregram of products of methyl – specific PCR for identification of promoter DNA–methylation of E–cadherin gene

Electrophoregramms with amplification products of target genes *Snail* and *Twist1* (A) and the control gene *β – actin* (B) are demonstrated on Figures 2 and 3. The product of amplification was observed in patients with CML, AML and ET, which indicate expression of transcription factors *Snail* and *Twist1* in patients with various forms of leukemia.

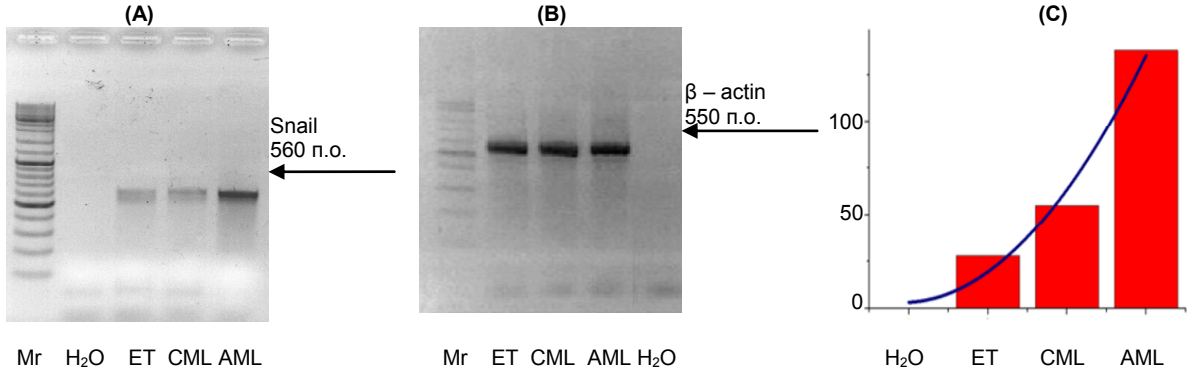

Fig. 2. Electrophoregramms with amplification products of target genes *Snail* (A) and the control gene *β – actin* (B) and graphical interpretation of the results (C)

Using densitometry analysis we revealed, that amount of amplified products of *Snail* gene increased at 2, 54 times, respectively, in patients with AML, in compared to patients with CML. The amount of referent gene *β – actin* was not significantly changed in all experimental samples.

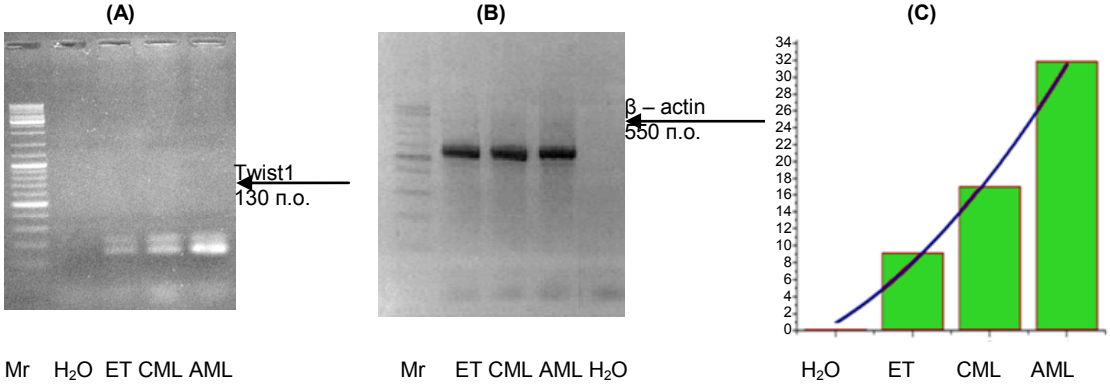

Fig. 3. Electrophoregramms with amplification products of target genes *Twist1* (A) and the control gene β – actin (B) and graphical interpretation of the results (C)

Using densitometry analysis we revealed, that amount of amplified products of *Twist1* gene increased at 1, 88 times, respectively, in patients with AML, in compared to patients with CML. The amount of referent gene *β – actin* was not significantly changed in all experimental samples.

Thus, using such methods, as bisulphate modification and methyl–specific PCR we indicate aberrant *CpG*-promoter methylation of E-cadherin gene in patients with CML and AML, whereas myeloproliferative disease essential trombocytosis (ET) has not this epigenetic modification. These results are similarly to the existing literature about the progression of metastatic tumors in various types of cancer [25-28], including the progression of leukemia [29, 30].

The mRNA expression of *Snail* and *Twist1* observed in progression of CML and AML. Chronic and acute forms of leukemia associated with reciprocal translocation t (9; 22) (q34; q11) between long arms of 9 and 22 chromosomes. This translocation results into fusion gene *bcr-abl* [31; 32]. Therefore, chimerical gene *bcr-abl* can be genetic marker of CML and AML, whereas ET has not this Ph-translocation.

Conclusion. Epigenetic transcriptional deregulation of E – cadherin gene at the expense of aberrant methylation of *CpG*-promoter in CML and AML, associated with expression's analysis of transcriptional factors *Snail* and *Twist1,* that modulated EMT, was shown.

Also we indicate that expression of *Snail* and *Twist1* significantly increase in progression of myeloproliferative diseases in such direction: essential thrombocythemia (ET); chronic myeloid leukemia (CML); acute myeloid leukemia (AML).

Aberrant hypermethylation of E – cadherine gene and expression of transcription factors *Snail* and *Twist1* play key role in progression of metastatic phenotype and might be considered as the target for a new cancer epigenetic therapy, include myeloproliferative leukemia.

References

1. Yang X, Hou J, Han Z et al. One cell, multiple roles: contribution of mesenchymal stem cells to tumor development in tumor microenvironment // Cell Biosci. – 2013 – Vol. 3, №5 – P. 1 – 6.

2. Jean Paul Thiery. Epithelial – mesenchymal transition in tumor progression // Nature Reviews Cancer – 2002 – Vol.2 – P. 442-454.

3. Tepass U, Truong K, Godt D, et al. Cadherins in embryonic and neural morphogenesis// Nat Rev Mol Cell Biol. – 2000 – Vol.1 – P. 91-100.

4. Cano A, Pérez-Moreno MA, Rodrigo I, et al. The transcription factor snail controls epithelial–mesenchymal transitions by repressing E-cadherin expression // Nature Cell Biol. – 2000 – Vol. 2 – P. 76-83.

5. Cichocki F, Miller JS, Anderson SK. Epigenetic regulation of NK cell differentiation and effector functions // Front Immunol. – 2013 – Vol.4, №55 – P. 1-8.

6. Hammoud SS, Cairns BR, Jones DA. Epigenetic regulation of colon cancer and intestinal stem cells // Curr Opin Cell Biol. – 2013 – Vol. 25, №2 – P. 177-83.

7. Cano A, Pérez-Moreno MA, Rodrigo I, et al. The transcription factor snail controls epithelial–mesenchymal transitions by repressing E-cadherin expression // Nature Cell Biol. – 2000 – Vol. 2 – P. 76-83.

8. Li L, Cole J, Margolin DA. Cancer stem cell and stromal microenvironment // Ochsner J. – 2013 – Vol. 13 – №1 – P. 109-18.

9. Misra A, Chattopadhyay P, Chosdol K, et al. Clonal mutations in primary human glial tumors: evidence in support of the mutator hypothesis // BMC Cancer – 2007 – Vol. 7, №190 – P. 1 – 13.

10. Li R, Sonik A, Stindl R, et al. Aneuploidy vs. gene mutation hypothesis of cancer: recent study claims mutation but is found to support aneuploidy // Proc Natl Acad Sci USA – 2000 – Vol.7 – P. 3236-41.

11. Collins FS, Morgan M, Patrinos A. et al. DNA methylation: an epigenetic pathway to cancer and a promising target for anticancer therapy // J Oral Pathol Med – 2002 – Vol. 31 – P. 443-9.

12. Guarino M, Rubino B, Ballabio G. The role of epithelial-mesenchymal transition in cancer pathology // Pathology. – 2007 – Vol. 39, №3 – P. 305-18.

13. Mareel M, Vleminckx K, Vermeulen S et al. Downregulation in vivo of the invasion-suppressor molecule E-cadherin in experimental and clinical cancer // Princess Takamatsu Symp. – 1994 – Vol. 24 – P. 63-80.

14. Hirohashi S. Inactivation of the E – cadherin – mediated cell adhesion system in human cancer // Am J Pathol. – 1998 – Vol.153, №2 – P. 333–339.

15. Wijnhoven BP, Dinjens WN, Pignatelli M. E-cadherin-catenin cell-cell adhesion complex and human cancer // Br J Surg. – 2000 – Vol. 87, №8 – P. 992-1005.

16. Berx G, Nollet F, van Roy F. Dysregulation of the E-cadherin/catenin complex by irreversible mutations in human carcinomas // Cell Adhes Commun. – 1998 – Vol. 6, №2-3 – P. 171-84.

17. Thiery JP. Epithelial-mesenchymal transitions in development and pathologies // Curr Opin Cell Biol. – 2003 – Vol. 15, №6 – P. 740-6.

18. Mareel M, Vleminckx K, Vermeulen S et al. E-cadherin expression: a counterbalance for cancer cell invasion // Bull Cancer. – 1992 – Vol. 79, №4 – P. 347-55.

19. Kim Y, Thanendrarajan S, Schmidt-Wolf IG. Wnt/beta-catenin: a new therapeutic approach to acute myeloid leukemia // Leuk. Res. Treat. 2011.

20. Nieto MA. The snail superfamily of zinc-finger transcription factors // Nat Rev Mol Cell Biol. – 2002 – Vol. 3 – № 3 – P. 155-66.

21. Puisieux A, Valsesia-Wittmann S, Ansieau S. A twist for survival and cancer progression // Br J Cancer. – 2006 – Vol. 94, №1 – P. 13-7.

22. Hirohashi S. Molecular aspects of adhesion-epigenetic mechanisms for inactivation of the E-Cadherin-mediated cell adhesion system in cancers // Verh Dtsch Ges Pathol. – 2000 – Vol. 84 – P. 28-32.

23. Teodoridis JM, Strathdee G, Brown R. Epigenetic silencing mediated by CpG island methylation: potential as a therapeutic target and as a biomarker // Drug Resist Updat. – 2004 – Vol. 7 – P. 267-78.

24. Logan CY, Nusse R. The Wnt signaling pathway in development and disease // Annu Rev Cell Dev Biol. – 2004 – Vol. 20 – P. 781-810.

25. Smit MA, Geiger TR, Song JY et al. A Twist-Snail axis critical for TrkB-induced epithelial-mesenchymal transition-like transformation, anoikis resistance, and metastasis // Mol Cell Biol. – 2009 – Vol. 29, №13 – P. 3722-37.

26. Jia J, Zhang W, Liu JY et al. Epithelial mesenchymal transition is required for acquisition of anoikis resistance and metastatic potential in adenoid cystic carcinoma // PLoS One. – 2012 – Vol. 7, №12 – P. 1 – 13.

27. McConkey DJ, Choi W, Marquis L et al. Role of epithelial-to-mesenchymal transition (EMT) in drug sensitivity and metastasis in bladder cancer // Cancer Metastasis Rev. – 2009 – Vol. 28, №3-4 – P.335-44.

28. Guarino M, Rubino B, Ballabio G. The role of epithelial-mesenchymal transition in cancer pathology // Pathology – 2007 – Vol. 39, №3 – P. 305-18.

29. Melki JR, Vincent PC, Brown RD et al. Hypermethylation of E-cadherin in leukemia // Blood. – 2000 – Vol. 95, №10 – P. 3208-13.

30. Corn PG, Smith BD, Ruckdeschel ES et. al. E-cadherin expression is silenced by 5' CpG island methylation in acute leukemia // Clin Cancer Res. – 2000 – Vol. 6, №11 – P. 4243-8.

31. Deininger M.W., Goldman J.M. et al. The molecular biology of chronic myeloid leukemia // Blood – 2000 – Vol. 96, №10 – P. 3343-3356.

32. Abdel-Wahab O., Manshouri T., et al. Genetic analysis of transforming events that convert chronic myeloproliferative neoplasms to leukemias // Cancer Research – 2010 – Vol. 70, №2 – P. 447-452.

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ПРОМОТОРНЕ ДНК-МЕТИЛЮВАННЯ ГЕНУ Е-КАДГЕРИНУ В МОЛЕКУЛЯРНОМУ ПАТОГЕНЕЗІ АДГЕЗИВНОГО КОМПЛЕКСУ ТА АКТИВАЦІЇ ЕМТ МАРКЕРІВ – *SNAIL* І *TWIST1* ПРИ МІЄЛОПРОЛІФЕРАТИВНИХ ЛЕЙКЕМІЯХ

Епітеліально-мезенхімальна трансдукція (ЕМТ) – це основна платформа мікрооточення пухлини. ЕМТ причинно пов'язана з раковою прогресією, модулюючи міграційний, інвазивний і метастатичний потенціали ракових клітин. Тому, ЕМТ є важливою метастазуючою нішою мікрооточення пухлини в остаточній агресивності ракових клітин. Більш того, ЕМТ лежить в основі індукції стовбурових ракових клітин та модуляції відповіді ракових клітин на хіміотерапію. Ранньою стадією в індукції ЕМТ є епігенетична дерегуляція Е-кадгерин-бета-катенін адгезивного сигналінгу. Аберантне ДНК-метилювання гену Е-кадгерина, як ключової адгезивної молекули, призводить до молекулярного "переключення" Е-кадгерин-бета-катенін адгезивного сигналінгу на канонічний бета-катенін-Wnt сигналінг, що пов'язується з трансдукцією або дедиференціацією епітеліальних клітин в мезенхімальні. Таким чином, епігенетична регуляція ЕМТ може розглядатись як таргетна мішень для нової стратегії у терапії раку.

Ключові слова: епітеліально–мезенхімальна трансдукція, Е–кадгерин, аберантне ДНК–метилування.

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ПРОМОТОРНОЕ ДНК-МЕТИЛИРОВАНИЕ ГЕНА Е-КАДГЕРИНА В МОЛЕКУЛЯРНОМ ПАТОГЕНЕЗЕ АДГЕЗИВНОГО КОМПЛЕКСА И АКТИВАЦИИ ЭМТ МАРКЕРО – *SNAIL* И *TWIST1* ПРИ МИЕЛОПРОЛИФЕРАТИВНЫХ ЛЕЙКЕМИЯХ

Эпителиально–мезенхимальная трансдукция – это основная платформа микроокружения опухоли. ЭМТ связана с опухолевой прогрессией, модулируя миграционный, инвазивный и метастатический потенциал раковых клеток. Поэтому, ЭМТ – это важная метастазирующая ниша микроокружения опухоли в окончательной агрессивности раковых клеток. Более этого, ЭМТ лежит в основе индукции стволовых раковых клеток и модуляции ответа раковых клеток на химиотерапию. Ранней стадией в индукции ЭМТ есть эпигенетическая дерегуляция Е-кадгерин-бета-катенин адгезивного пути. Аберрантное ДНК-метилирование гена Е-кадгерина, как ключевой адгезивной молекулы, приводит к молекулярному "переключению" Е-кадгерин-бета-катенин адгезивного пути на канонический Wnt-бета-катенин сигнальный путь. Именно он связан с трансдукцией или дедифференциацией эпителиальных клеток в мезенхимальные. Таким образом, эпигенетическая регуляция ЭМТ может быть мишенью для новой стратегии в терапии рака.

Ключевые слова: эпителиально–мезенхимальная трансдукция, Е – кадгерин, аберрантное ДНК-метилирование.

UDC 617.764.1 : 576.342 : 576.08 : 577.4

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PERMEABILIZED SECRETORY CELLS OF RATS EXORBITAL LACRIMAL GLANDS AS A MODEL FOR CA^{2+}-TRANSPORT SYSTEMS STUDY

The conditions of isolation and permeabilization of the rat exorbital lacrimal gland secretory cells were optimized. Optimal amount of digitonin required to exorbital lacrimal gland cells permeabilization was 50 μg / 0.5 million cells, and optimal incubation time – 10 min. Thus obtained permeabilized cells were stained with rhodamine 123 and chlortetracycline, indicating the functional integrity of mitochondria and endoplasmic reticulum. Permeabilized exorbital lacrimal gland cells is convenient model for study of Ca2+-transport systems functioning.

Keywords: lacrimal gland, secretory cells, isolation, permeabilization, digitonin, Ca2+-transport system.

Introduction. Research of lacrimal gland function in general, and their Ca^{2+}-transport systems particularly, are important and promising area of modern physiology. In particular, such common diseases as dry eye syndrome and Sjogren's syndrome are accompanied by lacrimation decrease [2, 26, 33], and it is known that lacrimal secretion is a calcium-dependent process [29, 33].

Comparatively with other exocrine glands, little is known about the Ca^{2+}-transport systems functioning of secretory cells of the lacrimal gland. Perhaps this is partly due to methodical difficulties in obtaining a sufficient number of functionally intact isolated secretory cells of lacrimal glands.

Permeabilized cells are a convenient system for study of many intracellular processes, including mechanisms of signal transduction in conditions that are close to the native ones, because in this case the correlation between Ca^{2+}-transport systems of different organelles are preserved. Cells membrane permeabilization makes intracellular Ca^{2+}-transport systems accessible for specific activators and inhibitors, that in normal conditions do not permeate through plasma membrane.

The aim of this work was to optimize the conditions of isolation and permeabilization of the rat exorbital lacrimal gland secretory cells and assess the possibility of their use for the intracellular Ca^{2+}-transport systems study.

Materials and methods. Experiments were performed on nonlinear white rats with 160-250 g weight, that were kept in a stationary conditions of vivarium. All manipulations with animals were carried out according to the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 18.III.1986) and the Law of Ukraine "On protection of animals from cruelty" (2006).

After chloroform anaesthesia and decapitation the exorbital lacrimal glands *(glandula orbitalis externa)* were quickly removed from the body and dissociated from the connective tissue. For lacrimal cells isolation modified method described by Herzog et al. was used [9].

The amount of cells was counted using camera Horyaeva. For this study the drop of investigated suspension was placed under the cover glass, put under a microscope and, if cells accommodated evenly, cells were counted in 225 large squares of the camera. The number of cells in 1 ml of suspension was calculated using the formula:

$$x = \frac{n}{225} \cdot 0.25 \cdot 2 ,$$

where x – number of million cells in 1 ml of suspension; 0.25 – conversion factor for volume; 2 – dilution by solution containing trypan blue; n – amount of cells in 225 large squares of the Horyaeva camera.

The integrity of the plasma membrane was controlled visually under a light microscope using dye trypan blue. For this purpose 0.2 % trypan blue solution, that was on the basis of nominal noncalcium extracellular medium, was mixed with a suspension of cells in the same volume and after 2–3 min examined under a microscope. Lack of cellular colour indicated the integrity of the plasma membrane.

Cells membrane permeabilization was accomplished by digitonin at 37 °C in medium that was close to intracellular. The concentration of free Ca^{2+} in the medium was assigned by Ca^{2+}/EGTA buffer and calculated using the program Maxchelator (http://maxchelator.stanford.edu).

After permeabilization cells were washed twice by intracellular solution without digitonin. Permeabilization grade was estimated visually using trypan blue.

The functioning of the Ca^{2+}-transport systems was estimated by changes of Ca^{2+} content in cells, after incubation with agonists or antagonists. Ca^{2+} content was determined using metalochromic staining agent arsenazo III. Ca^{2+}-ATPase activity was evaluated based on changes in the content of inorganic phosphate in the medium, which was determined by UV detection. Protein concentration was determined by Lowry method [18]. The lacrimal gland secretory cells respiration was recorded by polarographic method [4] using a propeller stirrer [19].

Mathematical-statistical data processing was performed using the software package Microsoft Excel. Statistical difference between groups was determined by t-Student's test, for statistically significant were taking changes with $P<0.05$.

Results and discussion. Optimization conditions of isolation and permeabilization of different tissues cells is an important task. Permeabilized cells are a convenient system for study of many intracellular processes, including mechanisms of signal transduction in conditions that are close to the native ones, because in this case the correlation between Ca^{2+}-transport systems of different organelles are preserved.

Optimization the conditions for isolation of exorbital lacrimal gland secretory cells. For lacrimal gland secretory cells isolation mostly trypsin or a mixture of trypsin, collagenase and hyaluronidase are used [9, 30, 32]. However, trypsin may cause disruption of membrane receptors. That is why it is advisable to use type IV of collagenase, which contains low tryptic activity. It is commonly used for islets and other applications where receptor integrity is crucial [34].

A high percentage of intact isolated secretory cells of lacrimal gland can be obtained using incubation with two extracellular media. Initially in 1) Ca^{2+} (1 mM)-containing media, which includes a mixture of collagenase (type IV) with lidase (hyaluronidase), and then in 2) EGTA (2 mM)-containing solution. This procedure was performed twice. A mixture of collagenase (690 U/ml) and lidase (400 U/ml) (dissolved in extracellular medium of the following composition (mM): NaCl – 119, KCl – 6, $MgCl_2$ – 1,2, HEPES – 10, $CaCl_2$ – 1, glucose – 10, $NaHCO_3$ – 25; pH 7.4) was injected to the gland. The gland was incubated 25 min in a water thermostat at 37 °C and moderate shaking. After incubation solution was changed to the extracellular containing EGTA (2 mM), and incubated in it for 5 minutes. Then again incubated in Ca^{2+}-containing extracellular medium with collagenase and lidase (15 min) and pipetted by tipped with a hole of different diameters in EGTA-containing extracellular medium, then again washed in Ca^{2+}-containing solution.

After the first incubation with collagenase and lidase, but before incubation with EGTA, the acinuses were associated in acinar complexes. After incubation in media with EGTA acinar complexes dissociated, and after the second incubation with collagenase and lidase and pipettation in EGTA-containing solution a suspension of individual cells and small groups of them was obtained.

The main, but not the only condition of cells intact is integrity of the plasma membrane. Lack of colour indicated the integrity of the plasma membrane. In addition, the cytoplasm of cells with damaged plasma membrane staining with trypan blue glows red under ultraviolet light.

Under influence of carbacholine (1 μM) after 15 min incubation of isolated secretory cells in medium with physiological Ca^{2+}concentration (1 mM) Ca^{2+} content in the studied cells decreased to 11,38 ± 2,33 % ($P<0,01$, n = 5) [14] and concentration of secreted protein (determined by Lowry) increased by 8 % (n = 3). In addition, registered store-operated Ca^{2+} entry in intact cells of lacrimal gland was inhibited by 2-APB [15]. In this way isolated cells retained mitochondrial respiration (n = 9).

It was found that the number of isolated cells and the percentage of intact cells among them depends on the concentration of collagenase in the incubation medium. After the first incubation with collagenase and lidase about 80 % of the cells were collected acinus or acinar complexes, which made it impossible counting their number and check their plasma membrane integrity. For the second incubation, the percentage of intact cells gradually increased with increasing concentrations of collagenase to 690 U/ml and is characterized by some decrease at 920 U/ml. A number of intact cells when incubated in a medium that did not contain collagenase (second incubation), were isolated under the influence of collagenase and lidase during the first incubation. The use of collagenase (230 U/ml) during the second incubation produced the 41,97 ± 8,07 % (n = 3) intact cells, 460 U/ml – 68,29 ± 5,83 % (n = 4) , 690 U/ml – 80,97 ± 1,15% (n = 8), 920 U/ml – 75,16 ± 4,64 % (n = 7). The remaining cells were damaged at the same time due to mechanical pipetting, which in this series of experiments was carried out the same amount of time regardless of the concentration of collagenase for the second incubation. However, in samples that contain lower concentrations of collagenase for cell isolation was necessary to apply more intensive pipetting that caused the violation of the integrity of their plasma membrane.

Thus the optimum concentration of collagenase IV for the second incubation is 690 U/ml, since in this case the output is the largest intact cells (Fig 1).

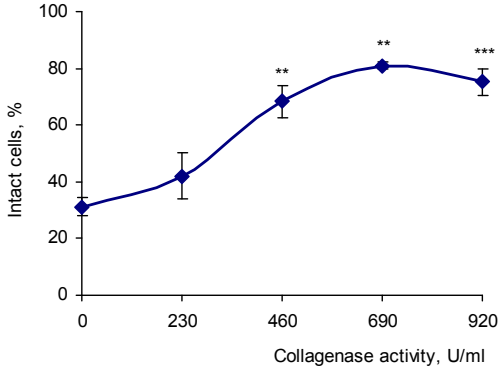

Fig. 1. Dependence of the amount of lacrimal gland secretory cells with integral plasma membrane on concentration of collagenase for the second incubation:
[NaCl] = 119 mM, [KCl] = 6 mM, [$MgCl_2$] = 1.2 mM, [glucose] = 10 mM, [$NaHCO_3$] = 25 mM, [HEPES] = 10 mM, [$CaCl_2$] = 1 mM, lidase activity – 400 U/ml; ** – difference compared with control reliable with $P < 0.01$, *** – $P < 0.001$

Optimization of conditions for permeabilization of exorbital lacrimal gland secretory cells. Cells membrane permeabilization makes intracellular Ca^{2+}-transport systems accessible for specific activators and inhibitors, that in normal conditions does not permeate through plasma membrane and enables to not use specific inhibitor of Ca^{2+}-transport systems of the plasma membrane. Cell membrane permeabilization with digitonin is commonly used for study of different cells functioning [10, 20, 21, 23, 28, 31].

Digitonin and other saponins can be used to selectively permeabilize the plasma membrane of a wide variety of cells without significantly affecting the gross structure and function of Ca^{2+}-sequestering organelles such as mitochondria and endoplasmic reticulum [8], obviously because

the cholesterol in intracellular membranes is much lower than in plasma membrane [6]. Because the plasma membrane of various cells differs significantly from cholesterol [1, 5, 6, 22, 25], the current density (concentration) of digitonin, permeabilization conditions and composition of solutions in each case are different. Therefore, it was necessary to adapt (optimize) this method to plasma membrane permeabilization of exorbital lacrimal gland secretory cells.

The optimal digitonin concentration was chosen experimentally. Plasma membrane permeabilization was performed by incubation with digitonin at 37 °C in the medium close by composition to the intracellular (mM): KCl – 140, $MgCl_2$ – 1.5, $CaCl_2$ – 0.0274, EGTA – 0.1 ($[Ca^{2+}] \approx 10^{-7}$ M), HEPES – 10; pH 7.2.

After permeabilization cells were washed twice by intracellular solution without digitonin. Permeabilization grade was estimated visually by trypan blue staining. In ultraviolet light cytoplasm of cells stained with trypan blue light red, indicating permeabilization of their plasma membrane (Fig. 2 *A, B*).

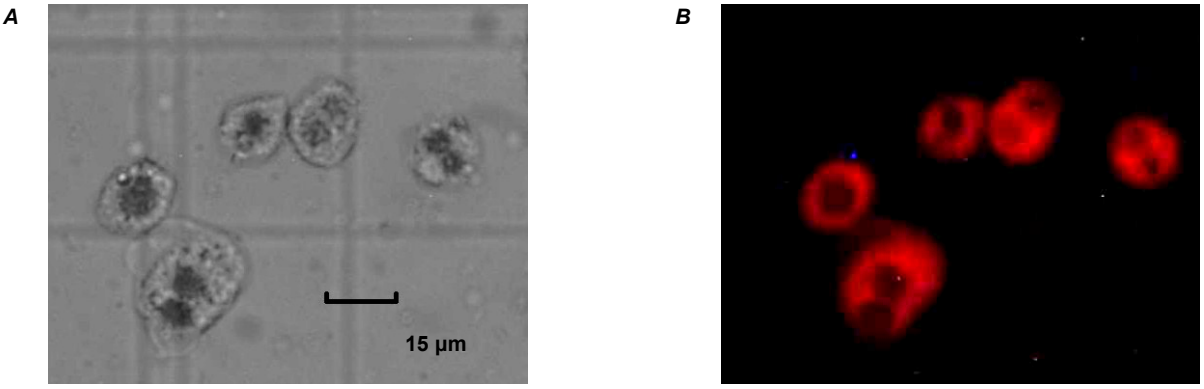

Fig. 2. Permeabilized secretory cells of the rat exorbital lacrimal gland (light microscope Nicon optiphot 2, Japan):
A – light microscopy; B – fluorescence registration (Filter Block G-2A, 590 nm); [KCl] = 140 mM, $[Ca^{2+}] = 10^{-7}$ M, $[MgCl_2]$ = 1.5 mM, [HEPES] = 10 mM, [trypan blue] = 0,1 %;

Digitonin forms insoluble complexes with cholesterol of plasma membrane, which leads to the formation of pores in the membrane. To avoid permeabilization (except plasma membrane) also intracellular membranes digitonin concentration and incubation time with it was selected on the basis that the minimum concentration of digitonin during the shortest incubation time obtain 95–97% of all cells being permeabilized.

Optimal amount of digitonin required to exorbital lacrimal gland cells permeabilization was 50 μg / 0.5 million cells, and optimal incubation time – 10 min. Under these conditions, about 95–97 % of the cells were trypan-positive (Fig. 3. *A, B*).

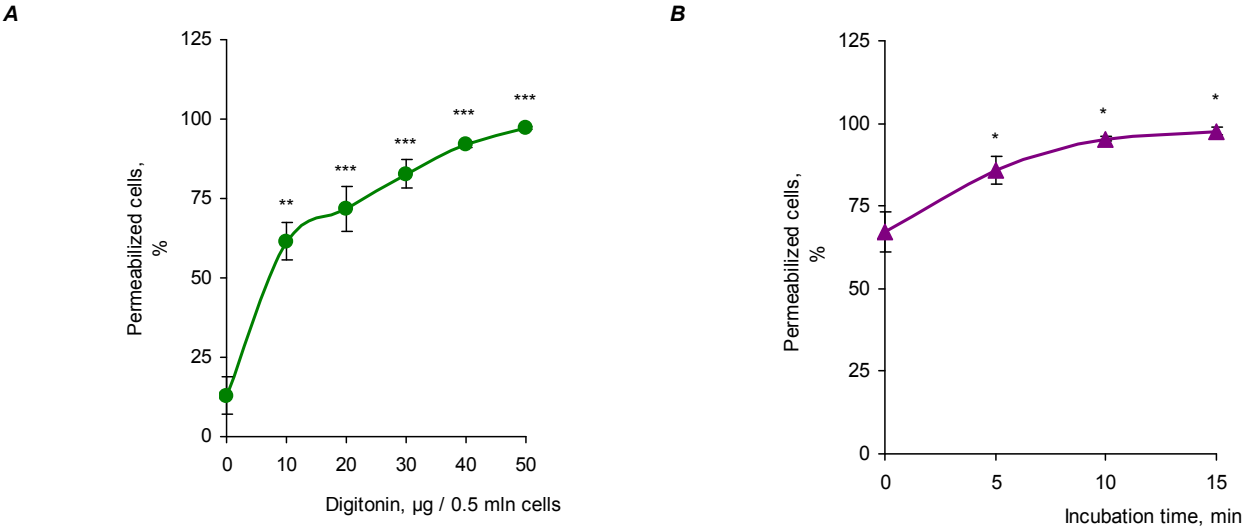

Fig. 3. Dependence of permeabilized cells of exorbital lacrimal gland of the rat on digitonin concentration and incubation time with it:
A – dependence of the percentage of permeabilized cells on digitonin concentration in the medium, incubation time – 10 min;
B – dependence of the percentage of permeabilized secretory cells on incubation time with digitonin, [digitonin] = 50 μg / 0.5 mln cells; in both [KCl] = 140 mM, $[Ca^{2+}] = 10^{-7}$ M, $[MgCl_2]$ = 1.5 mM, [HEPES] = 10 mM;
* – difference compared with control in appropriate group reliable with $P < 0.05$, ** – $P < 0.01$, *** – $P < 0.001$

The integrity of the membranes of intracellular organelles was assessed using chlortetracycline, which accumulated in intracellular organelles with a high concentration of ionized Ca^{2+}, where they form a complex compound [12, 13, 21, 24, 27]. Since the fluorescence of Ca^{2+}-chlortetracycline complex determined by the content of Ca^{2+} in intracellular stores, this suggests the integrity of endoplasmic reticulum membranes. In terms of chlortetracycline adding to the media with intact and permeabilized secretory cells in ultraviolet light cells glowed green, indicating integrity of the endoplasmic reticulum membranes.

Proof of endoplasmic reticulum integrity is that eosin Y (5–50 μM) reduces the content of Ca^{2+} in permeabilized cells by SERCA inhibiting in dose-dependent manner [17]. Reduction of Ca^{2+} content in permeabilized cells of studied glands was observed in the presence of IP_3 in medium (2 μM) [14].

In permeabilized exorbital lacrimal gland cells the AT-Pase activity was registered. It was found that the optimal incubation time for ATPase activity research of exorbital lacrimal glands secretory under *in situ* condition was 15 min. Maximum of Ca^{2+}-sensitive ATPase activity was observed with 2 mM of exogenous ATP and maximum eosin Y-sensitive ATPase activity was observed with 3 mM. Ca^{2+}-ATPase activity was effectively inhibited by eosin Y (10–20 μM) and thapsigargin (1 μM) [16]. Thus, a determination of the Ca^{2+}-Mg^{2+}-ATPase activity under *in situ* condition is an adequate and sensitive method for the study of the exorbital lacrimal glands [16].

Rhodamine 123 is a fluorescent cationic dye and distributed according to the negative potential of the inner mitochondrial membrane [3, 7, 11]. Permeabilized cells of the rat exorbital lacrimal gland in the presence of medium rhodamine 123 glowed green, indicating a functional integrity of mitochondria.

Conclusion. Permeabilized exorbital lacrimal gland cells are convenient model for the intracellular Ca^{2+}-transport systems study.

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References

1. Alam B.S., Alam S.Q. Studies on lacrimal gland lipids in essential fatty acid deficiency // Exp. Biol. Med. – 1979. – Vol. 161, № 2. – P. 199–203.

2. Beebe W.E., Esquivel E.D., Holly F.J. Comparison of lacrimation kinetics in dry eye patients and normals // Curr. Eye Res. – 1988. – Vol. 7, № 4. – P. 419–425.

3. Bindokas V.P., Lee C.C., Colmers W.F., Miller R.J. Changes in mitochondrial function resulting from synaptic activity in the rat hippocampal slice // The Journal of Neuroscience. – 1998. – Vol. 18, № 12. – P. 4570–4587.

4. Chance B., Williams G.R. Respiratory enzymes in oxidative phosphorylation I. Kinetics of oxygen utilization // J. Biol. Chem. – 1955. – Vol. 217. – P. 383–394.

5. Clarke M.S.F., Vanderburg C.R., Bamman M.M. et al. In situ localization of cholesterol in skeletal muscle by use of a monoclonal antibody // J. Appl. Physiol. – 2000. – Vol. 89, № 2. – P. 731–741.

6. Colbeau A., Nachbaur J., Vignais P.M. Enzymic characterization and lipid composition of rat liver subcellular membranes // Biochim. Biophys. Acta. – 1971. – Vol. 249, № 2. – P. 462–492.

7. Darzynkiewicz Z., Traganos F., Staiano-Coico L. et al. Interactions of rhodamine 123 with living cells studied by flow cytometry // Cancer Res. – 1982. – Vol. 42. – P. 799 – 806.

8. Fiskum G. Intracellular levels and distribution of Ca^{2+} in digitonin permeabilized cells // Cell Calcium. – 1985. – Vol. 6, № 1–2. – P. 25–37.

9. Herzog V., Sies H., Miller F. Exocytosis in secretory cells of rat lacrimal gland peroxidase release from lobules and isolated cells upon cholinergic stimulation // J. Cell Biol. – 1976. – Vol. 70. – P. 692–706.

10. Horbay R.O., Manko B.O., Manko V.V. et al. Respiration characteristics of mitochondria in parental and giant transformed cells of the murine Nemeth-Kellner lymphoma // Cell Biol. Int. – 2012. – Vol. 36, № 1. – P. 71–77.

11. Huang M., Camara A.K., Stowe D.F. et al. Mitochondrial inner membrane electrophysiology assessed by rhodamine-123 transport and fluorescence // Ann Biomed Eng. – 2007. – Vol. 35, № 7. – 1276–1285.

12. Kaul G., Sharma G.S., Singh B., Gandhi K.K. Capacitation and acrosome reaction in buffalo bull spermatozoa assessed by chlortetracycline and agglutinin fluorescence assay // Theriogenology. – 2001. – Vol. 55, № 7. – P. 1457–1468.

13. Kosterin S.A. Transport of calcium in smooth muscles. – Kyiv, 1990. (in Russian).

14. Kotliarova A., Manko V. The role of IP_3-sensitive Ca^{2+}-channels in Ca^{2+} signaling in secretory cells of the rat exorbital lacrimal gland // Proceedings of the V Symposium "Peculiarities of formation and physiological functions in human ontogenesis". – Cherkasy, 2012. – P. 46. (in Ukrainian).

15. Kotliarova A.B., Dorosh O.M., Kruk O.B., Manko V.V. Store-operated Ca^{2+} entry in secretory cells of the lacrimal gland // International scientific conference of students and young scientists dedicated to the 20th anniversary of the Kharkiv National University named after V.N. Karazin "Actual problems of modern medicine", Kharkov, 2013: book of abstracts. – Kharkov, 2013. – P. 106. (in Ukrainian).

16. Kotliarova A.B., Manko V.V. ATPase activity of isolated cells of the rat exorbital lacrimal gland under in situ condition // Visnyk of the Lviv University. Series Biology. – 2012. – Issue 60. – P. 270–282 (in Ukrainian)

17. Kotliarova A.B., Manko V.V. Ca^{2+} transporting systems in secretory cells of the rat exorbital lacrimal gland I. Ca^{2+} pumps of plasma membrane and endoplasmic reticulum // Studia Biologica. – 2012. – Vol 6, № 2. – P. 99–114 (in Ukrainian).

18. Lowry O.H., Rosebrough N.J., Farr A.L., Randall R.J. Protein measurement with the Folin phenol reagent // J.Biol.Chem. – 1951. – № 193. – P. 265–275.

19. Manko B., Voloshyn D., Manko V. Respiration of isolated acini of rat pancreas // Visnyk of the Lviv University. Series Biology. – 2013. – Issue 61. – P. 172–179. (in Ukrainian).

20. Manko B.O., Klevets M.Y., Manko V.V. An implication of novel methodology to study pancreatic acinar mitochondria under in situ conditions // Cell Biochem. Funct. – 2013. – №2. – P. 15–21.

21. Manko V.V. Ca^{2+} transporting systems in secretory cells of exocrinic glands: Monograph. – Lviv, 2011. (in Ukrainian).

22. Meldolesi J., Jamieson J.D ., Palade G.E. Composition of cellular membranes in the pancreas of the guinea pig II. Lipids // Journ. Cell Biol. – 1971. – Vol. 49. – P. 130–149.

23. Merlavsky V.M., Manko B.O., Ikkert O.V., Manko V.V. Energy processes in isolated hepatocytes at different duration of insulin action // Studia Biologica. – 2010. – Vol. 4, № 3. – P. 15–22 (in Ukrainian).

24. Owen N.E., Le Breton G.C. Ca^{2+} mobilization in blood platelets as visualized by chlortetracycline fluorescence // AJP. Heart. – 1981. – Vol. 241, № 4. – P. H613–H619.

25. Pankov R., Markovska T., Hazarosova R. et al. Cholesterol distribution in plasma membranes of beta1 integrin-expressing and beta1 integrin-deficient fibroblasts // Arch. Biochem. Biophys. – 2005. – Vol. 442, № 2. – P. 160–168.

26. Ramos-Casals M., Tzioufas A.G, Font J. Primary Sjogren's syndrome: new clinical and therapeutic concepts // Ann. Rheum. Dis. – 2005. – Vol. 64. – P. 347–354.

27. Rybalchenko V.K. The plasma membrane of smooth muscle cells: active transport of calcium, sodium-calcium exchange and reconstruction of ion conduction: Thesis. ... Dr. Biol. Sc. Dissertation – M., 1988. (in Russian).

28. Shlykov S.G., Babich L.G., Kosterin S.A. Suspension of smooth muscle cells treated with digitonin as a model for studying the myometrial endoplasmic reticulum calcium pump // Biochemistry. – 1997. – Vol.62. – P. 1666–1671 (in Russian).

29. Sundermeier T., Matthews G., Brink P.R., Walcott B. Calcium dependence of exocytosis in lacrimal gland acinar cells // Am. J. Physiol. Cell Physiol. – 2002. – Vol. 282. – P. C360–C365.

30. Ueda Y., Karasawa Y., Satoh Y. et al. Purification and characterization of mouse lacrimal gland epithelial cells and reconstruction of an acinar-like structure in three-dimensional culture // Invest. Ophthalmol. Vis. Sci. – 2009. – Vol. 50. – P. 1978 – 1987.

31. Velykopolska O.Yu., Manko B.O., Manko V.V. Endoplasmic-mitochonrial Ca^{2+}-functional unit: dependence of respiration of secretory cells on activity of ryanodine- and IP_3- sensitive Ca^{2+}-channels // Ukr. Biochem. J. – 2012. – Vol. 84, № 5. – P. 76–88 (in Ukrainian).

32. Voronov D., Gromova A. Liu D. et al. Transcription factors Runx1 to 3 are expressed in the lacrimal gland epithelium and are involved in regulation of gland morphogenesis and regeneration // Invest. Ophthalmol. Vis. Sci. – 2013; – Vol. 54, № 5. – P. 3115 – 3125.

33. Walcott B. The lacrimal gland and its veil of tears // News Physiol. Sci. – 1998. – Vol. 13, № 2. – P. 97–103.

34. Worthington K., Worthington V. Worthington Enzyme Manual // Worthington Biochemical Corporation. – 2011. (http://www.worthington-biochem.com/CLS/default.html).

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ПЕРМЕАБІЛІЗОВАНІ СЕКРЕТОРНІ КЛІТИНИ СЛІЗНИХ ЗАЛОЗ ЩУРІВ ЯК МОДЕЛЬНА СИСТЕМА ДЛЯ ДОСЛІДЖЕННЯ *Ca 2+* -ЗАЛЕЖНОЇ ТРАНСПОРТНОЇ СИСТЕМИ

Адаптовано метод ізолювання та пермеабілізації секреторних клітин зовнішньоорбітальної сльозової залози щура. Оптимальна концентрація дигітоніну для їх пермеабілізації становить 50 мкг / 0,5 млн клітин, оптимальний час інкубації – 10 хв. Отримані таким чином клітини зафарбовуються родаміном 123 та хлортетрацикліном, що свідчить про інтактність мітохондрій та ендоплазматичного ретикулуму. Пермеабілізовані клітини зовнішньоорбітальної сльозової залози щура є зручною моделлю для дослідження функціонування Ca2+-транспортувальних систем.

Ключові слова: сльозова залоза, секреторні клітини, ізолювання, пермеабілізація, дигітонін, Ca^{2+}-транспортувальні системи.

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ПЕРМЕАБИЛИЗИРОВАННЫЕ СЕКРЕТОРНЫЕ КЛЕТКИ ИЗ СЛЕЗНЫХ ЖЕЛЕЗ КРЫС КАК МОДЕЛЬНАЯ СИСТЕМА ДЛЯ ИССЛЕДОВАНИЯ *СА 2+* – ЗАВИСИМОЙ ТРАНСПОРТНОЙ СИСТЕМЫ

Адаптировано метод изолирования и пермеабилизации секреторных клеток внеглазничной слезной железы крысы. Оптимальная концентрация дигитонина для их пермеабилизации составляет 50 мкг / 0,5 млн клеток, оптимальное время инкубации – 10 мин. Полученные таким образом клетки окрашиваются родамином 123 и хлортетрациклином, что свидетельствует об интактности митохондрий и эндоплазматического ретикулума. Пермеабилизированные клетки внеглазничной слезной железы крысы являются удобной моделью для исследования функционирования Ca^{2+}-транспортных систем.

Ключевые слова: слезная железа, секреторные клетки, изолирование, пермеабилизация, дигитонин, Ca^{2+}-транспортные системы.

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DEVELOPMENT OF NON- AND VITRIFIED-THAWED PRONUCLEAR-STAGE MOUSE EMBRYOS CO-CULTURED WITH OVIDUCT EPITHELIAL CELLS

For optimize culture system for improving of the development the post-thaw survival in pronuclear-stage mouse embryos , we modificated culture conditions, using oviduct epithelial cells culture. The results suggest that co-culture with oviduct epithelial cells enhances the in vitro development of the vitrified-thawed mouse embryo with the hight quality.

Key words: pronuclear mouse embryos, vitrification*, *oviduct epithelial cells, co-culture.

Introduction. Epithelial cells of the mammalian oviduct play an important role in reproductive functions. Cultured MOEC show a wide variety of secretory activities and these secretory factors may influence early embryonic development and monolayer cultures of BOEC have been widely used for in vitro co-culture of bovine preimplantation embryos. The main function of the oviduct epithelial cells (OECs) is providing the successful transportation processes connected with fertilization, while secretory cells are known to secrete products called the embryotrophic oviduct-specific glycoprotein, especially in the first few days of the estrous cycle and during early pregnancy [5]. The growth factors synthesized by epithelial cells are important modulators in many reproductive processes that work in an autocrine or paracrine manner [12]. Culture oviduct epithelial cells contained ample glycogen which deposits caused dedifferentiation of the embryonic blastomeres or increased amounts of glucose in the culture medium which interprets as signs of dedifferentiation. The features of the oviduct epithelial cells like their changing protein profile, a high proliferative speed, specific growth cyclicity mentioning on their possible multipotential properties obtained from pushed away uterine mucus suggests that monolayer of mammalian oviduct epithelial cells possesses properties [3] that used in co-culture experiments to improve embryonic development of in vitro.

Aim. The effect of co-culturing non- and vitrified-thawed pronuclear-stage mouse embryos with mouse oviduct epithelial cell monolayer was investigated. Our purpose was to compare pronuclear-stage mouse embryos development after vitrified and non-vitrified embryos, followed by in vitro cultivation on oviduct epithelial cell monolayer at the blastocyst state, and with development of non- and vitrified-thawed pronuclear-stage mouse embryos after a single cultivation in cultural media KSMO.

Materials and methods. The oviducts from the same females were used as a source of MOECs. After PN embryos were flushed the oviducts transferred into Petri dishes with DMEM and 1% penicillin-streptomycin solution + 2% FBS. The oviduct ampoules were squeezed by eye-forceps to get the clamps of epithelial tissue. Concentrated suspension of epithelial cells was sucked throughout 200 µl pipette tip 10 times and squeezed throughout 27g surgical needle 25 times until almost single cell suspension was reached [1]. The cells have been left in the CO_2- incubator at 37^0C for 30 min to let them put down. The upper layer of the medium has been discarded then and fresh DMEM + 20% FBS (just in this step) added to reach a desirable rate of cell proliferation. The cells have been left in the CO_2-incubator at 37^0C for 3 days. After old condition medium was discarded and fresh DMEM + 10% FBS + 1% antibiotic solution were added again the MOECs culture was ready to use as a cell feeder substrate for developmental capacity of thawed PN embryos.

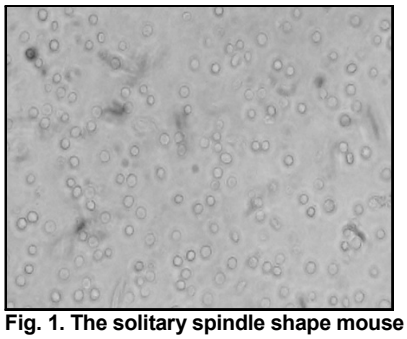

Fig. 1. The solitary spindle shape mouse oviduct cells lost cilia in culture. Their shape changed to round due to trypsin treatment, x 250.

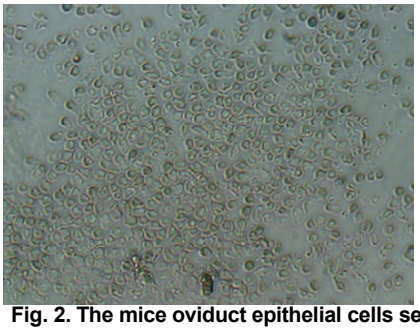

Fig. 2. The mice oviduct epithelial cells settled down on the bottom of Petri dish and started to create a cell monolayer.

Pronuclear-stage embryos were collected from superovulated mice after intraperitoneal injections of 5 IU of pregnant mare's serum gonadotropin (PMSG, Sigma), followed 48 hours later with 5 IU human chorionic gonadotrophin (hCG, Pregnyl) injection and were housed with the same strain males. Isolated from mice female the PN embryos were rinsed out cumulus cells into M2 medium with 80 M O /ml hyaluronidase to remove the cumulus cells. Zygotes were divided into two groups. Half of them (*Experimental group)* were vitrified on solid surface [4, 6] and after thawed were allocated into two A and B in vitro culture treatments. The remaining half (*Control group)* were cultured in vitro, with (Control A) or without (Control B) mouse oviduct epithelial cell monolayer (MOECs).

The A treatment were embryos culture in DMEM medium supplemented with 20% FCS with oviduct epithelial cell monolayer. The B treatment were embryos culture in KSOM (Specialty Media).

Experimental group. Exp. A. Vitrified zygotes were cultured in the presence of oviduct epithelial cell monolayer (vitrification^{+}/ MOECs $^{+}$).

Exp. B. Vitrified zygotes were cultured in the absence of oviduct epithelial cell monolayer (vitrification^{+}/ MOECs $^{-}$).

Control group. Control A. Control zygotes were cultured in the presence of oviduct epithelial cell monolayer (vitrification^{-}/ MOECs $^{+}$).

Control B. Control zygotes were cultured in the absence of oviduct epithelial cell monolayer in KSMO (vitrification^{-}/ MOECs $^{-}$).

The embryos in earch groups were cultured in 20 µl drops of culture medium supplemented with 20% FCS under mineral oil at 37°C in 5% CO_2 and 95% air atmosphere. The rates of embryo development to 2-cell, 4-8-cell, morula and blastocysts stages in the four groups were compared.

Results and discussion. The vitrified co-culture group (Exp A) had higher 2-cell development rates (82%) than the vitrified- thawed PN embryos were cultured in media KSMO only (Exp B) (80%) (Tab. 1). But the percentages of control PN embryos (Control B) cleaving to 2-cell stages was higher than the Control A co-culture group (96%, 92%, respectively). The rate of development PN embryos to 4-8-cell stage in the Exp B group was significantly higher than the vitrified co-culture Exp A group (78 % vs. 64 %, respectively). However, supplementation of the feeder epithelial cell monolayer for cultivation control fresh PN embryos (Control A) showed higher the rate of development to 4-8-cell stage embryos compared to the control group B (84 %; 80 %, respectively). The percentage cleaved to the morula stage (50%) and reached the blastocyst stage (36 %) of vitrified co-culture PN zygotes (Exp A) was higher than the Exp B vitrified group (48% and 34%, respectively).

Table 1. **Development of non-vitrified and vitrified- thawed pronuclear-stage mouse embryos when cultured in cultural media alone or co-cultured with mouse oviduct epithelial cell monolayers**

Groups	Number of replicates	Number of embryos (%)			
		2-cell	4-8-cell	morula	blastocysta
Exp A	50	41 (82,0)	32 (64,0)	25 (50,0)	18 (36,0)
Exp B	50	40 (80,0)	39 (78,0)	24 (48,0)	17 (34,0)
Control A	50	46 (92,0)	42 (84,0)	36 (72,0)	20 (40,0)
Control B	50	48 (96,0)	40 (80,0)	37 (74,0)	23 (46,0)

Exp A, vitrified-thawed PN co-culture with oviduct cells monolayer in culture media DMEM+10% FCS; Exp B, vitrified-thawed PN culture in media KSMO only; Con A, fresh PN co-culture with oviduct cells monolayer in culture media DMEM+10% FCS; Con B, fresh PN culture in media KSMO only.

The results of this study demonstrated that 2-cell, morula and blastocyst rates was significantly higher in oviduct monolyers cell co-culture (Exp. A) than in the cultural medium KSMO (Exp. B) (Fig. 3,4,5). In contradistinction to frozen/ thawed embryos, the higher embryo development observed when un-treatment control embryos cultivated in KSMO medium. The pronuclear-stage mouse embryos cultured in KSMO media had improved survival compared to those cultured with co-culture with oviduct epithelial cells (Cont. B). These results were consistent with reports by Nedambale T.L. [7] that used KSMO medium during the mouse embryo culture and found that medium induced early blastocysta formation without increasing cell proliferation.

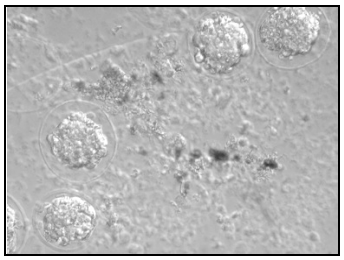

Fig. 3. Development of vitrified-thawed pronuclear-stage mouse embryos after co-cultured in mouse oviduct epithelial cell monolayers to the well-expanded blastocysts

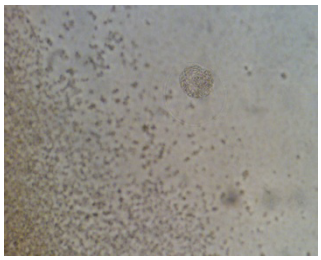

Fig.4. Vitrified-thawed blastocyst on the monolayers of oviduct epithelium cell culture

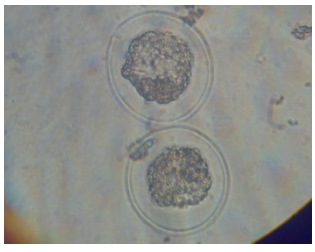

Fig 5. Development of vitrified-thawed mouse embryos after cultured without oviduct epithelial cells culture. In the area of view can be seen single granulose cells

Whereas, an alternative way of improving the developmental ability of embryos at the pronuclear stage after cryopreservation may be their cultivition in vitro to avoid the stresses [8] caused by the prevention of stresses that arise during vitrification.

Cultured MOEC show a wide variety of secretory activities and these secretory factors may influence early embryonic development. In the oviductal microenvironment have been identified regulatory molecules, protease inhibitors, growth factors, cytokines, binding proteins [9], enzymes and immunoglobulins which associates with the zona pellucida, perivitelline space and vitelline or blastomere membrane of ovulated eggs and preimplantation embryos. Oviduct epithelial cells secreted protein, the oviduct-specific, oestrogen-dependent glycoprotein that optimize the microenvironment for gamete maturation and transport, fertilization and early cleavage-stage embryonic development [11].

The feeder cells may have had more effective biophysical and biochemical properties, which assisted the embryo to continue its development [10]. These results suggest that co-culture of thawed PN embryos with oviduct epithelial cells in DMEM enhances a development of the vitrified-warmed mouse embryos and can result in conventional rate of the blastocyst stage.

Conclusions. Our study provides that co-culture with oviduct epithelial cells enhances the in vitro development of the vitrified-thawed mouse embryo. This co-culture model system may also provide a basis for future research into the improvement of subsequent culture conditions for embryos after cryopreservation.

References

1. Клітинні культури і можливості їх використання в ембріональній біотехнології: Навчально-методичний посібник / А.В. Мадіч, В.І. Шеремета, І.І. Гевкан та ін. за ред. А.В. Мадіч. – К.: АртЕконом, 2012.

2. Федорова С.В., Штапенко О.В. Ростові фактори у деконсервованій культурі епітеліальних клітин яйцепроводів як показники відновлення її морфофункціональних властивостей. // "Фундаментальні та прикладні дослідження в біології": Матер. II міжнар. конф. студентів, аспірантів та молодих учених, Донецьк, 2011. – Д., 2011. – С. 294.

3. Azadbakh M., Valojerdi M.R., Mowla S.J. Development of vitrified-warmed mouse embryos co-cultured with polarized or non-polarized uterine epithelial cells using sequential culture media // Anim. Reprod. Sci. – 2006. – Vol. 100, № 1-2. – P. 141 – 157.

4. Bagis H., Sagirkaya H., Odaman Mercan H. et al. Vitrification of pronuclear-stage mouse embryos on solid surface (SSV) versus in cryotube: Comparison of the effect of equilibration time and different sugars in the vitrification solution // Mol. Reprod. Dev. – 2004. – Vol. 67 – P. 186 – 192.

5. Buhi W. C. Characterization and biological roles of oviduct-specific, oestrogen-dependent glycoprotein // Reproduction. – 2002. – 123(3). – P. 355 – 362.

6. Dinneyes A., Yang XZ., Li X.L. et al. Solid surface vitrification (SSV): an efficient method for oocyte and embryo cryopreservation in cattle, pig and mouse // Cryobiology. – 2001. – V. 43. – P. 332 – 338.

7. Nedambale T.L., Dinnyés A., Groen W. et al. Comparison on in vitro fertilized bovine embryos cultured in KSOM or SOF and cryopreserved by slow freezing or vitrification // Theriogenology. – 2004. – Vol. 62, № 3-4. – P. 437 – 449.

8. Omar Farouk FN., Vlad M. In vitro development of mouse pronuclear embryos to blastocysts in sequential media with and without co-culture of autologous cumulus cells // J. Reprod. Dev. – 2008. – Vol. 54, № 5. – P. 385 – 390.

9. Posillico S., Kader A., Falcone T. et al. Ovarian tissue vitrification: modalities, challenges and potentials // Current Women's Health Reviews. – 2010. – Vol. 6. – P. 352 – 366.

10. Rizos D., Ward F, Boland M.P. et al. Effect of culture system on the yield and quality of bovine blastocysts as assessed by survival after vitrification // Theriogenology. – 2001. – Vol. 56, № 1. – P. 1 – 16.

11. Shirazi A., Nazari H., Ahmadi E. et al. Effect of culture system on survival rate of vitrified bovine embryos produced in vitro // Cryobiology. – 2009. – Vol. 59, № 3. – P.285 – 290.

12. Xu J.S., Cheung T.M., Chan S.T. et al. Temporal effect of human oviductal cell and its derived embryotrophic factors on mouse embryo development // Biol. Reprod. – 2001. – Vol. 65, № 5. – P.1481 – 1488.

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РОЗВИТОК НАТИВНИХ ТА КРІОКОНСЕРВОВАНИХ ЕМБРІОНІВ МИШЕЙ НА СТАДІЇ ДВОХ ПРОНУКЛЕУСІВ ПРИ СПІВКУЛЬТИВУВАННІ З ЕПІТЕЛІАЛЬНИМИ КЛІТИНАМИ ЯЙЦЕПРОВОДУ

Для удосконалення культуральної системи з метою підвищення розвитку відтаяних після вітрифікації ембріонів мишей на стадії пронуклеусів, в якості клітинної підтримуючої системи використано культуру клітин яйцепроводів. Отримані результати показали, що ко-культивування зародків на фідерному моношарі клітин яйцепроводів покращувало розвиток вітрифіковано-відтаяних ембріонів мишей.

Ключові слова: ембріони мишей на стадії пронуклеусів, вітрифікація, епітеліальні клітини яйцепроводів, ко-культивування.

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РАЗВИТИЕ НАТИВНЫХ И КРИОКОНСЕРВИРОВАННЫХ ЭМБРИОНОВ МЫШЕЙ НА СТАДИИ ДВУХ ПРОНУКЛЕУСОВ ПРИ КО-КУЛЬТИВИРОВАНИИ С ЕПИТЕЛИАЛЬНЫМИ КЛЕТКАМИ ЯЙЦЕПРОВОДА

Для оптимизации культуральной системы с целью улучшения развития размороженных эмбрионов мышей на стадии пронуклеусов после витрификации, в качестве клеточной поддерживающей системы было использовано культуру клеток яйцепроводов. Полученные результаты показали, что совместное культивирование эмбрионов на фидерном моношаре клеток яйцепроводов повышало уровень развития втрификовано-размороженых ембрионов мышей.

Ключевые слова: эмбрионы мышей на стадии пронуклеусов, витрификация, эпителиальные клетки яйцепроводов, совместное культивирование.

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COMPARATIVE PHENOTYPIC ANALYSIS OF POPULATIONS OF HEMOTOPOIETIC PROGENITOR CELLS WITH DIFFERENT EXPRESSION LEVEL OF CD34 DERIVED FROM PLACENTAL TISSUE AND UMBILICAL CORD BLOOD

The investigation of placental HPCs and compare their properties with other fetal and adult HPCs are necessary for the evaluation the possibility of their clinical applying. It has been shown that placental tissue contains three populations with different level of CD34 expression such as $CD34^{+++}CD45^{low}$, $CD34^{++}CD45^{low}$ and $CD34^{+/low}CD45^{low}$. Similar to fetal liver placenta contain both population of $CD34^{++}CD45^{low/-}$ and $CD34^{+}CD45^{low/-}$cells that suggested about hematopoiesis in placental tissue. $CD34^{++}CD45^{low/-}$ population also express CD133, almost negative by lineage markers and had lymphocyte-like morphology that give evidence that this population contain primitive HPCs potentially stem cells. Also among placental cells are later progenitors with phenotype $CD34^{+/low}CD45^{+}$ as majority of these cells express hematopoietic lineage markers. Population with phenotype $CD34^{+++}CD45^{low}$ was observed only in placenta that suggests generated perhaps only in the placental tissue or migrate from the other sites of hematopoiesis and change the level of CD34 expression. Enzymatic treatment has insignificant effect on level of some cell surface protein in FACS analyze that worth to take into account in such experiments.

Keywords: hematopoietic progenitor cells, placental hematopoiesis, the expression of CD34 marker, umbilical cord blood.

Introduction. Nowadays the serious problem of hematology remains a deficiency of donors of hematopoietic progenitor cells (HPCs) for the transplantation in cases of hematologic malignancies and congenital disorders of hematopoiesis. Thereby the search of the new additional sources of HPCs is important for medicine. Recently it was shown that the human placenta plays an important role in fetal hematopoiesis [2, 8]. On the other hand the immunophenotype of placental-derived HPCs and their multipotency are still insufficient studied. The investigation of placental HPCs and compare their properties with other fetal and adult HPCs are necessary for the evaluation the possibility of their clinical applying. Thus the aim of this study was compare the phenotype of placental HPCs with umbilical cord blood HPCs.

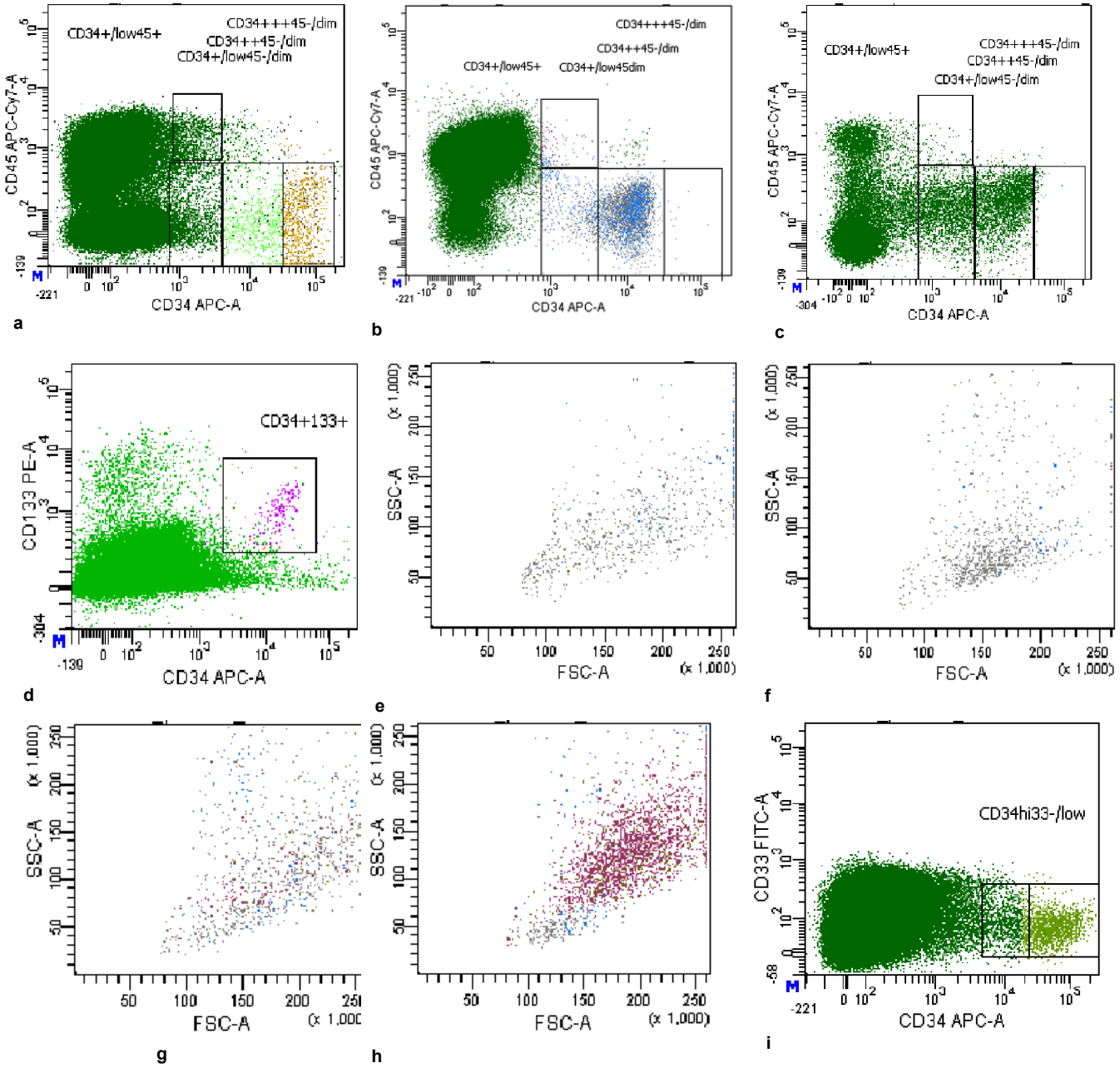
Materials and methods. *Placental and cord blood mononuclear cells isolation.* Placentas were obtained after natural childbirth or by Cesarean section on the 39 – 41 weeks in 23 – 36 years woman after informed consent. Umbilical cord blood was collected using standard cord blood collection techniques. All samples were tested on aerobic, anaerobic bacterial and fungal contamination. Maternal blood was tested for evidence of infection agent such as HIV-1/2, HCV, HBV, CMV and Treponema pallidum. Placental tissue was additional assay for Chlamidium trachomatis, Mycoplasma genitalis, Ureaplasma urealyticum and Ureaplasma parvum, HSV-1/2, CMV. The umbilical cord was cut and removed along with amniotic sac and deciduas from the placenta. Placenta was minced on small fragments by scissors. Remains placental blood was extensively washed from fragments of placental tissue on shaker until tissue became colorless. Placental tissue was treated with 0,2% collagenase I (Serva, Germany), 0,35 mg/ml hyaluronidase (Sigma, USA), 100 U/ml DNase I (Sigma, USA), 1 mg/ml BSA for 30-40 min at +37°C. After that placental cells were isolated by filtration through a 70μm cell strainer (Becton Dickinson, USA). Remaining tissue was incubated with fresh portion of enzymes for 20-40 min at +37°C. Mononuclear cells from placenta and umbilical cord were recovered by Ficoll density gradient fractionation (Density 1,077g/ml, Biochrome, Germany), washed twice, and filtered through a 40 μm cell strainer . Mononuclear cells from umbilical cord blood were treated with the same mix of enzymes for 50 min at +37°C and ones washed in PBS .

Flow cytometry analysis. For immunophenotyping placental and cord blood cells were stained with the following fluorochrome conjugated monoclonal antibodies (Becton Dickinson, USA): anti-CD34 APC, anti-CD90 FITC, anti-CD45 APC-Cy7, anti-CD105 PerCP-Cy 5.5, anti-CD73 PE, anti-CD14 Pacific Blue, anti-CD31 PE, anti-CD133 PE, anti-45RA FITC, anti-CD7 PE, anti-CD19 PE-Cy7, anti-CD33 FITC, anti-CD235a PE. Measurements were performed on cell sorter BD FACSAria (Becton Dickinson, США).

Statistical analysis. Results are expressed as mean with 95%-confidence interval. Statistical significance was determined using the U-Mann– Whitney test.

RESULTS. In previous investigation we have shown that ISHAGE protocol is more appropriate for FACS analysis of HPCs derived from native and cryopreserved placental tissue [9]. Analysis by this protocol shown that content of population HPCs that have phenotype $CD34^{+}CD45^{low}$ with lymphocyte-like morphology (SSC^{low}) among $CD45^{+}$ cells from native placental tissue was 0,60 % (0,39 – 0,86 %, n = 13). The percentage of $CD34^{+}CD45^{low}SSC^{low}$ among $CD34^{+}CD45^{low}$ was 78,5% (70,5 – 85,6%, n=13).We have shown that placental tissue consist of three populations that differ in expression level of CD34. We designate they as $CD34^{+/low}CD45^{low/-}$, $CD34^{++}CD45^{low/-}$ and $CD34^{+++}CD45^{low/-}$. Two populations $CD34^{+/low}CD45^{low/-}$ and $CD34^{++}CD45^{low/-}$ were determined both in placental tissue and in cord blood (Fig.1a,b). $CD34^{++}CD45^{low/-}$ population from placental tissue were also positive by CD133 (Fig.1d). The level of expression of CD14 on placental cells with phenotype $CD34^{+/low}CD45^{low/-}$ and $CD34^{++}CD45^{low/-}$ was 7,24 % (3,27 – 12,62%, n=4) and 3% (0,48 – 7,57%, n=4) in contrast to the same populations of cells from cord blood where it almost missing. Population of cells with phenotype $CD34^{+++}CD45^{low/-}$ was present in placental tissue and almost absent in both cord blood and fetal liver (Fig.1a-c). The percentage of $CD34^{+++}CD45^{low/-}$ cells among all viable mononuclear cells was 0,28% (0,05-0,70) in placental tissue and 0,006% (0,003-0,01) in cord blood. FASC analysis was shown that $CD34^{++}CD45^{low/-}$ cells isolated from placental tissue in general have lymphocyte-like morphology ($FSC^{low}SSC^{low}$) whereas both $CD34^{+}CD45^{low/-}$ and

$CD34^{+++}CD45^{low}$ cells are very heterogeneous by morphology (Fig.1e-g). Also increasing of expression of CD45 on $CD34^{+}$-cells lead to increasing their granularity and size (Fig.1h). The multiparameter flow cytometry analysis demonstrated that $CD34^{+++}CD45^{low}$ cells had immunophenotype $CD33^{-/low}$, $CD14^{-/low}$, $CD235^{-}$, $CD19^{-}$, $CD7^{-/low}$, $CD45RA^{-}$ (Fig.1i-n). The level of expression of CD14 on $CD34^{+++}CD45^{low}$ placental cells were 4,25 % (1,49 – 8,35%, n=4). Expression of CD14 was increased with decreasing of expression level of CD34 on $CD45^{low/-}$cells from placental tissue. Similarly the expression of CD7 and CD19 increased with decreasing expression level of CD34 on $CD45^{low/-}$cells from placental tissue and cord blood. Unlike cord blood and fetal liver placenta contain the population $CD34^{+/low}CD45^{+}$ cells. This population characterized by higher levels of lineage markers expression compare to population with lower level of CD45 and with higher level of CD34. The level of expression of CD14 together with CD33 on $CD34^{+/low}CD45^{+}$ cells was 73,5% (54,15 – 88,39%, n=4), the expression level of CD7 and CD19 on this population were 14,17% (8,38 – 21,18%, n=4) and 3% (1,38 – 5,24%, n=4) respectively. Furthermore level of expression hematopoietic lineage markers increased on placental CD34 positive cells with increasing expression level of CD45 and decreasing CD34 (Fig.1o-r).



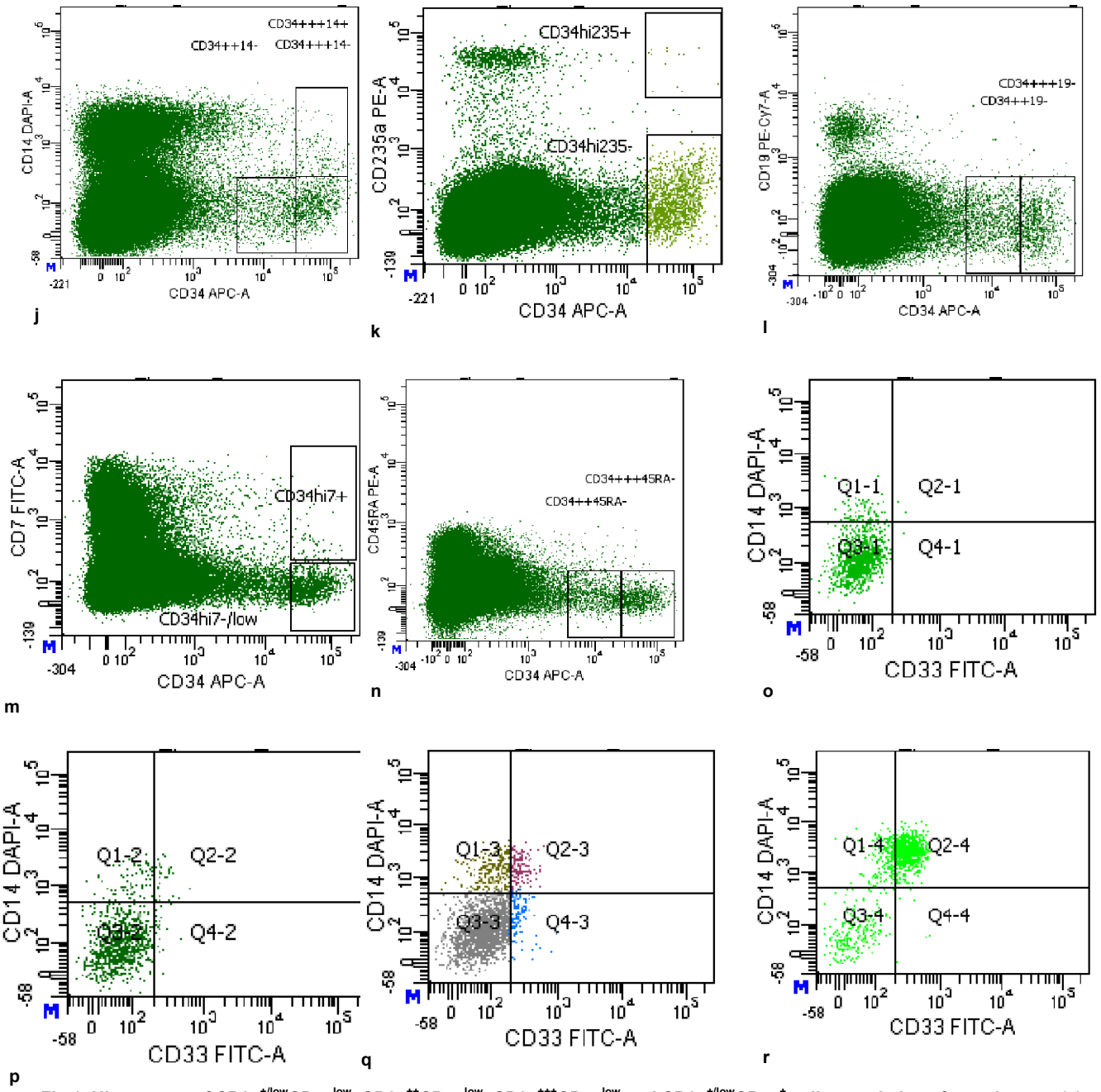


Fig 1. Histograms of CD34$^{+/low}$CD45^{low}, CD34^{++}CD45^{low}, CD34^{+++}CD45^{low} and CD34$^{+/low}$CD45^{+} cells populations from placenta (a), cord blood (b) and fetal liver (c). Histogram of expression of CD34 and CD133 in placental tissue (d). Histograms of FSC/SSC (morphology of cells) for populations with phenotype CD34^{+++}CD45^{low} (e), CD34^{++}CD45^{low} (f), CD34$^{+/low}$CD45^{low} (q) and CD34$^{+/low}$CD45^{+} (h) from placenta tissue. Histograms of expression of CD34 and lineage markers in placental tissue such as CD33 (i), CD14 (j), CD235 (k), CD19 (l), CD7 (m) and CD45RA (n). Histograms of expression of both CD14 and CD33 on cells populations with phenotype CD34^{+++}CD45^{low} (o), CD34^{++}CD45^{low} (p), CD34$^{+/low}$CD45^{low} (q) and CD34$^{+/low}$CD45^{+} (r) from placenta tissue. The results are representative of four experiments

We investigated the effect of enzymatic treatment that we used for isolation of placental cells on the expression level of some hematopoietic markers in umbilical cord blood. In most analysis we didn't observed a significant differences in the level of the expression of markers. However we have shown that the amount of cells in populations with phenotype CD7^{+}CD45RA^{+} CD45^{+}CD45RA^{+} were significant increased in enzymatically treated samples (Tab.1).

***Table* 1. The influence of enzymatic treatment on the selection of umbilical cord blood cells with some phenotype**

Markers	Level of markers expression in umbilical cord blood, %	Level of markers expression in umbilical cord blood after enzymatic treatment, %
CD7CD45RA	16,7 (10,4-24,3) *	25,2 (21,5-29,7) *
CD45CD45RA	40,4 (31,3-49,8) *	52 (44,7-59,1) *
CD33	12,6 (3,9-25,2)	19,6 (10,0-31,5)
CD34	0,9 (0,6-1,2)	1,1 (0,7-1,6)
CD14	10,2 (1,1-27,0)	17 (8,2-28,3)
CD19	19,5 (8,7-33,2)	16,2 (7,9-26,8)

* – significantly different, n=4, p≤0,05. All data not presented

Discussion. The analysis of the phenotype of placental mononuclear cell fraction once more confirmed that placental tissue contains hematopoietic progenitor cells. The percentage of $CD34^{+}CD45^{low}SSC^{low}$ among $CD34^{+}CD45^{low}$ that is lower than in cord blood evidence about necessity of gating the $CD34^{+}CD45^{low}$ cells by morphology as described in previous our work [9].

Placental tissue contains HPCs with different expression level of CD34 that we suggested evidence about different stage of immaturity. Similar to fetal liver placenta contain both population of $CD34^{++}CD45^{low/-}$ and $CD34^{+}CD45^{low/-}$cells that suggested about hematopoiesis in placental tissue. On the other hand among placental cells are more mature HPCs with phenotype $CD34^{+/low}CD45^{+}$ in contrast to fetal liver and cord blood. Majority of these cells express hematopoietic lineage markers that indicate they are later progenitors. We have shown that expression of lineage markers on HPCs such as CD14, CD7 and CD19 increased with decreasing of expression level of CD34. The fact that we find progenitors of different stage of differentiation into mature placental tissue suggests that such cells continue to generate in the placenta and/or migrate to the placental tissue and didn't disappear until the moment of birth.

Also we shown that CD133 mostly was expressed by placental and cord blood $CD34^{++}CD45^{low/-}$ that give evidence about primitively of this population of cells. Their stage of immaturity was confirmed by the level of lineage markers CD33, CD235, CD19, CD7 and CD45RA on the verge of absence however some cells among this population expressed CD14. Interestingly placenta contain $CD34^{+++}CD45^{low/-}$ cells that as well as $CD34^{++}CD45^{low}$ didn't express lineage markers but in contrast to $CD34^{++}CD45^{low/-}$ cells were negative for stem cells markers CD133 and have heterogeneous morphology. The absence of such cells in cord blood and fetal liver suggests that they perhaps generated only in the placental tissue or migrate from the other sites of hematopoiesis and change the level of CD34 expression. We propose that population of placental HPCs which have the very high level of cell-cell adhesion protein CD34 ($CD34^{+++}CD45^{low/-}$) allows them to interact with placental cell niche. It was studied at a single cell level the characteristics of a populations of $CD34^{+++}$ hematopoietic progenitor cells that autors defined in human umbilical cord blood and these cells have extensive proliferative and replating capacity *in vitro* [3].

Thus placenta contain primitive HPCs potentially stem cells with phenotype $CD34^{++}CD45^{low/-}$. These observations are in agreement with reports showing that cells with high level of CD34 contain primitive hematopoietic progenitors including stem cells and primitiveness of progenitor cells was closely related to levels of CD34 [4, 5, 6, 7, 10]. Barcena et al. found two population $CD34^{++}CD45^{low}$ and $CD34^{+}CD45^{low}$ in chorionic villi and in the chorioamniotic membrane of different stage of placental development. $CD34^{++}CD45^{low}$ cells express markers of multipotent primitive hematopoietic progenitors and hematopoietic stem cells and demonstrate myeloid and erythroid potential *in vitro*, generated $CD56^{+}$ natural killer cells and $CD19^{+}CD20^{+}sIgM^{+}$ B cells in polyclonal liquid cultures while $CD34^{+}CD45^{low}$ content more committed progenitors [2]. Fetal bone marrow cells also contain population of cells with different expression level of CD34 ($CD34^{hi}$ and $CD34^{lo}$) wherein only $CD34^{hi}$ cells has phenotype of the most primitive hematopoietic cells such as $Thy\text{-}1^{+}$,$HLA\text{-}DR^{low}$, $CD38^{low}$, $CD45RA^{-}$, also expressed low levels of antigens CD13 and CD33 but no detectable cell surface antigens of more mature cells (CD2, CD10, CD14, CD15, CD16, CD19, glycophorin A) in addition $CD34^{hi}$ cells support long-term B lymphopoiesis and myelopoiesis *in vitro* and mediate T, B, and myeloid repopulation of human tissues implanted into SCID mice [5]. In adult bone marrow CD34 antigen density decreased concurrently with maturation and increasing CD38 antigen density [10]. It was reported that fetal liver contain cells with high level of CD34 and they were enriched for $Thy\text{-}1^{+}$, $CD117^{+}$, $CD123^{+}$, $HLA\text{-}DR^{+}$, $CD7^{-}$, $CD38^{-}$, $CD45^{-}$, $CD71^{-}$,$CD115^{-}$ and able to reconstitute lymphoid and myeloid lineages in SCID-hu mice [6].

We have shown that enzymatic treatment that we used for placenta in some cases change the expression of some hematopoietic markers in umbilical cord blood that detected by FACS. Also it was reported that the intensity of expression of CD3, CD4, CD8, αβ and γδ T cell receptors that examined by FACS was decreased by 25-40% in peripheral blood lymphocyte that were incubated with collagenase type 1 A and Dispase [1]. It is worth noting that in our case the expression level was increased in the case of enzymatic treatment. Thus when comparing the expression of markers by FACS in the two samples, one of which was treated of enzymes while the other didn't it worth to take into account that the resulting difference may be caused only by the effect of enzymatic treatment. We consider that in such cases worth to treat all samples for obtaining reliable data. In our investigation we were treated the cells from cord blood with enzymes that we used for placenta and then analyzed by FACS.

Conclusions. Placental tissue contains three populations that differ in expression level of CD34 such as $CD34^{+/low}CD45^{low/-}$, $CD34^{++}CD45^{low/-}$ and $CD34^{+++}CD45^{low/-}$. Similar to fetal liver placenta contain both population of $CD34^{++}CD45^{low/-}$ and $CD34^{+}CD45^{low/-}$cells that suggested about hematopoiesis in placental tissue. $CD34^{++}CD45^{low/-}$ population also express CD133, almost negative by lineage markers and had lymphocyte-like morphology that give evidence that this population contain primitive HPCs potentially stem cells. Also among placental cells are later progenitors with phenotype $CD34^{+/low}CD45^{+}$ as majority of these cells express hematopoietic lineage markers. Population with phenotype $CD34^{+++}CD45^{low}$ was observed only in placenta that suggests generated perhaps only in the placental tissue or migrate from the other sites of hematopoiesis and change the level of CD34 expression. Enzymatic treatment has insignificant effect on level of some cell surface protein in FACS analyze that worth to take into account in such experiments.

References

1. Abuzakouk M., Feigherym C, O'Farrelly C. Collagenase and Dispase enzymes disrupt lymphocyte surface molecules // Joumal of Immunological Methods. – 1996. – 194. – P. 211-216.

2. Bárcena A., Muench M.O., Kapidzic M., Fisher S.J. A new role for the human placenta as a hematopoietic site throughout gestation // Reprod. Sci. – 2009 Feb. – 16(2). – P. 178-87.

3. By Li Lu, Mang Xiao, Rong-Nian Shen et al. Enrichment, Characterization, and Responsiveness of Single Primitive $CD34^{+++}$ Human Umbilical Cord Blood Hematopoietic Progenitors With High Proliferative and Replating Potential // Blood. – 1993. – Vol81, No 1 (January I). – P. 41-48.

4. Craig W., Kay R., Cutler R.L. et al. Expression of Thy-1 on human hematopoietic progenitor cells // Exp. Med. – 1993. – May 1; 177(5). – P. 1331–1342.

5. DiGiusto D., Chen S., Combs J., et al. Human fetal bone marrow early progenitors for T, B, and myeloid cells are found exclusively in the population expressing high levels of CD34 // Blood. – 1994. – Vol. 84, No 2 (July 15). – P. 421-432.

6. Marcus O. Muench, Roncarolo M. G., Namikawa R. Phenotypic and Functional Evidence for the Expression of CD4 by Hematopoietic Stem Cells Isolated From Human Fetal Liver // Blood. – 1997. – Vol 89, No 4 (February 15). – P. 1364-1375.

7. Muench M.O., Cupp J., Polakoff J., Roncarolo MG. Expression of CD33, CD38 and HLA-DR on $CD34^{+}$ human fetal liver progenitors with a high proliferative potential // Blood. – 1994. – 83 (11) – P.3170-81.

8. Serikov V., Hounshell C., Larkin S. et al. Human Term Placenta as a Source of Hematopoietic Cells // Experimental Biology and Medicine. – 2009. – 234. – P. 813 – 823.

9. Shablii V.A., Kuchma M.D., Kyryk V.M. et al. Cryopreservation human placental tissue as source of hematopoietic and mesenchymal stem cells // Cell Transplantology and Tissue Engineering. – 2012. – Vol. VII, №1. – P. 54-62.

10. Terstappen L.W., Huang S., Safford M. et al. Sequential generation of hematopoietic colonies derived from single nonlineage-committed CD34/CD380 progenitor cells // Blood. – 1991. – Mar 15;77(6). – P. 1218-27.

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ПОРІВНЯЛЬНИЙ ФЕНОТИПІЧНИЙ АНАЛІЗ ПОПУЛЯЦІЙ ГЕМОПОЕТИЧНИХ ПРОГЕНІТОРНИХ КЛІТИН З РІЗНИМ РІВНЕМ ЕКСПРЕСІЇ *CD34*, ОТРИМАНИХ З ТКАНИНИ ПЛАЦЕНТИ І ПУПОВИННОЇ КРОВІ

Дослідження плацентарних гемопоетичних прогеніторних клітин (ГПК) і порівняння їх з властивостями ГПК плоду і дорослого організму необхідні для оцінки можливості їх клінічного застосування. Було показано, що тканина плаценти містить три популяції з різним рівнем експресії CD34, такі як $CD34^{+++}CD45^{low}$, $CD34^{++}CD45^{low}$ і $CD34^{+/low}CD45^{low}$. Як і в фетальній печінці, в плаценті містяться популяції з фенотипом $CD34^{++}CD45^{low/-}$ і $CD34^{+}CD45^{low/-}$, що дозволяє говорити про кровотворення в плацентарній тканині. $CD34^{++}CD45^{low/-}$ популяція також експресує CD133, практично негативна по лінійним маркерами і має лімфоцитоподібну морфологію. Це свідчить про те, що така популяція містить примітивні ГПК, потенційно – стовбурові клітини. Також серед плацентарних клітин присутні більш пізні прогенітори з фенотипом $CD34^{+/low}CD45^{+}$. Більшість таких клітин експресує гемопоетичні лінійні маркери. Популяція з фенотипом $CD34^{+++}CD45^{low}$ спостерігається лише в плаценті, клітини з таким фенотипом, імовірно, утворюються тільки в плацентарній тканині і/або мігрують з інших сайтів кровотворення, змінюючи при цьому рівень експресії CD34. Ферментативна обробка має незначний вплив на рівень експресії поверхневих білків при FACS аналізі, що варто враховувати в подібних експериментах.

Ключові слова: гемопоетичні прогеніторні клітини, плацентарний гемопоез, експресія CD34 маркера, пуповинна кров.

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СРАВНИТЕЛЬНЫЙ ФЕНОТИПИЧЕСКИЙ АНАЛИЗ ПОПУЛЯЦИЙ ГЕМОПОЭТИЧЕСКИХ ПРОГЕНИТОРНЫХ КЛЕТОК С РАЗЛИЧНЫМ УРОВНЕМ ЭКСПРЕССИИ *CD34*, ПОЛУЧЕННЫХ ИЗ ТКАНИ ПЛАЦЕНТЫ И ПУПОВИННОЙ КРОВИ

Исследование плацентарных гемопоэтических прогениторных клеток (ГПК) и сравнение их со свойствами ГПК плода и взрослого организма необходимы для оценки возможности их клинического применения. Было показано, что ткань плаценты содержит три популяции с различным уровнем экспрессии CD34, такие как $CD34^{+++}CD45^{low}$, $CD34^{++}CD45^{low}$ и $CD34^{+/low}CD45^{low}$. Как и в фетальной печени, в плаценте содержатся популяции с фенотипом $CD34^{++}CD45^{low/-}$ и $CD34^{+}CD45^{low/-}$, что позволяет говорить о кроветворении в плацентарной ткани. $CD34^{++}CD45^{low/-}$ популяция также экспрессирует CD133, практически отрицательна по линейным маркерам и имеет лимфоцито-подобную морфологию. Это свидетельствует о том, что такая популяция содержит примитивные ГПК, потенциально стволовые клетки. Также среди плацентарных клеток присутствуют более поздние прогениторы с фенотипом $CD34^{+/low}CD45^{+}$. Большинство таких клеток экспрессирует гемопоэтические линейные маркеры. Популяция с фенотипом $CD34^{+++}CD45^{low}$ наблюдается только в плаценте, клетки с таким фенотипом, предположительно, образуются только в плацентарной ткани и/или мигрируют из других сайтов кроветворения, изменяя при этом уровень экспрессии CD34. Ферментативная обработка оказывает незначительное влияние на уровень экспрессии поверхностных белков при FACS анализе, что стоит учитывать в подобных экспериментах.

Ключевые слова: гемопоэтические прогениторные клетки, плацентарный гемопоэз, экспрессия CD34 маркера, пуповинная кровь.

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DEVELOPMENT OF PIG CUMULUS-OOCYTE COMPLEXES AT CONSTANT AND OSCILLATING TEMPERATURE AND PH

It is shown that replacement of constant temperature and pH of culture conditions by oscillating ones does not significantly decrease diameter of COCs as a result of their maturation in in vitro culture for 24 hours. Increase in content of follicular fluid from 10 % to 20 % in the medium of their maturation NCSU does not influence the gain in COCs diameter.

Key words: cumulus-oocyte complex, follicular fluid.

Introduction. Improving the growth and development of the live object remains relevant because the problem associated with the increase of its productivity and improvement of its viability. You can select two opposite to each other, strategic approaches to its solution: genetic and epigenetic, which, like any opposites, deny and complement each other and are interconnected by passages. Gene expression takes place only in certain environments [23]. It is pointed out, in particular, by such concepts as expressivity and penetrance. The result of the development of a biological object is not only preformed, in particular, in the form of genetic program but also is determined by the epigenesis, in particular, by the influence of external conditions. And then, by the creation of conditions of the envi-

ronment, more adequate to the nature of the living object, one can (too) enhance its growth-development, contribute to the recovery of its (re)productive capacity. In this regard, previously unsolved, experimentally and theoretically, part of the overall problem of improving growth and development of a living object, the question remained, what should be a favorable external environment adequate, constant or inconstant, and what the character they should be if they are not constant.

In our view, between all known means of using the external environmental conditions for this purpose, the most adequate to nature of the living object, is the method of application, or at least maintaining namely biorhythmic oscillations of them. Namely it, as the literature data and our own research show, enables us to dramatically improve the growth-development and performance-viability of a living object, to remove it (re) productive potential.

Around the world, preimplantation embryos of mammals are continued to culture, in the hole, at constant conditions, which are trying to stabilize as strong as possible, – to prevent smallest changes, using a routine method which was developed 50 years ago [25].

It has been shown that the use of forced pH oscillations of culture medium, instead of it forced stabilization, significantly ($p < 0.001$) improves the development in vitro of 1 to 4-cell pig embryos ($n = 788$) flushed out from the organism: by 3.2 to 3.9 times more blastocysts are formed, and they grow to more advanced stages of embryonic development [4; 5]. Actually the survival rate of embryos increases.

As a result, the analysis of recent studies and publications, in which solving problems started, it was discovered that the theory and practice of application of the environment conditions oscillations, as opposed to the use of constant conditions, takes in the whole world more and more development and distribution.

It is shown that (biorhythmically) oscillating environmental conditions can be useful for improving the growth-development, performance-viability of the microorganisms, plants, crustaceans, amphibians [13], fish [9; 12], birds, [19; 20] and mammals [8].

For the present, oscillating environmental conditions have restricted use during in vitro culture of gametes, cells and mammalian preimplantation embryos [27; 28].

Here up to the present, they do not use oscillations of temperature and pH, indicating a sporadic and not a conscious application of oscillation on these biological objects, limited understanding of their role in the structures-functions of living object.

The aim of the work was to reveal the regularities of cumulus-oocyte complexes (COCs) growth in the maturation mediums in vitro – NCSU and 199 – with 10 and 20 % follicular fluid (FF) at biorhythmically oscillating temperature and pH in comparison with constant ones.

Materials and methods. The OCC were selected as an object of the study. Such a choice is conditioned by the need to increase the quantity and improve the quality of the oocytes in vitro maturation (in the form of COCs), from which embryos should further be get in vitro. The latest are used in large quantities for many scientific and practical purposes.

Of the dead at slaughterhouse pigs, the ovaries were withdraw and delivered to the laboratory, where the COCs and FF were received from the follicles.

Maturation of the COCs was done in the NCSU medium which were prepared with our own hands from Sigma reagents as described in literature, and in the medium 199, Sigma catalog number 5085 M.

10% (common) or 20% of the FF (in an attempt to make a step in the direction of the conditions that occur in vivo), 10 IU/ml of human chorionic gonadotropin, 10 IU/ml of horse chorionic gonadotropin, 0.53 mmol cysteamine and 20 mcg/ml gentamicin sulfate were added to the medium.

The in vitro culture at constant temperature and pH was carried out by a common (not principally modified for our purposes) method [25].

Cups with COCs in the appropriate medium, were put in gas chambers, – 100 ml of medical graded bottles with wide mouth, – blown through with gas mixture of carbon dioxide and air, which as a result of interaction with the medium has provided the last a constant pH around 7.4 units and tightly sealed.

The pH of the medium was controlled by measuring it in parallel chambers, which do not include the COCs. For this purpose, aliquots of the medium were taken and the pH of it was measured.

Temperature oscillation with 40-minute period was created by our own method [10; 17]. Air thermostat TC-80 was converted for this purpose into thermooscillator. Namely, the water in plastic bottles was placed into it and it was programmed to turn on and off using the timers of Feron and Brilux firms. The temperature changes in the chamber with COCs were judged from this in the parallel one, with a thermometer inserted into it, which pierces the wall of the thermooscillator chamber and shows of which can be watched at any time. Amplitude of temperature oscillation was changed from 37 °C to 39 °C by changing the amount of water in the thermooscillator. In another thermostat TC-80, temperature was maintained stable from 38.9 °C to 39.0 °C.

pH oscillation with daily period was created by our own method [11; 18]. To do this, specially constructed gas aluminum cameras with pipes of the silicone rubber half penetrated for gas were used.

COCs passed into the glass chambers with the maturation medium on which the vaseline oil was previously stratified. The initial pH of the medium, equal 8.0 to 8.2 unites, was created. These cameras put into gas chambers. The last were blown through with a mixture of carbon dioxide and the air, what led to a decrease in the medium pH up to 7.2. After 24 hours, the pH of the medium came up again the level of 8.0 to 8.2 unites owing to the back coming out of the carbon dioxide from the medium, and then doing so from the gas chamber, through the tube of silicone rubber, by the gradient of it concentration.

The stability of constant temperature in the range of 38.9 °C to 39.0 °C was guaranteed by thermostat TC-80. The COCs development was estimated by the change in magnitude of its diameter as a result of culture for one day. The diameter of the COCs was measured before and after culture using the eyepiece-micrometer of binocular microscope MBS-9. The relative growth rate, measured as a percentage, was counted by the formula Brody [15]:

$$y \equiv 2 \times 100 \times \frac{M_2 \div M_1}{M_2 \oplus M_1},$$

where y is the value of the y relative growth rate, expressed as a percentage, M1 is the diameter of the COCs before culture, M2 is the diameter of the COCs after 24 hours from the beginning the culture.

Biometrical processing was carried out on the computer using programs Excel and STATISTICA 6. All research was conducted in the Laboratory of Physiology of the Pig Breeding and Agro-Industrial Production Institute of NAAS of Ukraine.

Results and their discussion. 22 in vitro cultures were performed and 1249 COCs cultured. It is believed

that the sizes of living object related to the growth are distributed normally. In six of the ten studies, more than 100 COCs were generally employed.

This is the value which permits using parametrical statistics. On the other hand, it is known that nonparametric statistics have less statistical power (less sensitive) than their parametric competitors [16]. For these reasons, it was decided to be limited by the use of parametric statistics only.

As it can be seen from the results of this study, the percentage of the increase in the COC diameter in the NCSU medium with 10 % of FF at constant culture conditions does not differ significantly from that obtained as a result of culture in the same medium, as with 10 % of FF so with 20 % one, at oscillating temperature, separately – at oscillating pH, and received as a result of culture in the same medium with 10 % of FF at the oscillating temperature together with the oscillating pH (table 1).

***Table* 1. A comparison of the increase in the COCs diameter at constant and oscillating conditions in different maturation mediums contained different percentage of FF**

Culture conditions	Culture medium	Number of culture, n_1	Number of COCs, n_2	FF, %	Gain in COCs diameter, %	Cv, %
Constant	NCSU	13	185	10	48,28±3,98^{a}	28,52
	NCSU	5	75		50,92±6,97^{a}	27,36
	199	4	135		20,04±4,05^{c}	34,98
Oscillating temperature	NCSU	13	177	10	46,47±4,31^{ав}	32,06
	NCSU	5	77	20	48,67±7,21^{ав}	29,63
	199	4	139		33,50±4,59^{св}	23,70
Oscillating pH	NCSU	13	173	10	44,11±4,03^{ав}	31,63
	NCSU	5	80	20	42,57±4,15^{ав}	19,50
	199	1	46		32,93	-
Oscillating temperature an pH	NCSU	12	162	10	51,14±5,93^{a}	38,50

Note: the values with different superscripts differ significantly.

The percentage of increase in the COCs diameter in the medium 199 with 10 % of FF at constant culture conditions too differed not significantly from that obtained as a result of culture in the same medium with 20 % of FF at oscillating temperature. But the result obtained at oscillating temperature, had a distinct trend (although $p > 0.05$) to prevail that, resulted from constant conditions.

The percentage of increase in the COCs diameter in the medium NCSU with 10 % of FF at constant culture conditions was significantly greater ($p < 0.001$) from such derived as a result of culture in the medium 199 at the same culture conditions and with the same FF concentration. And at oscillating temperature, it differed not significantly.

The percentage of increase in the COCs diameter in the medium NCSU with 10 % of FF at oscillating temperature do not significantly differed from that obtained from culture in the same medium, but with 20 % of FF. The same applies to the culture at oscillating pH. Therefore, increasing the content of FF from 10 % to 20 % in the COCs maturation medium, NCSU, do not influenced negatively on increase of the diameter.

The data obtained are consistent with conclusion made [24], according to which the addition of FF to COCs maturation culture medium, prepared on the base of medium 199, can be beneficial, if is not more than 25%. So, in this way, one can save reagents in preparing medium for in vitro culture and the money at buying them.

It is interesting to compare the dimensions of the COCs and the magnitude of the diameter gain in our experiments with such in the works of other researchers. The average initial diameter of the COCs, which we took at the first thirteen cultures, was 15 points, or 210 microns. The average final diameter of the COCs was in the range from 25 units to 29 units, or from 350 mcm to 364 mcm. And the increase in the diameter of the COCs (Brody formulae) was in the range from 44 % to 51 %.

In experiments [22] the average diameter of the original COCs which were taken on culture in the medium that was also prepared on the medium NCSU, at the same concentration of FF (10 %), was equal to 240 microns, the average end – 340 microns.

In this case, the percentage increase in the diameter of the COCs in their investigation came up to the magnitude of 33 % which was less than that of received by us at least 11 %. However, they spent the measurement not after 24 h from the beginning of culture, but after 22 h.

But, they measured the largest diameter of the COCs, and we calculated the middle one. With this comparison, we can conclude that the culture conditions in our experiments were quite good.

The final diameter of COCs cultured by us (from 350 mm to 364 mm) coincides with this found [14]: diameter of the COCs, which had more than five – six layers of cumulus cells, composed from 210 microns to 350 microns. The following COCs are in the medium and large follicles.

At all culture conditions, a size of final diameter of the COCs strongly and almost everywhere, reliably correlated with the initial one (table 2). The regression coefficient also shows that practically all of the magnitude of the correlation is determined by the magnitude of the initial diameter of the COCs.

Low performance results of the COCs culture in the medium 199 at constant conditions can be explained by the fact that in this case it was less adequate to their needs than the medium NCSU, but the application of temperature oscillations (and, separately, also pH) significantly increased its adequacy.

The existence of differences in the expansion of cumulus depending on which medium is used for in vitro culture is known from the literature [21].

***Table* 2. The dependence of the final value of the average diameter of the COCs on the magnitude of their original middle diameter under different culture conditions**

# from the row-com	Culture conditions	Number of cultured COCs	The dependence of the final value of the average diameter of the COCs from the magnitude of the initial one		
			Correlation coefficient, r	Trustworthiness, p	Regression coefficient, r^2
1	constant, NCSU, 10 % of FF	185	0,82	0,0006	0,67
2	oscillating temperature, NCSU, 10 % of FF	177	0,94	0,0000	0,89
3	oscillating pH, NCSU, 10 % of FF	173	0,86	0,0002	0,74
4	oscillating temperature and pH, NCSU, 10 % of FF	162	0,88	0,0002	0,77
5	constant, medium 199, 10 % of FF	135	0,76	0,24	0,58
6	oscillating temperature, medium 199, 20 % of FF	139	0,92	0,085	0,84
7	constant, NCSU, 10 % of FF	75	0,98	0,0027	0,97
8	oscillating temperature, NCSU, 20 % of FF	77	0,93	0,0226	0,86
9	oscillating pH, NCSU, 20 % of FF	80	0,90	0,0386	0,81

It can be brined a few potential reasons of why the expected overwhelm in impact of oscillating parameters of culture medium is not detected in comparison with the influence of the constant ones.

One of them is a short duration of culture, for only the entire day. Usually, as the results of the literary review show, the positive impact of the factor is manifested after five or six days of the further culture. However, this culture duration was enough to show tendentious best influence of oscillating parameters of COCs culture medium, 199, compared with the influence of constant ones. The best effect of oscillating temperature on the increments of living mass, in comparison with the influence of constant one, was showed on young fish [9].

The second possible reason is no resonance (rhythm) changes in temperature in relative to the rate of growth and development of COCs (resonance rhythm is unknown).

The third one is the application of too large period of pH biorhythmic changes; it is desirable to experience the one hour rhythm of the pH oscillation in the future.

The fourth one is suboptimal amplitude of temperature and pH oscillations in some cultures of this searching research.

The fifth one is the use of medium designed for culture in namely constant conditions.

Comparison of the percent of the COCs diameter gain in medium NCSU and 199 led us to the realization of the fact that all, without exception (!), to the best of our knowledge, the existing culture medium for any cells and tissues, gametes and mammalian preimplantation embryos are designed with the aim of culturing them at stable constant conditions.

First of all we have in mind the medium 199 and the medium NCSU.

It may be one of the most important reasons of why it is difficult to show that the culture at oscillating conditions can be much more useful than the culture at constant ones.

Such utility should be displayed on medium, specifically constructed for this purpose, or on the medium that contains a significant amount of liquid of biological origin.

We think that the liquid of biological origin certainly needs to be such that reflects the biorhythmicity of the processes in the living body and its cells, and reflects the diversity of living objects at the cellular and subcellular level.

In our conception, in other to promote the culture at oscillating conditions, the medium would consist not only of those components, which are consumed by cells under conditions that occur near the position of conditional equilibrium around which medium conditions are oscillating on some of its parameter but also with those which are consumed by cells at the edges of the range of the environmental changes of the cells around the points of maximum deviations of these environmental conditions the cell from a conditional equilibrium, in extremes of their (conditions) sinusoidal changes.

Namely from these positions, it can easily be explained the tendentious preference applying oscillating parameters over constant ones only in the medium 199, which is much more complicated for the medium NCSU.

Why oscillation conditions of oocyte culture medium (also cells and embryos) can be more useful than constant ones?

In quantitative terms, oscillation of medium conditions is the transition from maximum concentration of a substance or maximum of medium parameter to minimum one or minimum of medium parameter, and vice versa, which is performed around conditional position of equilibrium, around which the oscillation takes place, around the average concentration, the average value of the parameter.

In qualitative terms, oscillation is the transition into relatively opposite state, for example: from acidic state to alkaline one and vice versa, if we talk about concentration of hydrogen ions, and hydroxyl ions; from warm to cold and vice versa, if we talk about the temperature.

It is well known that the rhythm is a universal feature of the movement of matter [1], each parameter of the body oscillates multiperiodically [6], oscillation is observed from early ontogeny: entrance of sperm cell into oocyte generates oscillation, in particular, calcium ions [29], and the external (environmental) conditions are oscillating and fluctuating too [3].

According to our vision, the creation of (biorhythmically) oscillating medium conditions, pH, temperature and other parameters, can synchronize processes in living object, to promote the maturation of eggs in time – alternate greater strengthening the growth and development, the anabolism and catabolism. After all, it is known that oscillations are necessary for cells to be passed from one extreme of the physiological state in which outweigh the anabolic processes in the second, where the katabolic ones is dominated [2].

(Biorhythmic) oscillatory mutual transitions of opposite states of medium (environmental) conditions (for example, temperature maximum and minimum or pH maximum and minimum) can contribute to the same (biorhythmic) oscillatory mutual transitions of the expression each of any opposites of any structure-function of the gametes (and also the cell, embryo, organism).

In contrast to constant conditions of environment, especially to those constancy of which stabilize at full strength, overly, oscillation extends the ranges of parameter changes, diversifies the environment on parameter values and, respectively, quality.

During the culture of 1 – 4-cell pig embryos in vitro constant pH was managed to hold within a range of ± 0.1 units, while oscillating pH changes in the range of 7.3 to 8.3 units [5].

It is well known that many different enzymes, as well as a variety of hormones, are presented in the cells of the organism by isomeric forms.

In the environmental conditions, (biorhythmically) oscillating in the normally width range (biologically diverse), unlike the constant ones, overly stabilized, narrowed, more different genes, whose products are formed isomeric forms, may be exposed to the expression.

In this case, in oscillating medium, the probability of one or other biochemical reactions processing may be increased.

If some gametes (or cells of an embryo or organism), have genetically altered some of the genes that are responsible for the formation of the isomeric form, enzyme can be at least is not active in constant environment (medium).

In the oscillating medium, same isomeric form can be formed, although only during some phases of the oscillatory period (cycle). And the reaction will be processed periodically.

In oscillatory conditions, a cell, perhaps, can live with the participation of a greater diversity of their genes, products of their expression, with a greater diversity of structures, built from these products, a greater variety of functions that are inherent to these structures.

In the oscillating (and fluctuating) medium conditions, biological diversity can be helpful on all structural and functional levels.

It can be assumed that, together with a temperature and pH oscillations, which we created in our work, the concentration of calcium ions was oscillated too what, as was shown by researchers, promotes gene expression in the cell [26].

As we expect, oscillatory culture, in comparison to culture at constant medium conditions, will give the possibility to develop normally in vitro to a larger percent of the total number of COCs taken on culture.

Still, replacing of constant culture medium parameters for oscillating expands conditions of environment and in this way allows to survive those biological objects, which need for their development environmental conditions that differ from those that are provided by the culture at constant, overly narrowed, stabilized conditions.

Not for all COCs optimal cultivation condition may be, for example, a pH of 7.4 units because all COCs are slightly different by nature. Has no doubt that discovered [7] biological non-equivalence and differential quality of reproductive cells, apply in the full measure to the COCs too.

The prospects for further development in this direction are large and multisided.

Still, in the case of the finding for optimal regimes of biorhythmic oscillation of medium culture conditions it can be used the new powerful nonspecific factors for in vitro development of oocytes and embryos, sperm capacitation, in vitro fertilization, inducement the oocytes in vitro to parthenogenetic development.

In this way is made, only the first, but very important steps: it is shown that the processes of COCs growth associated with getting embryos in vitro, can go under oscillating conditions at least at the same level as they do under constant ones.

The next step will be to find those ranges and amplitude of oscillation parameters of culture medium that will provide the best results with in vitro embryo obtaining from those that are received at constant conditions.

This step is already done for the preimplantation embryos received in vivo and cultured at pH, oscillated with the daily period [5].

In this work we leaned on biorhythms that are known for the somatic cells of the organism.

In further work, it would need to rely on the biorhythms that are place in oocytes during their maturing, but what we are not yet known.

Assume that the study on the culture at any (biorhythmically) oscillating medium parameters of any cells, taken from any plants and animals, will be perspective.

Such medium conditions could, in particular, to reduce the concentration of inoculate, to increase performance and viability of the cells.

An extremely promising is the direction of development the nutrient mediums for culture namely in oscillatory conditions.

Another promising line of research can be related with the development of equipment that can provide (biorhythmic) oscillation of temperature (thermooscillator instead of thermostat), pH (CO_2 incubator-oscillator instead of CO_2 incubator), lighting, electromagnetic field, concentration of gases in the atmosphere…

Conclusions: 1. The gain in COCs diameter, obtained after 24 h of culture in the medium NCSU at oscillating temperature and pH, both separately and together, does not trustworthily differ from the gain, received at constant conditions.

2. The gain in COCs diameter, obtained after 24 h of culture in the medium 199 at oscillating temperature has expressive trend (although $p > 0.05$) to be greater than the gain, received at constant conditions.

3. The gain in COCs diameter, obtained after 24 h of culture in the medium NCSU at constant conditions is significantly more than the gain, received as a result of culture in medium 199 at the same conditions.

4. Enhancement of the FF content from 10 % to 20 % in the maturation medium of the COCs, NCSU, does not influenced negatively on the increase of their diameter.

5. The advantage of oscillating culture conditions over constant ones expressively manifests itself in a relatively more complicated medium 199 compared to medium NCSU.

References

1. Алякринский Б.С., Степанова С.И. По закону ритма. – М., 1985.

2. Бюннинг Э. Биологические часы // Биологические часы. – М., 1964. – С. 11-26.

3. Григор'ев П.Є. Зв'язок інфрадіанної ритміки фізіологічних процесів у тварин з варіаціями геліогеофізичних факторів : автореф. дис. канд. біол. наук. – Сімферополь, 2005.

4. Денисюк П.В., Мартыненко Н.А. Принципиально новый метод культивирования доимплантационных эмбрионов млекопитающих // Доповіді Нац. АН України. – 1995. – № 11. – С. 148-149.

5. Денисюк П.В. Вплив pH середовища на розвиток in vitro доімплантаційних ембріонів свині : автореф. дис. … канд. біол. наук. – Харків, 1997.

6. Доскин В.А., Лаврентьева Н.А. Ритмы жизни. – М., 1991.

7. Квасницкий А.В. Половые клетки с.-х. животных, их биологическая неравноценность и разнокачественность // Науч. тр. Полтавский с.-х. ин-т. – 1949. – Вып. 4. – С. 79-92.

8. Квасницкий А.В., Мартыненко Н.А. Использование пульсирующей гипобарии для стимуляции развития эмбрионов у свиней и повышения их выживаемости // Физиол. журн. – 1980. – Т. 26, № 6. – С. 830-834.

9. Константинов А.С., Зданович В.В. Влияние осцилляции температуры на рост и физиологическое состояние молоди карпа (Cyprinus Carpio L.) // Доклады АН СССР. – 1985. – Т. 282, – № 3. – С. 760-764.

10. Корчан Н., Денисюк П. Розвиток ооцит-кумулюсних комплексів (ОКК) in vitro за осцилюючої температури // Вісн. проблем біол. і мед. – Полтава, 2011. – Вип. 1. – С. 250-252.

11. Корчан Н., Денисюк П. Розвиток ооцит-кумулюсних комплексів (ОКК) in vitro за осцилюючого pH // Вісн. проблем біол. і мед. – Полтава, 2012. – Вип. 1 (91). – С. 77-80.

12. Кузнецов В.А. Константинов А.С., Лукиянов С.В. Влияние колебаний pH на эмбрионально-личиночное развитие щуки Esox lucius // Усп. совр. биол. – 2009. – Т. 129, – № 3. – С. 286–293.

13. Лобачёв Е.А. Влияние колебаний экологических факторов на эмбрионально-личиночное развитие земноводных: дисс. канд. биол. наук. – Саранск, 2008.

14. Лобченко В.А. Влияние физиологических факторов на качество ооцитов свиньи и их созревание вне организма : автореф. дис. канд. біол. наук. – Харьков, 1991.

15. Brody S. Bioenergetics and growth. With special reference to the efficiency complex in domestic animals. – N. Y., 1945.

16. Непараметрическая статистика и подгонка распределения [Електронний ресурс]. – Режим доступу : http://www.statsoft.ru/home/textbook/modules/stnonpar.html.

17. Патент 62419, Україна, МПК (2011.01), A01N63/00. Спосіб культивування поза організмом ооцит-кумулюсних комплексів (ОКК) за температури, осцилюючої з одногодинним періодом / Корчан Н.О., Денисюк П.В.; заявник і патентоутримувач Інститут свинарства ім. О.В. Квасницького НААН; опубл. 25.08.2011, Бюл. № 16.

18. Патент 68013, Україна, МПК (2012.01), C12N 5/00, A61M 1/00. Застосування примусової біоритмічної осциляції pH середовища культивування поза організмом як способу, призначеного для збільшення міри розростання ооцит-кумулюсних комплексів (ОКК) / Корчан Н.О., Денисюк П.В.; заявник і патентоутримувач Інститут свинарства і агропромислового виробництва НААН. – № u 2011 10439; заявл. 29.08.2011; опубл. 12.03.2012, Бюл. № 5.

19. Патент RU № 2070387, Россия, A01K 41/00. Способ инкубации яиц сельскохозяйственной птицы / Фандеев Е.И. и др.; заявители и патентообладатели Фандеев Е.И. и др. – № 94028901/15; заявл. 03.08.1994; опубл. 20.12.1996.

20. Патент RU № 2107434, Россия, A01K 41/00. Способ инкубации яиц сельскохозяйственной птицы и устройство для его осуществления / Фандеев Е.И. и др.; заявители и патентообладатели Фандеев Е.И. и др. – № 94035822/13; заявл. 27.09.1994; опубл. 27.03.1998.

21. Abeydeera L.R. Development and viability of pig oocytes matured in protein free medium containing epidermal growth factor // Theriogenology. – 2000. – Vol. 54. – P. 787–797.

22. Beek J., Bijttebier J., Maes D. et al. The importance of cumulus expansion for monospermic fertilization of porcine cumulus-oocyte complexes // Reprod. Fertil. Dev. – 2011. – V. 23, N. 1. – P. 202.

23. Booth F.W., Chakravarthy M.V., Spangenburg E.E. Exercise and gene expression: physiological regulation of the human genome through physical activity // J. Physiol. (Lond). – 2002. – Vol. 543, Is. 2. – P. 399-411.

24. Brauch A., Keisler D.H. Effect of follicular fluid supplementation on in vitro maturation of porcine oocytes [Електронний ресурс] // Undergraduate Research and Creative Achievements Forum (MU) : collection. – 2007 [163]. – Режим доступу : http://hdl.handle.net/10355/1572.

25. Brinster R.L. A method for in vitro cultivation of mouse ova from two-cell to blastocyst // Exp. Cell Res. – 1963. – Vol. 32. – P. 205-208.

26. Dolmetsch R.E., Xu K., Lewis R.S.. Calcium oscillations increase the efficiency and specificity of gene expression // Nature. – 1998. – Vol. 392. – P. 933–936.

27. Mizobe Y., Yoshida M., Miyoshi K. Enhancement of cytoplasmic maturation of in vitro-matured pig oocytes by mechanical vibration // J. Reprod. Dev. – 2010. – Vol. 56, N. 2. – P. 285-290.

28. Ozil J. The parthenogenetic development of rabbit oocytes after repetitive pulsatile electrical stimulation // Development. – 1990. – Vol. 109. – P. 117-127.

29. Swann K., Lai F.A. A novel signaling mechanism for generating $Ca^2(+)$ oscillations at fertilization in mammals // BioEssays. – 1997. – V. 19. – P. 371-378.

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РОЗВИТОК ООЦИТ-КУМУЛЮСНИХ КОМПЛЕКСІВ СВИНІ ЗА ПОСТІЙНИХ І ОСЦИЛЮЮЧИХ ТЕМПЕРАТУРИ Й *РН*

Установлено, що заміна постійних температури й pH культивування на осцилюючі достовірно не зменшує приріст діаметра ооцит-кумулюсних комплексів (ОКК) у результаті їх дозрівання в культурі in vitro протягом доби. Підвищення вмісту фолікулярної рідини від 10 % до 20 % у середовищі їх дозрівання – NCSU, не впливає на величину приросту діаметра ОКК.

Ключові слова: ооцит-кумулюсних комплексів, фолікулярна рідина.

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РАЗВИТИЕ ООЦИТ-КУМУЛЮСНЫХ КОМПЛЕКСОВ СВИНЬИ ПРИ ПОСТОЯННЫХ И ОСЦЕЛИРУЮЩИХ ТЕМПЕРАТУРЫ И *РН*

Выявлено, что замена постоянных температуры и pH культивирования на осцилирующие достоверно не уменьшает прирост диаметра ооцит-кумулюсных комплексов (ОКК) в результате их дозревания в культуре in vitro на протяжение суток. Повышение содержания фоликулярной жидкости от 10% к 20% в среде их дозревания – NCSU, не влияет на величину прироста диаметра ОКК.

Ключевые слова: ооцит-кумулюсный комплекс, фолликулярная жидкость.

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EVIDENCE OF OXIDATIVE STRESS DEVELOPMENT IN PANCREATIC CELLS OF RATS WITH CHRONICALLY SUPPRESSED GASTRIC ACID SECRETION

Long-term hypochlorhydria is sometimes associated with mild pancreatitis development. Up to date, there is no clear evidence on mechanisms involved in pancreatic damage upon these conditions. The aim of study was to estimate the intensity of free-radical processes in rat pancreatic cells upon experimental hypochlorhydria. The increased hydrogen peroxide content (1,8 times), Nos2 gene mRNA level (2,9 times), total NO-synthase (4 times), thioredoxin reductase (1,6 times) and mitochondrial superoxide dismutase (1,5 times) activities, as well as decreased content of total (1,3 times), protein-bound (1,3 times) and nonprotein (1,4 times) SH-groups in rat pancreas were established. Thus, there is evidence of oxidative stress development in rat pancreatic cells upon long-term suppression of gastric acid secretion, suggesting the involvement of disturbed redox balance in pathophysiologic mechanisms of mild pancreatitis development upon these conditions.

Key words: oxidative stress, hypochlorhydria, dysbiosis, pancreas.

Introduction. Hypochlorhydria is defined as a state of low hydrochloric acid content in gastric juice with complex etiology: it develops as a consequence of pharmacologic suppression of gastric acid secretion, as a natural process during aging of digestive tract, as a complication upon some disease (atrophic gastritis, autoimmune disordrs, *H. Pylori* infection, achylia etc.) and in rare cases it has genetic causes [13].

In contrast to hyperacidic states, low stomach acidity is substantially harder to diagnose due to less prominent symptoms. At the same time, 10-15% of adult population and up to 50% people of retirement age in developed countries has hypochlorhydria [30]. Loss of gastric juice bactericidal properties is accompanied by bacterial overgrowth in different regions of gastrointestinal tract (GIT). Formation of dysbiotic bacterial biofilms in duodenum leads to initiation of endogenous inflammation that can involve associated organs, such as pancreas [3-4].

Although development of pancreatic disorders upon hypochlorhydria isn't unambiguous, there is some evidence of mild acute pancreatitis development (AP) upon long-term use of gastric H^+/K^+-ATPase inhibitors, such as omeprazole

(one of the most common causes of hypochlorhydria) [6, 27, 31]. At the same time, data on exact pathophysiologic and biochemical mechanisms of initiation and propagation of AP upon long-term hypochlorhydria are absent.

The imbalance between pro- and antioxidant factors with significant intensification of free-radical processes in pancreatic cells upon different experimental models of AP was shown during last two decades [15]. Oxidative stress (OS) is known to be a non-specific biochemical mechanism of cellular damage upon different pathological states [26]. Thus, determination of pro-/antioxidant balance indices can be used as a markers of pancreatic damage, and may help in elucidation of involved pathologic mechanisms in pancreatitis-like signs development upon long-term hypochlorhydria.

The **aim** of current study was to estimate the intensity of free-radical processes and state of antioxidant systems in rat pancreatic cells upon long-term suppression of gastric acid secretion by proton-pump inhibitor omeprazole.

Materials and methods. All experiments were performed on white non-strain male rats with initial weight around 180-200 g, which were kept on standard diet. International recommendations on performance of medical and biological investigations with the use of animals according to "European Convention for the Protection of Vertebrate Animals Used for Experimental and other Scientific Purposes" were followed.

All animals were divided into two groups. Rats injected abdominally with 0,2 ml of physiological solution and 0,5 ml of water were used as a control (first group). Hypochlorhydria (second group) was modeled by abdominal injection of omeprazole (14 mg/kg once a day) during 28 days [37].

Total nitric oxide synthase (NOS) activity was estimated by method [24]. In brief, this assay is based on determination of nitric oxide aerobic oxidation products. The content of hydrogen peroxide (H_2O_2) stable products was measured by sorbitol/xylenol orange assay [21]. Thioredoxin reductase (TRR) activity was determined by rate of NADPH-dependent reduction of Ellman's reagent with use of sodium aurothiomalate as TRR inhibitor [10]. The content of total and nonprotein SH-groups was estimated with Ellman's reagent in whole homogenates and protein-free fraction, respectively [7]. Superoxide dismutase (SOD) activity was measured in mitochondrial (corresponds to Mn-SOD activity) and cytosolic (corresponds to Cu,Zn-SOD activity) fractions of pancreatic cells by competition with nitroblue tetrazolium for electrons generated in NADH / phenazine methosulfate mixture [38]. Total protein concentration in whole homogenates and separate fractions was determined by Lowry assay [16].

RNA was isolated following Chomczynski et al. [5]; cDNA was synthesized in 20 µl of reaction mix upon such conditions: 70°C – 5 min, further 37°C – 5 min, 42°C – 1 hour. Amplification of DNA fragments was carried out upon such temperature conditions: initiative denaturation 94ºC – 4 min; further 35 cycles: DNA denaturation 94ºC – 45 s; hybridization of primers 52ºC – 45 s for *Nos2* (440 b.p.), and 49ºC – 40 s for *Actb* (521 b.p.) (internal control of reaction); chain extension 72ºC – 1 min 15 s for *Nos2* and 1 min for *Actb*. Further fill-in of PCR products was performed upon 72ºC for 5 min. Such primer sequences were used in reactions – for *Nos2*: forward GTGTTCCACCAGGAGATGTTG, and reverse – CTCCTGCCCACTGACTTCGTC; for *Actb*: forward – TGGGACGATATGGAGAAGAT, and reverse – ATTGCCGATAGTGATGACCT. Separation of PCR products was performed electrophoretically with following semiquantitative analysis of amplicons expression based on densitometry the ImageJ 1.45s program was used. Indices of mRNA expression were calculated for each sample following Konturek et al. [14].

Statistical processing of experimental data was performed with analysis of varience. Probability of difference between control and test measurements was assessed with Student's t-test. The difference between compared data was treated as probable if $p < 0,05$. All calculations and graph plotting were carried out in "OriginLab Origin 8.6" and "Microsoft Excel 2003" programs.

Results and discussion. To establish the state of pro-/antioxidant balance in rat pancreas upon long-term experimental hypochlorhydria such indices were assessed:

1) Prooxidants: hydrogen peroxide content, total NOS activity, expression of *Nos2* gene.

2) Antioxidants: SOD activity (mitochondrial isoform), total TRR activity, content of total, protein-bound and nonprotein SH-groups.

Hydrogen peroxide is a type of reactive oxygen species, which is produced upon two-electron reduction of oxygen or as a consequence of superoxide radical dismutation. Biological effects of H_2O_2 are concentration-dependent: in low concentrations H_2O_2 acts as a signal molecule, while in high experiments – as a cytotoxic oxidant [12].

It was established, that the level of H_2O_2 stable products in rat pancreas upon long-term hypochlorhydria was 1,8 times higher in comparison with the control. Observed increase in hydrogen peroxide level indicates the intensification of free-radical processes in rat pancreas and general depletion of cellular antiperoxide ability. The source of H_2O_2 production upon these conditions, probably, is a dismutation of superoxide radicals upon action of SOD and high XO activity. The increase in superoxide anion level and XO activity in pancreatic cells upon hypochlorhydria was shown in our recent study [34], thus confirming the probable source of hydrogen peroxide synthesis in these conditions. Hydrogen peroxide is known not only for its possibility to directly oxidize biomolecules, but also for its ability to split into highly toxic kind of ROS – hydroxyl radical ($\cdot OH$), which can rapidly oxidize lipids and proteins, and also damage cellular genome.

NOS is a group of NADPH-, FAD-, FMN- and BH_4-dependent oxidoreductases that catalyze conversion of L-arginine and O_2 to citrulline and nitrogen oxide (NO) [8]. There are 3 isoforms of NOS: endothelial, neuronal and inducible (iNOS). Expression of constitutive NOS isoforms was shown in pancreatic acinar cells of rodents [15] in normal circumstances, and there is also data on low basal level of iNOS expression in rat acini [29]. Depletion of constitutive isoforms expression with simultaneous elevation of iNOS level was established upon different experimental models of AP [1, 15].

It was established, that total pancreatic NOS activity in rats treated only with omeprazole for 28 days was 4 times higher in comparison with the control. Observed elevation of total NOS activity may reflect the activation of both constitutive and inducible isoforms of NOS, but according to a literature data activation of inducible NOS isoform is more probable and justified in stress conditions [8, 15]. To resolve this issue the level of iNOS gene (*Nos2*) expression in rat pancreatic tissue was estimated.

Table 1. **Indices of prooxidant factors in rat pancreas upon long-term suppression of gastric acid secretion (M ± m, n = 10)**

Parameter \ Group	Control	Omeprazole
Total NO-synthase activity, nmol × min^{-1} × mg of $protein^{-1}$	2,41 ± 0,23	9,73 ± 0,95*
Hydrogen peroxide content, mmol × gram of $tissue^{-1}$	2,61 ± 0,28	4,82 ± 0,45*

Remarks: * – $p < 0,05$ in relation with control.

According to Vaquero et al., there is low level of *Nos2* gene expression in pancreatic acinar cells in rats [29]. Moreover, it's known that different NOS isoforms are expressed in vascular endothelium and nerves spanning the pancreas [15]. Significant increase in *Nos2* gene expression upon different models of experimental AP was shown recently [1, 15, 19].

The investigation of *Nos2* gene expression level showed that in the control the mRNA level of this gene was 0,326 ± 0,034 (Fig. 1). In animals with long-term hypochlorhydria, the level of *Nos2* mRNA was 2,9 times higher than control values.

The observed existence of iNOS mRNA in pancreatic cells of control rats is in accord with data of Vaquero et al. [29]. Significantly increased level of *Nos2* gene expression in rat pancreas upon long-term suppression of gastric acid secretion was established in our study. According to papers of different authors, high level of iNOS expression was observed upon arginine-, taurocholate and caerulein-induced AP models [1, 15, 19]. The expression of iNOS was located mainly in vascular endothelial and muscle cells of pancreas in these experiments [15]. Unfortunately, the conditions of our study don't allow us to fix the exact place of iNOS expression, since the contamination of analyzed pancreatic tissue with fragments of vessels is possible. However, the increased level of *Nos2* mRNA can be responsible for earlier indicated high total NOS activity (table 1).

Enormous level of nitric oxide (synthesized by iNOS) in pancreatic cells favors nitration and nitrosilation of cellular proteins and DNA, that can lead to structural and functional damage of the pancreas [35]. Moreover, it can be suggested the generation of toxic peroxynitrite ($ONOO^-$) – condensation product of nitric oxide and superoxide anion ($\cdot O_2^-$) – upon these conditions, since the increase of $\cdot O_2^-$ level was shown recently [33-34]. Peroxynitrite is known to induce damage of wide spectra of biomolecules, such as oxidation of NH- and SH-groups in proteins, peroxidation of membrane lipids and one-chain brakes in DNA [15].

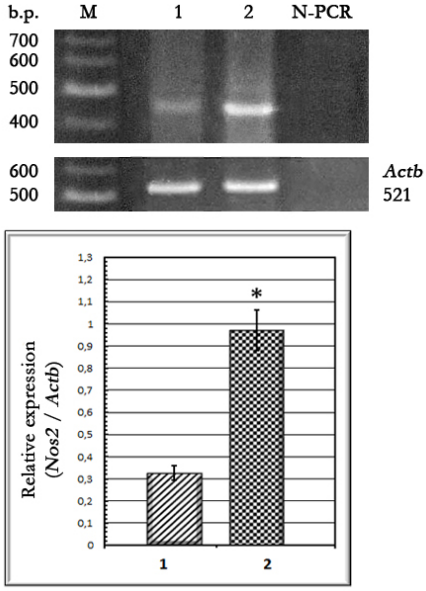

Fig. 1. Level of *Nos2* gene mRNA in rat pancreas upon long-term gastric hypochlorhydria

Remark: M – molecular mass marker; 1 – control; 2 – omeprazole; N-PCR – negative PCR control; * – $p < 0,05$ in relation with control

All aerobic organisms have evolved complex antioxidant systems (AOS) aimed on counteraction to free-radical processes and maintaining cellular redox homeostasis. AOS is a dynamic compound formation including both high-molecular enzymes and low-molecular non-enzymatic substances of different chemical nature [36]. Disturbance of AOS function in rat pancreas upon AP and inflammation was observed in a whole series of investigations [15, 23, 28]

Enzymes from SOD family constitute the first line of antioxidant cellular defense. SOD activity is crucial for effective inactivation of superoxide radicals and, thus, for whole control of free-radical processes in the cell. There are 3 isoforms of SOD in mammalian tissues: Cu,Zn-SOD (cytosolic), Mn-SOD (mitochondrial) and EC-SOD (extracellular) [18]. Regulation of *sod* genes expression, as well as enzymatic activity and compartmentalization of SOD isoforms is important for maintaining of stable ROS concentration in cells.

It was established that SOD activity in mitochondrial fraction (corresponds to Mn-SOD activity) of rat pancreas upon long-term gastric hypochlorhydria was 1,5 times higher in comparison to the control (table 2).

Table 2. **Indices of antioxidant system in rat pancreas upon long-term suppression of gastric acid secretion (M ± m, n = 10)**

Parameter / Group	Control	Omeprazole
Superoxide dismutase activity in mitochondrial fraction, units × min^{-1} × mg of $protein^{-1}$	0,194 ± 0,018	0,298 ± 0,027*
Thioredoxin reductase activity, μmol × min^{-1} × mg of $protein^{-1}$	5,512 ± 0,491	8,643 ± 0,780*
Total SH-groups content, mmol × mg of $protein^{-1}$	0,337 ± 0,032	0,255 ± 0,024*
Protein-bound SH-groups content, mmol × mg of $protein^{-1}$	0,229 ± 0,021	0,176 ± 0,016*
Nonpreotein SH-groups content. mmol × mg of $protein^{-1}$	0,108 ± 0,010	0,079 ± 0,008*

Remarks: * – $p < 0,05$ in relation with control

Thus, activation of second SOD isoform upon long-term suppression of gastric acid secretion were observed. Activation of Mn-SOD may be a consequence of increased superoxide radicals generation by mitochondrial electron-transporting chain, since $\cdot O_2^-$ is a specific substrate of SOD. On the other hand, transcription factors NF-κB and AP-1 stimulating *sod2* gene (encoding mitochondrial isoform of SOD) expression are usually upregulated as a result of proinflammatory cytokine stimulation and upon oxidative stress [15, 18].

Mammalian TRR is a key enzyme in so called "thioredoxin AOS", which also includes NADPH and thioredoxin [2]. Main function of this system is reduction of disulfide bonds in proteins and maintaining protein-bound SH-groups in reduced state. There 2 isoforms of TRR in mammalian cells – cytosolic/nuclear and mitochondrial.

Determination of TRR activity in rat pancreatic tissue indicated that total TRR activity upon long-term suppresiion of gastric acid secretion was 1,6 times higher in comparison with the control. On the basis of these data it may be suggested that increased TRR activity leads to high level of reduced thioredoxin in rat pancreatic cells upon long-term hypochlorhydria. Thioredoxin is a main cellular denitrosilating and disulfide reducing agent, thus increased thioredoxin level may be a compensatory reaction to enhanced NO generation and oxidation of protein-bound SH-groups in pancreatic cells in these conditions. This idea is supported by indicated above elevation of total NOS activity, as well as by shown earlier increased level of NO upon these conditions [33]. In order to estimate cellular thiols state the content of total, protein-bound and nonprotein SH-groups in rat pancreatic homogenates was determined.

It was established that protein-bound SH-groups level in control rat pancreatic homogenates was more than 2 times higher than nonprotein SH-groups content (table 2); these data is in accord with general distribution of SH-groups in tissues [25]. In pancreatic homogenates of rats with long-term hypochlorhydria the content of total, protein-bound and nonprotein SH-groups was, respectively, in 1,3; 1,3 and 1,4 times higher in relation to the control.

Observed decreased nonprotein SH-groups content is in accord with our earlier obtained data on reduced glutathione depletion in rat pancreas upon these conditions [32], since reduced glutathione is a main non-protein thiol in pancreatic cells [36]. Thus, decreased level of total, protein-bound and nonprotein SH-groups in rat pancreas upon long-term hypochlorhydria indicates general shift of redox-balance in pancreatic cells towards activation of oxidative processes and oxidative stress development.

On the basis of these and earlier obtained results [32-34] the following biochemical mechanism of oxidative stress development in rat pancreas upon long-term hypochlorhydria can be suggested. Long-term administration of omeprazole leads to dysbiosis development in duodenum – the nearest to pancreas part of GIT. Secretory or cellular components of dysbiotic microbiota and (or) stimulation by proinflammatory cytokines (caused by dysbiotic bacteria) near the pancreas lead to development of inflammatory cycle in pancreatic tissue [17]. Pancreatic cells are known to express some types of Toll-like receptors (TLR), which are sensitive to bacterial cell wall components [20]. Upon bacterial colonization of duodenum or pancreatic ducts, activation of TLR and inflammation initiation seems to be probable. At that, release of proinflammatory cytokines, such as TNF-α, IL-1, IL-8 and PAF with simultaneous IL-10 depletion may occur [9]. These cytokines stimulate expression of cell adhesion molecules (CAM) on the surface of neutrophils and endotheliocytes leading to neutrophils migration from blood vessels into pancreatic tissue [22]. Activation of neutrophils leads to further increase in proinflammatory cytokines production, CAM expression, vascular permeability and ROS generation in acinar cells [9, 17]. Cytokine release and high CAM expression favor even greater leukocyte migration into acini – thus develops inflammatory cycle [9]. As a result of this cycle, powerful leukocytic infiltration into pancreatic tissue develops with further ROS generation and intensive OS [15]. Elevation of ROS and RNS content, lipid and protein oxidation products level, as well as depletion of AOS enzymes in rat pancreatic cells upon long-term hypochlorhydria were observed in our recent researches, thus (together with results of this study) confirming OS development upon these conditions.

Conclusions. The elevation of prooxidant factors (hydrogen peroxide content, total NOS activity, expression of *Nos2* gene) and depletion of many antioxidants (total, protein-bound and nonprotein SH-groups content, TRR activity) in rat pancreas upon long-term experimental hypochlorhydria were established in this study. Obtained results undoubtedly indicate oxidative stress development in pancreatic cells of rats upon these conditions suggesting the important role of cellular redox homeostasis disequilibrium in pancreatitis-like signs development. Elucidation of initiating events underlying observed results requires further investigations.

References

1. Al-Mufti R.A., Williamson R.C., Mathie R.T. Increased nitric oxide activity in a rat model of acute pancreatitis // Gut. – 1998. – Vol. 43. – P. 564-570.

2. Cai W., Zhang L., Song Y., et al. Small molecule inhibitors of mammalian thioredoxin reductase // Free Rad, Biol, Med. – 2012. – Vol. 52. – P. 257-262.

3. Canani R., Terrin G. Gastric acidity inhibitors and the risk of intestinal infections // Curr. Opin. Gastroenterol. – 2010. – Vol. 26, № 1. – P. 31-35.

4. Chey W., Spiegel B. Proton pump inhibitors, irritable bowel syndrome, and small intestinal bacterial overgrowth: coincidence or Newtons third law revisited? // Clin. Gastroenterol. Hepatol. – 2010. – Vol. 8, № 6. – P. 480-482.

5. Chomczynski P., Sacchi N. Single-step method of RNA isolation by acid guanidiniumthiocyanate-phenol-chloroform extraction // Anal. Biochem. – 1987. – Vol. 162. – P. 156-159.

6. Eland I., Huerta Alvarez C., Stricker B., Garcia Rodriguez L. The risk of acute pancreatitis associated with acid-suppressing drugs // Br. J. Clin. Pharmacol. – 2000. – Vol. 49. – P. 473-478.

7. Ellman G. Tissue sulfhydryl groups // Arch. Biochem. Biophys. – 1959. – Vol. 82, №1. – P. 70-77.

8. Forstermann U., Sessa W.C. Nitric oxide synthases: regulation and function // Eur. Heart. J. – 2012. – Vol. 33 (7). – P. 829-837.

9. Frossard J.L., Pastor C.M. Experimental acute pancreatitis: new insights into the pathophysiology // Front. Biosci. – 2002. – Vol. 7. – P. 275-287.

10. Hill K.E., McCollum G.W., Burk R.E. Determination of thioredoxin reductase activity in rat liver supernatant // Anal. Biochem. – Vol. 253. – P. 123-125.

11. Holmgren A., Lu J. Thioredoxin and thioredoxin reductase: current research with special reference to human disease // Biochem. Biophys. Res. Commun. – 2010. – Vol. 396. – P. 120-124.

12. Kanta J. The role of hydrogen peroxide and other reactive oxygen species in wound healing // Acta Medica (Hradec Kralove). – 2011. – Vol. 54 (3). – P. 97-101.

13. Kitchen J. Hypochlorhydria: a review – Part 1 // Townsend Let. Doct. Pat. – 2001. – P. 56-60.

14. Konturek P.C., Brzozowski T., Pierzchalski P. et. al. Activation of genes for spasmolytic peptide, transforming growth factor alpha and for cyclooxygenase (COX)-1 and COX-2 during gastric adaptation to aspirin damage in rats // Aliment. Pharmacol. Ther. – 1998. – Vol. 12. – P. 767-777.

15. Leung P.S., Chan Y.C. Role of oxidative stress in pancreatic inflammation // Antioxid. Redox Signal. – 2009. – Vol. 11 (1). – P. 135-165.

16. Lowry O.H., Rosebrough N.J., Farr A.L., Randal R.I. Protein measurement with Folin phenol reagent // J. Biol. Chem. – 1951. – Vol. 193, № 1. – P. 265-275.

17. McClave S. Drivers of oxidative stress in acute pancreatitis: the role of nutrition therapy // J. Parenter. Enteral. Nutr. – 2012. – Vol. 36 (1). – P. 24-35.

18. Miao L., Clair D.K. Regulation of superoxide dismutase genes: implications in diseases // Free Radic. Biol. Med. – 2009. – Vol. 47 (4). – P. 344-356.

19. Motoo Y., Su S.B., Xie M.J., et al. Effect of herbal medicine keishito (TJ-45) and its components on rat pancreatic acinar cell injuries in vivo and in vitro // Pancreatology. – 2001. – Vol. 1. – P. 102–109.

20. Nakamura T., Ito T., Oono T., et al. Bacterial DNA promotes proliferation of rat pancreatic stellate cells through TLR-9: potential mechanisms for bacterially induced fibrosis // Pancreas. – 2011. – Vol. 40 (6). – P. 823-831.

21. Nourooz-Zadeh J., Tajaddini-Sarmadi J., Wolff S.P. Measurement of plasma hydroperoxide concentrations by the ferrous oxidation – Xylenol Orange assay // Anal. Biochem. – 1994. – Vol. 220. – P. 403-409.

22. Pham C.T. Neutrophil serine proteases: specific regulators of inflammation // Nat. Rev. Immunol. – 2006. – Vol. 6 (7). – P. 541-550.

23. Rau B., Poch B., Gansauge F., et al. Pathophysiologic role of oxygen free radicals in acute pancreatitis: initiating event or mediator of tissue damage? // Ann. Surg. – Vol. 231 (3). – P. 352-360.

24. Salter M., Knowles R.G., Moncada S. Widespread tissue distribution, species and changes in activity of Ca^{2+}-depend and Ca^{2+}-independent nitric oxide syntases // FEBS Lett. – 1991. – Vol. 291, № 1. – P. 145-149.

25. Sedlak J., Lindsay R. Estimation of total, protein-bound and nonprotein sulfhydryl droups in tissue with Ellman's reagent // Anal. Biochem. – 1968. – Vil. 25 (1). – P. 192-205.

26. Storz G., Imlay J. Oxidative stress // Curr. Opin. Microbiol. – 1999. – Vol. 2. – P. 188-194.

27. Sundstrom A., Blomgren K., Alfredson L., Wiholm B. Acid-suppressing drugs and gastroesophageal reflux disease as risk factors for acute pancreatitis – results from a Swedish Case-Control Study // Pharmacoepidemiol. Drug Saf. – 2006. – Vol. 15, № 3. – P. 141-149.

28. Tsai K., Wang S.S., Chen T.S., et al. Oxidative stress: an important phenomenon with pathogenetic significance in the progression of acute pancreatitis // Gut. – 1998. – Vol. 42 (6). – P. 850-855.

29. Vaquero E., Gukovsky I., Zaninovic V., Gukovskaya N., Pandol S. Localized pancreatic NF-κB activation and inflammatory response in taurocholate-induced pancreatitis // Am. J. Physiol. Gastrointest. Liver Physiol. – 2001. – Vol. 280. – P. 1197-1208.

30. Wright J. Why stomach acid is good for you: natural relief from heartburn, reflux and GERD. – M. Evans & Company, 2001. – 285 p.

31. Youssef S.S., Iskandar S.B., Scruggs J., Roy T.M. Acute pancreatitis associated with omeprazole // Int. J. Clin. Pharmacol. Ther. – 2005. – Vol. 43 (12). – P. 558-561.

32. Дворщенко К., Вакал С., Гайда Л., Остапченко Л. Антиоксидантні ефекти мультипробіотику Симбітер у підшлунковій залозі щурів за умов гіпоацидного стану // Вісник Київського національного університету імені Тараса Шевченка. – №61. – 2012. – С. 42-45.

33. Дворщенко К., Вакал С., Драницина А., Остапченко Л. Вільнорадикальні процеси у підшлунковій залозі за умов тривалої гіпоацидності та при дії мультипробіотика Симбітер // Вісник КНУ імені Тараса Шевченко. Серія: біологія. – 2013. – № 63. – С. 26-28.

34. Дворщенко К.О., Вакал С.Є., Драницина А.С., Сенін С.А., Остапченко Л.І. Стрес-респонсивні системи підшлункової залози щурів в умовах тривалої шлункової гіпохлоргідрії та за введення мультипробіотика "Симбітер®" // Український біохімічний журнал. – 2013. – Т. 85, № 2. – С. 68-77.

35. Лазебник Л.Б., Дроздов В.Н., Барышников Е.Н. Роль оксида азота (NO) в патогенезе некоторых заболеваний органов пищеварения // Эксперим. и клин. гастроэнтерол. – 2005. – № 2. – С. 4-11.

36. Харченко В.В. Природні антиоксиданти та печінка // Сучасна гастроентерологія. – 2006. – № 38. – С. 79-85.

37. Цирюк О.І., Берегова Т.В. Вплив омепразол-викликаної гіпергастринемії на базальну шлункову секрецію у щурів // Вісник проблем біології і медицини. – 2007. – Вип. 3. – С. 38-43.

38. Чевари С., Чаба И., Секей Й. Роль супероксиддисмутазы в окислительных процессах клетки и метод определения ее в биологических материалах // Лаб. дело. – 1985. – № 11. – С. 678-681.

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РОЗВИТОК ОКИСНОГО СТРЕСУ В КЛІТИНАХ ПІДШЛУНКОВОЇ ЗАЛОЗИ ЩУРІВ ЗА УМОВ ХРОНІЧНОГО ПРИГНІЧЕННЯ СЕКРЕЦІЇ ШЛУНКОВОГО СОКУ

Тривала гіпохлоргідрія інколи супроводжується розвитком помірного панкреатиту. На сьогодні точно невідомі механізми, що залучені до пошкодження підшлункової залози за цих умов. Метою дослідження було оцінити інтенсивність вільнорадикальних процесів у клітинах підшлункової залози щурів при експериментальній гіпохлоргідрії. Було встановлено підвищення вмісту пероксиду водню (у 1,8 рази), мРНК гена Nos2 (у 2,9 р.), загальної NO-синтазної (у 4 р.), тіоредоксинредуктазної (у 1,6 р.) та мітохондріальної супероксиддисмутазної (у 1,5 р.) активностей, а також зниження вмісту загальних (у 1,3 р.), білок-зв'язаних (у 1,3 р.) та небілкових (у 1,4 р.) SH-груп у підшлунковій залозі щурів. Отже, встановлено факт розвитку окисного стресу у клітинах підшлункової залози щурів за умов тривалої гіпохлоргідрії, що вказує на залучення порушення про-/антиоксидантного балансу до патофізіологічних механізмів розвитку помірного панкреатиту за даних умов.

Ключові слова: окисний стрес, гіпохлоргідрія, дисбіоз, підшлункова залоза.

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РАЗВИТИЕ ОКИСЛИТЕЛЬНОГО СТРЕССА В КЛЕТКАХ ПОДЖЕЛУДОЧНОЙ ЖЕЛЕЗЫ КРЫС В УСЛОВИЯХ ХРОНИЧЕСКОГО УГНЕТЕНИЯ СЕКРЕЦИИ ЖЕЛУДОЧНОГО СОКА

Длительная гипохлоргидрия иногда сопровождается развитием умеренного панкреатита. На сегодняшний день точно не известны механизмы задействованные в повреждение поджелудочной железы в этих условиях. Целью исследования было оценить интенсивность свободно-радикальных процессов в клеток поджелудочной железы крыс при экспериментальной гипохлоргидрии. Было установлено повышение содержания пероксида водорода (в 1,8 раз), мРНК гена Nos2 (в 2,9 р.), общей NO-синтазной (в 4 р.), тиоредоксинредуктазной (в 1,6 р.) и митохондриальной супероксиддисмутазной (в 1,5 р.) активностей, а также снижение содержания общих (в 1,3 р.), белок-связанных (в 1,3 р.) и небелковых (в 1,4 р.) SH-групп в поджелудочной железе крыс. Таким образом, установлен факт развития окислительного стресса в клетках поджелудочной железы крыс в условиях длительной гипохлоргидрии, что указывает на вовлечение нарушения про-/антиоксидантного баланса в патофизиологические механизмы развития умеренного панкреатита в данных условиях.

Ключевые слова: окислительный стресс, гипохлоргидрия, дисбиоз, поджелудочная железа.

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BIOLOGICAL EFFECTS OF CARBON NANOSTRUCTURE FULLERENE C_{60} IN LEUKEMIC CELLS

The ability of fullerene C_{60} both to produce reactive oxygen species and to modulate Ca^{2+}- homeostasis inside leukemic cells after UV-visible photoexcitation is demonstrated. It is shown that L1210 cells of leucosis origin are characterized by decreased store-operated Ca^{2+} entry as compared with normal lymphoid cells (primary rat thymocytes). After irradiation of L1210 cells pre-incubated with 10^{-5} M C_{60} the concentration of free cytosolic calcium and the relative value of endoplasmic reticulum (ER) Ca^{2+} pool were found to be increased, indicating on initiation of signalling events leading to Ca^{2+}-dependent apoptosis.

Key words: fullerene C_{60}, L1210 cells, ROS, cytosolic calcium, ER-pool.

Introduction. Nowadays searching for a new nanoscale chemically inert compounds that would selectively impair tumor and would not affect normal cells is a very important task in biology. Recent progress in nanobiotechnology has arised interest in biomedical application of fullerene C_{60}. Due to its unusual structure C_{60} exhibits unique physicochemical properties and biological activity. The spherical form of the C_{60} molecule, its nanosize (0.72 nm in diameter) and lipophilicity ensures a steric compatibility with biological molecules and effective accommodation inside hydrophobic regions of cell membranes. It takes a few milliseconds for C_{60} molecule to be incorporated into artificial bilayer lipid membrane and several hours to accumulate in a cytoplasm of cells [13]. Due to extended π-conjugated system of molecular orbitals It is fullerene C_{60} absorb strongly UV and blue light but the tail of absorbtion does stretch into red region. In a cell-free models the ability of photoexcited C_{60} to generate superoxide anion and single oxygen with almost 100% quantum yield is detected [2].

Intracellular prooxidant-antioxidant balance is known to be a regulator of cell proliferation and growth, but intense ROS production can lead to oxidative stress and cell death. Another messenger which also plays critical role in controlling cell prolifeartion, cell death and carcinogenesis is calcium. Changing the level of $[Ca^{2+}]_i$ in cells can occur by two mechanisms – increased Ca^{2+} entry through the plasma membrane or cation release from intracellular stores. In non-excitable cells the main mechanism of Ca^{2+} entry from the extracellular space is store-operated (pool-controlled) calcium entry (SOCE). This mechanism is controlled by the filling of ER calcium pool, providing Ca^{2+} entry through the plasma membrane only when ER content is essentially depleted.

Ca^{2+}-signalling in cancer cells is supposed to be remodulated – low basal filling of intracellular Ca^{2+} pool allows cancer cells to bypass Ca^{2+}-dependent apoptotic pathway. Many of Ca^{2+} -transporters and signalling molecules ,are the targets of ROS [6] and redox regulation of their activity seems to be one of the ways to modulate Ca^{2+} compartmentalization and signalling in cancer cells.

The aim of the study was to determine the relative value of store-operated Ca^{2+} entry in normal (Wistar rat thymocytes) and leukemic (L1210 cell lines of leucosis origin) cells and to estimate ROS production, the level of free cytosolic calcium and Ca^{2+}-pool of ER in leukemic cells after treatment with fullerene C_{60} following UV-visible light exposure.

Materials and methods. Rat thymocytes were isolated from Wistar rats (150-180 g). Thymus was removed and passed through nylon mesh into RPMI 1640 medium. The isolated cells were washed by centrifugation at 600 g. The leukemia L1210 cell line was obtained from Bank of Cell Line from human and animal tissue of the Kavetsky Institute of Experimental Pathology,Oncology and Radiobiology (NAS of Ukraine). L1210 cells were cultured in RPMI 1640 medium supplemented with 5% fetal calf serum, 10 U/ml penicillin and 10 μg/ml streptomycin.

Fullerenes C_{60} were synthesized in Technical University of Ilmenau (Germany). A stable water colloidal solution of 10^{-4} M fullerene C_{60} (purity > 99.5%), contains C_{60} clusters with the size of 12-50 nm [9].

Cells were incubated for 1 h with or without fullerene C_{60} (10^{-5} M). Fullerene C_{60} photoactivation was done by UV/Vis irradiation of probes with mercury-vapor lamp (320-600 nm, irradiance 200 mW/cm^2, distance 2 cm, during 2 mintes).

ROS production was measured using fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA), wich was added to the cell incubation medium (2×10^6 cells/ml) in concentration 5 mM. The fluorescent intensity was measured in real time on spectrofloumeter Shimadzu 150 RF (Japan) λ excitation – 480 nm, λ emission – 520 nm [10].

The concentration of free cytosolic Ca^{2+} was measured with fluorescent probe Indo-1 (Sigma, USA). Cells (3×107/ml) in buffer A consisting of (mM): KCl – 5, NaCl – 120, $CaCl_2$– 1, glucose – 10, $MgCl_2$ – 1, $NaHCO_3$ – 4, HEPES – 10, pH 7.4 were loaded with Indo-1AM in the presence of 0.05% Pluronic F-127 (Sigma, USA) for 40 min at 25 °C, then washed twice from excessive fluorescent probe by centrifugation (600 g, 10 min) and resuspended in a Ca^{2+}-free buffer A, containing 0.1 mM EGTA. Cells, loaded with Indo-1, were incubated at 37 °C for 1 h with or without fullerene C60 (10^{-5} M). Indo-1 fluorescence in cells was recorded using spectrophotometer (Shimadzu RF-510, Japan), λ excitation – 350 nm, λ emission – 410 and 495 nm. The concentration of free cytosolic Ca^{2+} was calculated as described in [8]. Data processing and plotting were performed by IBM PC using specialized applications Excel 2003 and Origin 7.0.

Results and discussion. The first task was to determine whether the leukemic cells are characterized by remodeling of Ca^{2+}-buffering system and, in particular, reduced basal filling of endoplasmic reticulum Ca^{2+}-stores and decreased SOCE. Ca^{2+}-content of endoplasmic reticulum was measured indirectly using thapsigargin (TG) which inhibits ER Ca^{2+} pump allowing Ca^{2+} to release from the ER pool. Indo-1 loaded cells in Ca^{2+}-free medium were first depleted of ER Ca^{2+} with 1 μM TG. In the absence of Ca ions in the extracellular medium calcium pool of ER cannot be replenished, so the increase of calcium probe fluorescence corresponds to Ca^{2+} released from the endoplasmic reticulum. It was shown that 1 μM TG caused a gradual increase in $[Ca^{2+}]_i$, which was hold on the plateau within 10 min, indicating that calcium pool of ER was depleted (Fig. 1). The value of TG-induced $[Ca^{2+}]_i$ increase above the basal level was considered as the relative value of ER Ca^{2+}-pool. Store-operated Ca^{2+} entry was examined by adding 1 mM $CaCl_2$ to cells, which were treated with TG in Ca^{2+}-free medium. The value of Ca^{2+}- induced $[Ca^{2+}]_i$ increase above the $[Ca^{2+}]_i$ level in TG-treated cells was considered as the relative value of SOCE. We found that dynamics of

TG-induced Ca^{2+}-release from ER was slower and the relative value of SOCE was lower in in L1210 cells than in thymocytes ($\Delta [Ca^{2+}]_i$ = 102±8 and 360±15 respectively (Fig. 1) The obtained data agree with the assumption about genetically determined remodulation of calcium homeostasis: in tumor cells [1, 12].

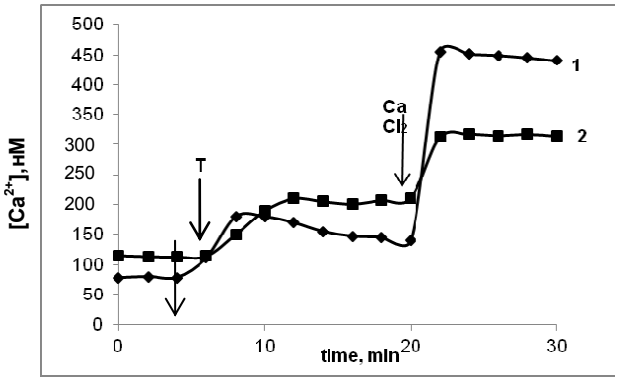

Fig.1. Dynamics of thapsigargin-induced Ca^{2+} release from ER and store-operated Ca^{2+} entry in thymocytes (1) and L1210 cells (2) loaded with Indo-1

The next step of the study was to determine whether photoexcited fullerene C_{60} is able to generate ROS inside L1210 cells and to affect Ca^{2+} homeostasis. A sensitive and specific method for ROS production evaluation in cells is the use of fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). ROS production evaluation is based on the ability of the DCFH-DA to penetrate into the cell, where it is deacyleted to no fluorescent form DCFH by intracellular esterases. Upon iteraction of DCFH with intracellular ROS DCFH is oxidized to DCF, which is characterized by high fluorescence. Therefore, the increase of the fluorescence intensity shows the dynamics of DCF entry into the cells as well as the level of endogenous ROS. Fluorescent probe DCFH-DA is mostly sensitive to relative amount of hydroxyl radical [14], which is increased during the chain of redox reactions initiated by superoxide anion radical.

The production of ROS under the action of photoactivated C_{60} was studied on cells preincubated with 10^5M C_{60} within 1 hour, irradiated in the UV-visible range and incubated within 3 hours. When DCFH-DA was added to untreated cells the increase of fluorescence after 10min incubation was detected corresponding to the endogenous ROS level. (Fig.2). Cells respond to to light irradiation by increase of ROS production, but more strongly pronounced increase of fluorescent signal was detected when the treatment with C_{60} and light irradiation were combined. These data indicate that fullerene C_{60} is able to penetrate into L1210 cells and produce ROS under condition of its photoactivation.

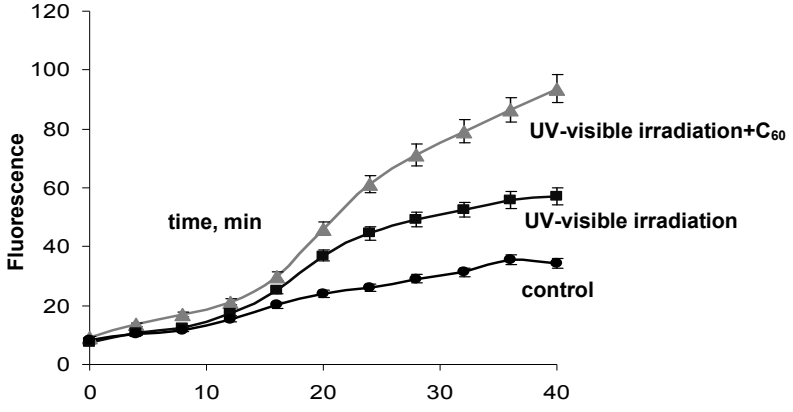

Fig.2. The dynamic of ROS generation in L1210 cells under action of photoexcited fullerene C_{60}

Functionally important targets of ROS In cells are components of calcium homeostasis – Ca^{2+}-ATPases, IP_3 receptors in the ER membrane, mitochondrial voltage-dependent anion channel [4,7]. To elucidate the possible mechanisms of photoexcited fullerene C_{60} influence on the system of Ca^{2+}-signaling,the concentration of free cytosolic Ca^{2+} and ER Ca^{2+}-pool in leukemic cells after fullerene C60 photoexcitation was measured.

The concentration of free cytosolic calcium in L1210 cells was measured with using of fluorescent probe Indo-1., control values were 112±10 nM, which did not change within 1-hour preincubation of cells and after addition of C_{60} in the incubation medium. In L1210 cells treated with photoactivated fullerene C_{60} the value of $[Ca^{2+}]_i$ was increased by 2 and 3,5 times at 1 and 3 hours respectively (Fig. 3 A). Thus, the ability of photoexcited fullerene C_{60} to increase the concentration of free cytosolic calcium in leukemic cells was demonstrated. Long-term increase of $[Ca^{2+}]_i$ is supposed to be one of the inducers of apoptosis [5]. The increase of $[Ca^{2+}]_i$ was observed in the early stages of apoptosis in thymocytes and lymphocytes induced by glucocorticoids, tributultin, antibodies to CD3 as well as in response to the treatment of cells with thapsigargin and calcium ionophore. It was shown that treatment of the cells with ionophore A23187 or ionomicin leaded to apoptosis in thymocytes, mouse S49 lymphoma cells and B-lymphocytes [3].

Increase of free cytosolic Ca^{2+} concentration in leukemic cells under combined action of fullerene C_{60} and irra-

diation could be provoked by two main reasons: 1) uncontrolled cation entry from the extracellular space via the plasma membrane channels 2) the release of Ca^{2+} in the cytosol from intracellular Ca^{2+} stores. Thus, the endoplasmic reticulum Ca^{2+} – content as one of the main indicators of calcium homeostasis and possible initiator of SOCE in cells was evaluated. It was shown that treatment of leukemic cells with photoexcited C_{60} is followed by the increase of the ER Ca^{2+}-pool, which at 3 hour was 1,7 times higher than that in control (Fig. 3 B).

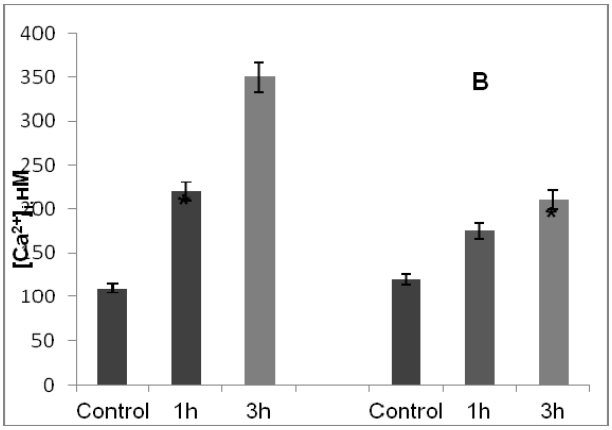

Fig. 3. Quantification of free cytosolic calcium concentration (A) and ER Ca^{2+} pool (B) in L1210 cells

Modulating of Ca^{2+} homeostasis in L1210 cells by photoexcited fullerene can be explained by the membranotropic properties of C_{60} and its ability to produce ROS which effect the activity of targets, containing ROS-sensitive thiol groups- Ca^{2+}-ATPase of ER and IR_3 receptors [4]. It is known that Ca^{2+} release from the ER stores in the areas localized near mitochondria, especially the combination of two factors – increased concentrations of both cation and ROS, could be followed by the fall of mitochondrial potential and release of proapoptotic factors from mitochondria to cytosol [11].

Conclusions. A comparative study of store-operated calcium ions entry showed that the relative value of SOCE in L1210 cells was substantially lower than that in thymocytes, thus confirming the remodulation of Ca^{2+} -signalling in cancer cells. The ability of photoexcited fullerene C_{60} to generate ROS inside the cells and to increase $[Ca^{2+}]_i$ and Ca^{2+}-pool of ER in L1210 cells indicates the possibility of using carbon nanostructure to affect the system of Ca^{2+}-signaling and to initiate events leading to apoptosis of leukemic cells.

References

1. Antunes F., Cadenas E., Brunk U. // Biochem. J. – 2001. – 356. – P. 549-555.

2. Bosi S., Da Ros T., Spalluto G.et al. Fullerene derivatives: an attractive tool for biological applications // Eur. J. Med. Chem. – 2003. – Vol. 38. – P.913–923.

3. Cheng H. Calcium Sparks / H. Cheng, W. J. Lederer // Phys Rev. – 2008. – Vol. 88, № 4. – P. 1491–1545.

4. Csordas G., Hajnoczky G. SR/ER-mitochondrial local communication: Calcium and ROS // J. Physiol. – 2009. – Vol. 529. – P. 11–21.

5. Eisner D.iA., Diaz M. E., Li Y. Stability and instability of regulation of intracellular calcium // Exp. Physiol. – 2004. – Vol. 90, № 1. – P.i3–12.

6. Flouraki M. Insights into Ca^{2+} homeostasis of advanced prostate cancer cells / M. Flouraki, F. Matthieu, P. Natacha // Biochimica et Biophysica Acta. – 2009. – Vol. 17. – P. 1105–1109.

7. Foyer C., Noctor G. Redox homeostasis and antioxidant signaling. A metabolic interface between stress perception and physiological response // Plant Cell. – 2005. – Vol. 17, № 5. – P. 1866–1876.

8. Grynkiewicz G., Poenie M., Tsien R.Y. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties // J. Biol. Chem. – 1985. – Vol. 260. – P. 3440 – 3450.

9. Grynyuk I., Grebinyk S., Prylutska S., Mykhailova A., Franskevich D., Matyshevska O., Schutze C., Ritter U. Photoexcited fullerene C60 disturbs prooxidant-antioxidant balance in leukemic L1210 cells// Mat.-wiss. u.Werkstofftech. – 2013. – 44, No. 2-3. – P. 139-143.

10. LeBel C.P., Ischiropoulos H., Bondy S.C. // Chem. Res. Toxicol. – 1992 – 5. – P. 227–231.

11. Roderick H. L., Cook S. Ca^{2+} signalling checkpoints in cancer: remodelling Ca^{2+} for cancer cell proliferation and survival // Nat Rev Cancer. – 2002. – Vol. 8, № 5. – P. 361–375.

12. Stone J.R. // Arch. Biochem. Biophis. – 2001. – 422. – P. 119-124.

13. Tabata Y., Ikada Y. Biological functions of fullerene // Pure Appl. Chem. – 1999. – Vol.71. – P. 2047-2053.

14. Tarpey M., Wink D., Grisham M. Methods for detection of reactive metabolites of oxygen and nitrogen: in vitro and in vivo considerations // Am. J. Phys. – 2004. – Vol. 286, № 3. – P. 431–444.

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БІОЛОГІЧНІ ЕФЕКТИ КАРБОНОВОЇ НАНОСТРУКТУРИ ФУЛЕРЕНУ *С60* У ЛЕЙКОЗНИХ КЛІТИНАХ

Показана здатність фотозбудженого фулерену C_{60} продукувати активні форми кисню та модулювати Ca^{2+} – гомеостаз у лейкозних клітинах. У лейкозних клітинах (клітини L1210) вхід Ca^{2+} за ємнісним механізмом є нижчим, ніж у нормальних (тимоцити щура). Фотозбуджений фулерен C_{60} призводить до збільшення рівня вільного цитозольного кальцію та кальцієвого пулу ендоплазматичного ретикулуму, що може призвести до кальцій залежного апоптозу.

Ключові слова: фулерен C_{60}, L1210, АФК, цитозольних кальцій, кальцієвий пул.

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БИОЛОГИЧЕСКИЕ ЭФФЕКТЫ КАРБОНОВОЙ НАНОСТРУКТУРЫ ФУЛЛЕРЕНА *С60* В ЛЕЙКОЗНЫХ КЛЕТКА

Показана способность фотовозбужденных фуллеренов C_{60} продуцировать активные формы кислорода и модулировать Ca^{2+} – гомеостаз в лейкозних клетках. В лейкозних клетках (клетках L1210) вход кальция по емкостному механизму- ниже, чем в нормальных (тимоцитах крысы). Фотовозбужденный C_{60} приводить к увеличению уровня цитозольного кальция и кальциевого пула эндоплазматического ретикулума, что может привести к кальций зависимому апоптозу.

Ключевые слова: фуллерен C_{60},L1210, АФК, цитозольный кальций, кальциевый пул.

UDC 611.013 + 575

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DEVELOPMENTAL CAPACITY OF HUMAN EMBRYOS POSSESSING NUMERICAL CHROMOSOMAL ABNORMALITIES

The rate of numerical chromosomal abnormalities in the sample of 720 preimplantation human embryos was estimated and subsequently the developmental patterns of pathologic specimen were studied on cleavage and blastulation stage. Low prognostic value of morphologic criteria usage for noninvasive selection of euploid embryos in vitro was testified.

Key words: preimplantation genetic screening, embryo, morphology.

Introduction. In vitro fertilization (IVF) technique gave us the option of preimplantation human development investigation including different aspects of genetics and embryo development interrelation. During its early growth embryo should pass the number of crucial key points successfully to survive to the stage relevant to implantation [4]. As far as every step of ontogenesis is predetermined by the genetic component, aneuploidy may negatively affect even the earliest stages of embryos' growth being the cause of delayed development, morphologic disturbances and embryo inability to implant [2].

The newly developed preimplantation genetic screening (PGS) enables us to check whether the embryos obtained during the in vitro fertilization treatment carry chromosomal disorders and after that transfer only euploid samples to the patient's uterus. PGS helps to avoid the miscarriage and pregnancy termination induced by fetus aneuploidy therefore and contributes to the highest pregnancy and delivery rates in poor prognosis patients [8]. Inasmuch as PGS is an invasive, time-consuming and expensive procedure [2] the search for other criteria for euploid embryos selection during the embryo culture in vitro is continued. The investigation of developmental characteristics inherent to embryos possessing chromosomal anomalies appears to be the basis of elaboration of morphological criteria to distinguish euploid samples for the transfer without PGS.

Thus, **the aim** of the study was to analyze the morphological characteristics of aneuploid embryos on cleavage and blastulation stage in order to determine their actual developmental capacity.

Materials and methods. Between September 2010–December 2012 in Reproductive Genetics Institute (Kyiv) 925 embryos obtained in 82 infertility treatment cycles were subjected to preimplantation genetic screening for the most frequent numerical chromosomal abnormalities. Poor prognosis patients [6] who entered such treatment cycles didn't possess genetic reproductive risks and contraindications to therapy.

Insemination of the oocytes obtained in the IVF+PGS cycles was performed with intracytoplasmic sperm injection. Oocytes were checked for the presence of two pronuclei and second polar body in 16-18 h after insemination procedure. Overall, during the study 1058 mature oocytes were retrieved (12,86 ± 6,1/treatment cycle) and the rate of fertilization success was 86,28 %. Obtained zygotes were cultured in droplets of commercial media (MediCult, Denmark) for 5 days till the specimen considered to be euploid according to PGS results were transferred to the recipient's uterus. During the cleavage stage the number of blastomeres and the presence of morphological defects were investigated whilst the blastula structure was evaluated according to Gardner's tripartite scoring system [1].

Blastomeres for preimplantation genetic screening were biopsied on the 3rd day of embryo culture as at the cleavage stage embryonic cells maintain totypotency and loss of one blastomere can be compensated by the adjacent blastomeres division [3]. Embryos possessing ≥ 6 blastomeres were used for investigation [5], but slow developing samples (≤ 5 cells at the moment of biopsy) were also examined as a control.

Retrieved cells were hypotonically treated and their nuclei were fixated with the ethanol/acetic acid mixture [7]. A two-step FISH protocol was used to examine the number of chromosomes 13, 16, 18, 21, 22 (PB Multivysion, Vysis, USA), X and Y (CEPX, CEPY, Vysis, USA). The signal analysis was performed with the help of computer program ISIS (MetaSystems,Germany).

Results and discussion. Preimplantation genetic screening was held successfully for 720 embryos which were assigned to one of the categories depending on the diagnostic results [9]:

1. Euploid samples carrying 2 copies of every autosome investigated and the set of sex chromosome specific to males (XY) or females (XX);

2. Embryos with numerical chromosomal abnormalities. These are: а) samples with lack of chromosome (monosomic and nullisomic embryos); b) samples with an additional chromosome (presence of extra chromosome of certain pair); в) samples with complex chromosomal abnormality (disorders involving ≥2 pairs of chromosome simultaneously).

The study revealed that 474 embryos examined (65,83 ± 1,77 %) possessed chromosomal disorders. The major part among the diagnosed embryos (Figure 1) comprised specimen with complex aberrations of chromosomal set (212 cases, 29,44 ± 1,7 % of the group). The quantity of mono- and polysomic embryos was almost equal and constituted 11,25 ± 1,18 % and 11,53 ± 1,19 % of the research group. Moreover, 13,61 ± 1,28 % of examined specimen were considered to be haploid (bearing one copy of each chromosome investigated) or polyploid (with a multiple increase in chromosomal sets). Notably, that only 34,17 ± 1,77 % of studied sample possessed two copies of target chromosomes, that gives us 3,0 ± 1,06 euploid embryos/diagnostic cycle. Established rate of normal embryos is slightly lower than the corresponding results obtained by other authors (44 % according to Munne [7], 39 % in Magli's research [6]) that maybe the consequence of sample formation bias or the natural attribute of our nation [3].

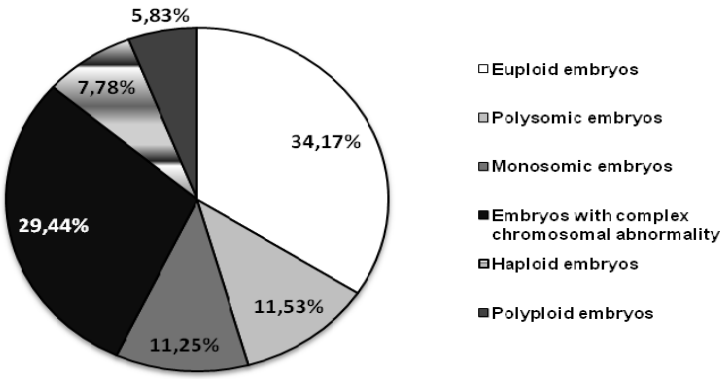

Figure 1. Assignment of embryos examined according to the results obtained during the chromosomal screening

Analysis of pathologic embryos' morphology on the 2nd day of their development revealed that major part of the sample comprised specimen with 4 cells, that constituted 54,33 ± 2,29 % of the group (Figure 2). Furthermore, at rate of 18,6 ± 1,79 and 14,8 ± 1,63 % the embryos consisting of 2 or 3 blastomeres were detected. As a result after 40–42 h of culture the optimal tempo of cleavage [1] was inhered by 87,55 ± 1,52 % of pathologic embryos, while 59 samples on the 2nd day of development had ≥ 6 cells and so transcend the expected speed of division. The average number of blastomeres was equal to 3,66 ± 1,02 while the structural aberrations such as cell fragmentation or uneven blastomeres were encountered at a rate of 6,96 ± 1,17 %.

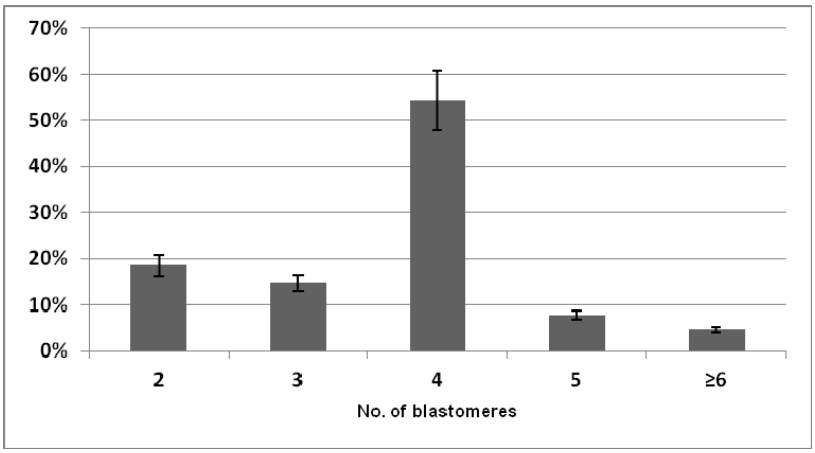

Figure 2. Distribution of aneuploid embryos in relation to the cellular state on the 2nd day of culture

In 64–66 h after fertilization embryos possessing 8 blastomeres prevailed among the pathologic group (35,65 ± 2,2 %). Overall, optimal morphological characteristics [1] were attributable to 68,99 ± 2,12 % of examined embryos (Figure 3), moreover 3,59 ± 0,85 % of sample had more than 10 cells or even formed morula. At the same time, 29 abnormal embryos comprised from ≤ 4blastomeres, while the development of 0,84 ± 0,42 % of specimen was arrested. On the average, there were 7,09 ± 1,66 blastomeres in every embryo on the 3rd day of embryo culture. Noteworthy, structure abnormalities were detected in 53,38 ± 2,29 % of examines specimen. Among them cell size inequality predominated (31,22 ± 2,13 % of group) and the estimated rates of other morphology defects were almost similar (7,17 ± 1,19 for fragmentation and 7,38 ± 1,22 for cytoplasmic bulbs).

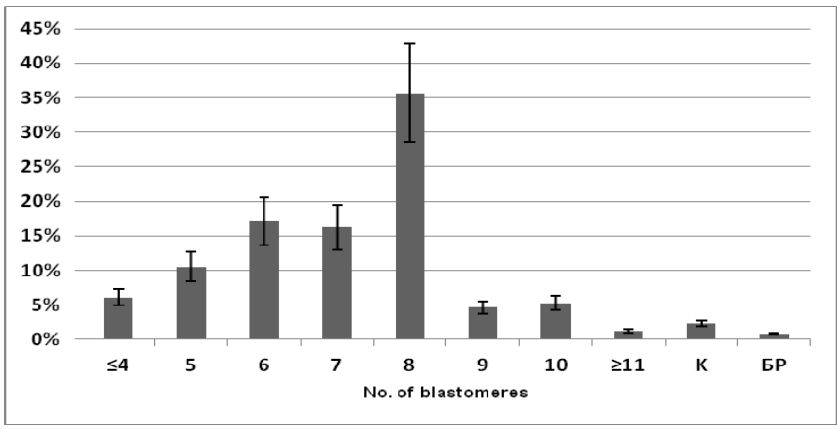

Figure 3. Percentage of aneuploid embryos in relation to the cellular state (64–66 h after fertilization) with M for morula and DA for developmental arrest

On the 4th day of development aneuploid embryos were graded according to their developmental stage (table 1). More specifically, the lowest grade "0" was conferred to embryos that didn't exhibit signs of progressive development during last 24 h of observation including samples with distinct marks of degradation. On the contrary, fully developed blastocyst that successfully completed the hatching process were rated as "9" grade.

***Table* 1. Embryo grading depending on its morphological characteristics in 88-114 h after fertilization**

Grade	Developmental characteristics of embryo
0	No evidence of progressive development for last 24 h of culture
1	Embryo possesses 8-16cells, it's development is impaired, but no signs of degeneration are observed
2	Morula – embryo contains 12-16 tightly packed cells, thus resembling a mulberry
3	Compactisation – the gap junctions are formed, cells are bound tightly, no distinct outlines of particular cells can be seen
4	Cavitation is the initial step of blastocoels formation. The cavity size is less than half of the embryo. Corresponds to grade 1 of Gardner embryo classification [1]
5	Blastocoel enlarges and occupies more than a half of the blastocyst flattening the surrounding cells. Gardner's grade 2
6	Blastocoel is formed completely. It's of round shape. Embryoblast and trophectoderm are clearly seen. The latter is comprised of numerous tightly packed cells. Zona pellucida is stretched. Grade 4 in Gardner embryo classification
7	Expanded blastocyst with very thin zona pellucida. Gardner's grade 4.
8	Hatching blasctocyst. Gardner's grade 5.
9	Embryo managed to leave the zona and is ready for implantation. Corresponds to grade 6 of Gardners embryo classification.

According to proposed arrangement in 88-90 h after insemination average developmental grade of aneuploid embryos was 1,87 ± 1,07 (Figure 4). Among the studied samples 36,21 ± 2,21 % formed morulae, 28,63 ± 2,07 % demonstrated the initial steps of compactisation process whereas 68 specimen failed to develop steadily (14,32 ± 1,61 % of the group). On the 5th day of culture the development of almost 38,48 ± of pathologic embryos was ceased, thus lowering the average developmental grade index to 3,07 ± 2,1 (corresponds to compaction stage). But still 48,99 ± 2,36 % of sample reached the blastocyst stage. Among them 70 embryos proceeded the hatching and 44 were of highest quality according to Gardner's classification. This implies that taking into consideration the morphologic characteristics solely almost 15,6 % of abnormal embryos would be the samples of choice for the transfer in corresponding IVF treatment cycles [4].

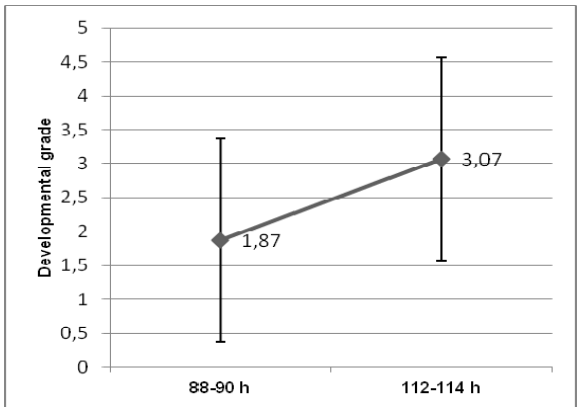

Figure 4. Aneuploid embryos' development on the blastulation stage

Conclusions. The preimplantation genetic screening revealed that 65,83 % of studied human preimplantation embryos possessed numerical chromosomal abnormalities the majority of which constituted combined aneuploidy. The detrimental effect of detected chromosomal abnormalities wasn't visible on the cleavage stage as majority of pathologic samples corresponded to high quality developmental criteria. Till the 5th day of culture 38,5 % of aneuploid embryos degraded but still 15,0 % chromosomally unbalanced sample were able to form morphologically optimal blastocysts. The obtained results prove that morphological evaluation of early embryos is ineffective for euploid embryos' selection, therefore the preimplantation chromosomal investigation is recommended for poor prognosis patients' treatment.

References

1. Bączkowski T., Kurzawa R., Głąbowski W. Methods of embryo scoring in in vitro fertilization // Reprod Biol. – 2004. – Vol. 4, № 1. – P. 5–22.

2.Current value of preimplantation genetic aneuploidy screening in IVF / Donoso P., Staessen C., Fauser B. C. et al. // Hum. Reprod. Update. – 2007. – Vol. 13, № 1 – P. 15–25.

3. Delhanty J. D. A. Preimplantation genetics: an explanation for poor human fertility? / Annals of human genetics. – 2001. – Vol. 65. – P. 331–338.

4. Embryo morphology and development are dependent on the chromosomal complement / M. C. Magli, L. Gianaroli, A. P. Ferraretti et al. // Fertility and Sterility. – 2007. – Vol. 87, No. 3. – P. 534–540.

5. ESHRE PGD Consortium 'Best practice guidelines for clinical preimplantation genetic diagnosis (PGD) and preimplantation genetic screening (PGS)' / A.R. Thornhill, C.E. deDie-Smulders, J.P. Geraedts et al. / Hum. Reprod. – 2005. – Vol. 20, № 1. – P. 35–48.

6. Incidence of chromosomal abnormalities from a morphologically normal cohort of embryos in poor-prognosis patients / M. C. Magli, L. Gianaroli, S. Munné et al. // J. Assist. Reprod. Genet. – 1998. – Vol. 15(5). – P. 297–301.

7. Maternal age, morphology, development and chromosome abnormalities in over 6000 cleavage-stage embryos / S. Munné, S. Chen, P. Colls et al. // Reprod Biomed Online. – 2007. – Vol. 14(5). – P. 628-634.

8. Preimplantation genetic diagnosis reduces pregnancy loss in women 35 and older with a history of recurrent miscarriages / S. Munné, S. Chen, J. Fischer et al. // Fertility and Sterility – 2005. – Vol. 84. – P. 331–335.

9. Scoring criteria for preimplantation genetic diagnosis of numerical abnormalities for chromosomes XY, 13, 16, 18 and 21 / S. Munné, C. Marquez, M. C. Magli et al. // Mol. Hum. Reprod. – 1998. – Vol.4. – P. 863–870.

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ПРЕІМПЛАНТАЦІЙНИЙ РОЗВИТОК ХРОМОСОМНО НЕЗБАЛАНСОВАНИХ ЕМБРІОНІВ ЛЮДИНИ

Встановлено частоту виникнення кількісних хромосомних аномалій різних типів серед преімплантаційних ембріонів людини, вказані особливості розвитку патологічних зразків на стадії дроблення та бластуляції. Доведена низька прогностична цінність дослідження морфологічних характеристик культивованих in vitro зразків для неінвазивної селекції еуплоїдних ембріонів перед ембріотрансфером.

Ключові слова: преімплантаційний генетичний скринінг, ембріон, морфологія.

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ПРЕИМПЛАНТАЦИОННОЕ РАЗВИТИЕ ХРОМОСОМНО НЕСБАЛАНСИРОВАННЫХ ЭМБРИОНОВ ЧЕЛОВЕКА

Установлена частота возникновения количественных хромосомных аномалий разного типа среди преимплантационных эмбрионов человека, проведена оценка особенностей их развития на стадии дробления и бластуляции. Показана низкая прогностическая ценность исследования морфологических характеристик культивируемых in vitro эмбрионов с целью неинвазивной селекции хромосомно сбалансированных образцов перед эмбриотрансфером.

Ключевые слова: преимплантационный генетический скрининг, эмбрион, морфология.

UDC 575

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KARYOTYPE DISORDERS AMONG MEN AND WOMEN DIAGNOSED WITH INFERTILITY

This article shows the frequency of chromosomal abnormalities in women and men with reproductive disorders and infertility.

Key words; karyotype, infertility, cytogenetic studies, translocation, inversion, mosaicism.

Introduction. Infertility problem has become increasingly relevant lately: according to WHO data every fifth couple in the world is infertile. At the same time a lot of breakthroughs referring to infertility correction have been developed recently. For example, intracytoplasmic sperm injection (ICSI) elaboration significantly contributed to infertility treatment practice. Genetic disorders those are responsible for natural conception failure can be inherited by progeny conceived with the help of assisted reproductive technologies (ART). Therefore attention should be paid to the genetic factor of patients undergoing infertility treatment as the central task of ART is healthy child birth [10].

For the selection of appropriate treatment approach complete diagnostic examination is necessary. One of the most important genetic tests is karyotyping that studies the numerical and structural characteristics of chromosomal set. Numerous researches report that the most frequent chromosomal disorders found in the group of infertile patients are: numerical abnormalities of sex chromosomes; 2. balanced reciprocal translocations; 3. Robertsonian translocations; 4. inversions; 5. marker chromosomes; 6. mosaic forms of pathologies mentioned above [5].

Generally the rate of chromosomal abnormalities (CA) in population is low and accounts for 0,5-3,0%, while corresponding CA frequency among infertile patients is about 4,3 – 9,6% though its phenotypical manifestation is rare. [4]. According to some authors [6,2] CA rate among patients undergoing ICSI procedure exceeds13,1%.

Risk of conceiving a child with physical and mental disorders (including severe mental retardation) is significantly higher for carriers of balanced structural aberrations in comparison with population [7]. Recurrent pregnancy loss is also common in patients carrying chromosomal aberrations. Consequently the karyotype exmination is extremely important for counseling of infertile couples or patients with spontaneous abortions. That is why methods of prenatal diagnostics of chromosomal disorders are evolving rapidly nowadays and its practical application in public health service helps to prevent the birth of severely affected children [3].

As chancing the parents' karyotype is impossible the awareness of such problem gives patients the opportunity to do the genetic examination of fetus in time thus revealing and solving the problem after careful consideration. Being well-informed about all the risks considering clinical prognosis couple can plan the pregnancy. Some of them apply for donor programs. In case of IVF treatment the option of preimplantation genetic testing can be the subject for a debate [1].

So the aim of this research is determination of CA rate among female and male patients diagnoswd with infertility and its consequent comparison with regard to patent's sex.

Materials and methods. The primary information search was conducted between 2009–2012 years in the cytogenetic laboratory of Reproductive Genetics Institute clinic headed by Illin I.E., MD. The results of cytogenetic tests of 2495 patients (1135 males and 1360 females) were processed.

Standard procedure of peripheral blood lymphocytes culture and chromosome smears preparation was used. The blood for cytogenetic testing were obtained from cubital vein and added to the culture media. The specimen were incubated at +37°C for 72 hours. For metaphase harvesting colchicine was added into the culture tubes. Than the specimen were centrifuged and treated with hypotonic solution of 0,075M potassium chloride for no longer than 20 min at +37°C. The cells were fixated by means of three replacements of iced to +4°C fixative made ex tempore of ethanol and acetic acid in the ratio of 3:1. After the last centrifugation and supernatant aspiration, cell suspension was dropped on wet cold slides which then were air-dried. The chromosome preparations were GTG-banded [9]. 15-30 metaphase spreads at 550 bands resolution analyzed for every patient [11].

In order to detect differences of CA distribution between males and females the sampling ratios of groups were compared [8].

Results and discussion

The study included the results of karyotyping conducted for 2495 patients (1135 males and и 1360 females).

Among the studied females 208 patients possessed chromosome abnormalities that accounted for 15.3% out of all the specimen studied (table 1).

Table 1. **Quantitative characteristic of karyotype abnormalities in female patients' group**

	2009	2010	2011	2012	Total
Total number of patients undergoing karyotype study	*130*	*209*	*427*	*594*	*1360*
Number of patients with karyotype features	14	22	76	96	208
%	10.8	10.5	17.8	16.2	15.3
Translocations %	4 *3.10*	6 *2.90*	4 0,94	6 *1.01*	20 *1,47*
Inversions %	- *0*	5 *2.3*	10 *2.34*	14 *2,36*	29 *2,13*
Variations of autosome heterochromatic regions %	2 *1.5*	3 *1.4*	30 *7.02*	45 *7.58*	80 *5.88*
Increasing of stalk length and satellite size of acrocentric chromosomes %	5 *3.9*	6 *2.9*	20 *4.68*	21 *3,54*	52 *3.82*
Changes of X chromosome number %	3 *2.3*	1 *0.5*	15 *3.51*	14 *2,36*	33 *2.43*
Unbalanced karyotype: deletions , duplications and markers %	- *0*	- *0*	1 *0.23*	4 *0.67*	5 *0.38*

Cytogenetic studies revealed such patterns of chromosome structure and number variations: translocations (1,47%), inversions (2,13%), polymorphism of autosome heterochromatic regions (5,88%), increasing of stalk length and satellite size of acrocentric chromosomes (3,82%), numerical changes of chromosome X copies (2,43%), unbalanced karyotype involving deletions , duplications and markers (0,38%).

Table 2. **Quantitative characteristic of karyotype abnormalities detected among female patients**

	2009	2010	2011	2012	всего
Infertility	39	68	203	309	619
Missed abortion	55	53	66	112	286
Multiple miscarriages	17	28	44	26	115
Children with multiple congenital abnormalities in anamnesis	12	8	12	3	35
Family planning	7	52	102	144	305
Toltal	*1360*				

Karyotype studies were performed on the basis of such indications: infertility (45,51%), missed abortion (21,03%), multiple miscarriages (8,46%), family planning (22,43%), children born with multiple congenital abnormalities (2,57%). (Table 2). CA were detected in every of the aforementioned groups. The highest rate of karyotype disorders was identified in the sample of female with multiple miscarriages in anamnesis (15.3%), the lowest one – among women whose karyotype was examined because of fetus malformations detected in previous pregnancies (4.3%).

In the group of male patients various features of chromosomal set were detected in 202 specimen constituting almost 17,8% of all the cases studied. (Table 3)

Table 3. **Quantitative characteristic of karyotype abnormalities in male patients' group**

	2009	2010	2011	2012	всего
Total number of patients undergoing karyotype study	146	195	322	472	1135
Number of patients with karyotype features	20	29	64	89	202
%	13.7	14.9	19.9	18.9	17.8
Translocations *%*	3 *2.05*	3 *1.54*	2 *0.62*	5 *1.06*	13 *1.15%*
Klinefelter syndrome *%*	1 *0.68*	3 *1.54*	-	2 *0.42*	6 *0.53%*
Инверсии *%*	4 *2.74*	6 *3.08*	9 *2.79*	14 *2.97*	32 *2.82%*
Variations of autosome heterochromatic regions *%*	2 *1.37*	5 *2.56*	21 *6.52*	29 *6.14*	57 *5.02%*
Increased heterochromatin of Y chromosome *%*	-	3 *1.54*	12 *3..72*	9 *1.91*	24 *2.11%*
Small heterochromatic region in the long arm of Y chromosome *%*	5 *3.42*	6 *3.08*	9 *2.79*	14 *2.97*	34 *2.99%*
Increasing of stalk length and satellite size of acrocentric chromosomes *%*	7 *4.79*	8 *4.11*	13 *4.04*	19 *4.03*	47 *4.14%*

Cytogenetic studied conducted on the peripheral blood of male patients' revealed such patterns of chromosome structure and number changes: translocations (1,15%), Klinefelter syndrome (0,53%), inversions (2,91%), variations of heterochromatic region in the long arm of Y chromosome (5,10%), increasing of stalk length and satellite size of acrocentric chromosomes (4,14%).

Some of the mentioned CA are presented on fig. 1, 2, 3.

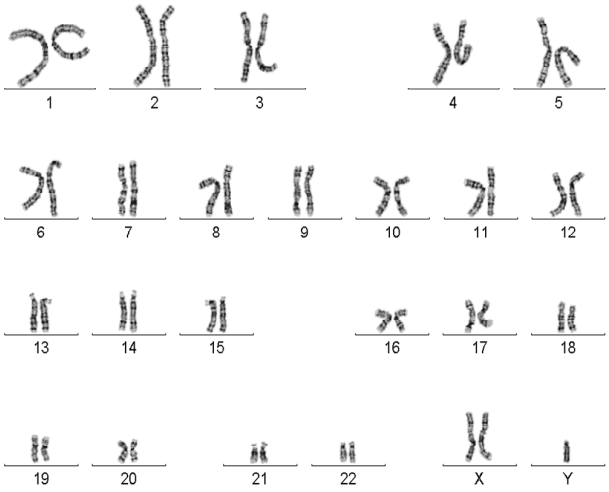

Fig.1. Karyotype with two chromosomes X and one chromosome Y

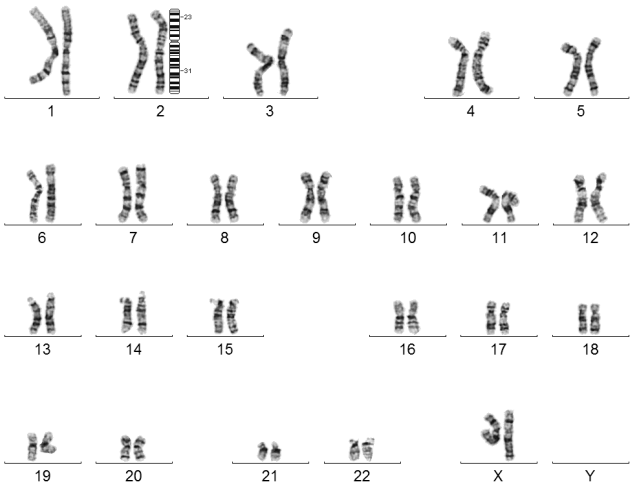

Fig.2. Female karyotype with chromosome 2 pericentric inversion

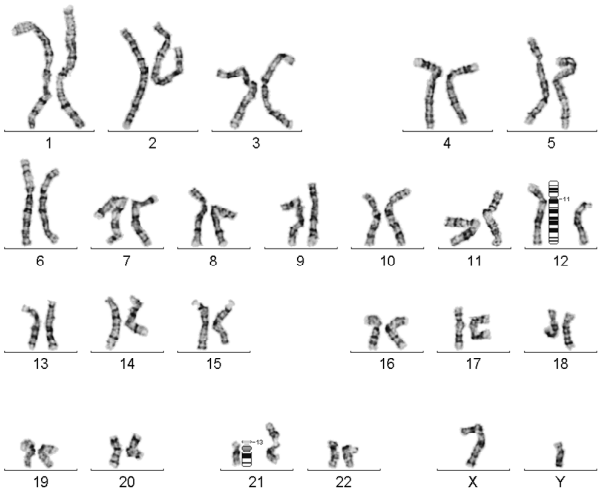

Fig.3. Male karyotype with reciprocal translocation involving chromosome 12 and chromosome 21

Statistical comparison of karyotype features rate between male and female group asserted the absence of statistical difference between values considered (p≥0,001). This observation testifies that CA occurrence is not affected by the sex.

Conclusions. The high frequency of chromosomal abnormalities among patients with fertility requires more attention from doctors of reproductive medicine. Peripheral blood cells' karyotyping appears to be one of the most important steps in couple's examination held before their enterance the in vitro fertilization program. This test is crucial for the calculation of risk of chromosomally abnormal child conceiving and subsequent choice of the most appropriate treatment approach involving additional diagnostic methods, e.g. preimplantation genetic diagnosis. This procedure allows to avoid the embryotransfer of aneuploid or chromosomally unbalanced embryos into the uterus what is extremely helpful in case of one of the parents is a carrier of balanced chromosomal aberrations or other abnormality.

References

1. Chantot-Bastaraud S, at al. Underlying karyotype abnormalities in IVF/ICSI patients // Reprod Biomed Online. – 2008. – Vol.16, № 4: – P.514-22.
2. Düzcan F at al. Cytogenetic studies in patients with reproductive failure// Acta Obstet Gynecol Scand. – 2003 – Vol. 82, № 1. – P.53-6.
3. Gekas J at al. Chromosomal factors of infertility in candidate couples for ICSI: an equal risk of constitutional aberrations in women and men // Hum Reprod. – 2001. – Vol. 16, № 1. – P. 82-90.
4. Gekas J at al. Value of karyotyping women patients of couples referred for sterility// Gynecol Obstet Fertil. – 2003. – Vol. 3, № 1. – P: 66-9.
5. Morel F at al. Chromosomal abnormalities in couples undergoing intracytoplasmic sperm injection. A study of 370 couples and review of the literature// Int J Androl. – 2004. – Vol. 27, № 1. – P. 178-82.
6. Peschka B, at al. Type and frequency of chromosome aberrations in 781 couples undergoing intracytoplasmic sperm injection // Hum Reprod. – 1999. – Vol. 14. № 9. – P. 2257-63.
7. Radojcić Badovinac A at al. Chromosome studies in patients with defective reproductive success// Am J Reprod Immunol. – 2000. – Vol. 44, № 5. – P. 279-83.
8. Атраментова Л.А., Утевская О.М. Статистические методы в биологии: Учебник. – Горловка: ЧП "Видавництво Ліхтар", 2008.
9. Ворсанова С.Г., Юров Ю.Б., Чернышов В.Н. – Хромосомные синдромы и аномалии. // Ростов-на Дону – 1999. – с.155-156.
10. Глинкина Ж.И. Комплексное генетическое обследование мужчин: программа ИКСИ// Гинекология, том08/N, М. – 2006. – с. 28-30.
11. Зерова-Любимова Т.Е., Горовенко Н.Г. Стандарты анализа препаратов хромосом человека (методические рекомендации) // К., 2003. – с.10.

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ПОРУШЕННЯ КАРІОТИПУ СЕРЕД ЧОЛОВІКІВ І ЖІНОК З ДІАГНОЗОМ БЕЗПЛІДДЯ

Дана стаття висвітлює частоту хромосомних аномалій серед жінок і чоловіків з порушенням репродуктивної функції та безпліддям.
Ключові слова: каріотип, безпліддя, цитогенетичні дослідження, транслокації, інверсії, мозаїцизм.

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НАРУШЕНИЕ КАРИОТИПА СРЕДИ МУЖЧИН И ЖЕНЩИН С ДИАГНОЗОМ БЕСПЛОДИЕ

Данная статья освещает частоту хромосомных аномалий у женщин и мужчин с нарушениями репродуктивной функции и бесплодием.
Ключевые слова: кариотип, бесплодие, цитогенетические исследования, транслокации, инверсии, мозаицизм.

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FEATURES CONDITIONS SELECTION AND CULTIVATION MOUSE BONE MARROW ADHEZIVE FRACTION MONONUKLEAR CELLS

Was established that the parameters centrifugation of mouse bone marrow cells in density gradient fikol-verohrafin 1,074 by centrifugal force of 300 g is provide getting an adhesive cells fraction, the most enriched on mesenchymal stem cells. The conditions for the allocation of mouse bone marrow cells, in which a mixture contributes most proliferative activity of mesenchymal stem cells (MSC).

Key words: mesenchimal stem cells, fikol-verohrafin gradient, bone marrow, mouse.

Introduction. For the formation and growth of the colonies of mononuclear bone marrow cells requires some concentration and ratio of substances and factors that stand in the environment in the life of most cells. For a variety of techniques and correlation of these substances varies, which in turn affects the formation and growth of colonies of cells.

It is known that the cells of organism need to exchange information with each other to regulate its development of the tissue, to control growth process and division, to coordinate functions. The structure of the bone marrow are fibroblasts, macrophages, adipocytes, osteoblasts, osteoclasts, endothelial cells, mesenchymal and hematopoietic stem cells and their descendants [3,4].

Vital cells in culture greatly simplified compared to the functioning of the body, and is usually limited to division. Interactions of bone marrow cells supports stay mesenchymal stem cells in the inactive phase of mitosis (G0). The output of this phase of the transition in the mitotic cycle is regulated by changes in intracellular signals [5,1]. Intercellular interaction occurs through hormones, neurotransmitters, and histohormoniv. Obviously, the latter type of intercellular signaling is present between the cells of the bone marrow, and the signals received by the bone marrow cells, and report violations of homeostasis in the body, coming through hormonal and neurotransmitter substances. These signals and give the command to exit stimulates a number of stem cells from dormantnoho state, which then become the path of differentiation and migrate to the area of the pathological process under the action of the same intercellular signals [2,3]. The same signaling molecules can cause target cells unequal response. In some cases this is due to the fact that the signaling molecule binds to various proteins, receptors, in other cases, they bind to the same protein receptors, but activated in different cells different mechanisms of response. Some reaction cells are gradual and increase in direct proportion to the increase ligand. However, there is another group of cells that responds to an increase in the concentration of signaling substances on the principle "all or nothing", in this case, the target cells are not observed any changes until the ligand concentration reaches a threshold level, and the achievement of it – immediately starts maximal response [6].

As already noted, one of the hallmarks of MSCs is their high adhesive ability to culture dishes by culturing. It is

possible, in due time, O.Frydenshteyn separate MSCs from hematopoietic cells. Note that when using this property resultant cell population is heterogeneous and contains other cells that also have adhesive properties and which can, according to some researchers, inhibit proliferative activity receiving MSCs [7, 8].

Materials and methods. The aim of the study was to determine the optimal conditions for isolation and cultivation mouse bone marrow mononuklear cells fraction with high proliferative activity.

We have studied 16 Number of combinations of a cell mononuklernoyi fraction of mouse bone marrow high proliferative activity. It was studied four combinations of method separating suspensions of bone marrow cells in a 4 density gradient fikol-verohrafin (1,074, 1,076, 1,078, 1,080) with the magnitude of the centrifugal force of 300 g and cell culture in vitro on culture medium DMEM, RPMI, 199 with Earle salts, 199 Hepes, with the addition of 20% FBS (fetal calf serum), 10 mkl/sm3 – antibiotic-antifungal.

Cells were seeded in to culture dishes with a density 250 x 10^3 per cm^2. Cultivation of cells was carried out in Petri dishes (d = 60 mm) in the CO_2 incubator with temperature 37 ^oC and CO_2 concentration – 5%. Evaluating the effectiveness of methods for isolation of mononuclear cell fraction of bone marrow was performed based on the formation of colonies and proliferative activity of cells. Evaluation of adhesive properties of cell growth and colony formation, monolayer formation of mononuclear cell fraction of bone marrow was performed every day.

Results and discussion. For various combinations of methods of separating suspensions primary material in a 4 fikol density gradient have unequal number of mononuclear cell fraction of bone marrow. The indicators fikol density gradient and mononuclear cell fraction of bone marrow are directly proportional.

On the 4-5th day of cultivation primary material we have seen the emergence of single cells r sprawled on the bottom of culture dishes and entered the mitotic division (Fig. 1).

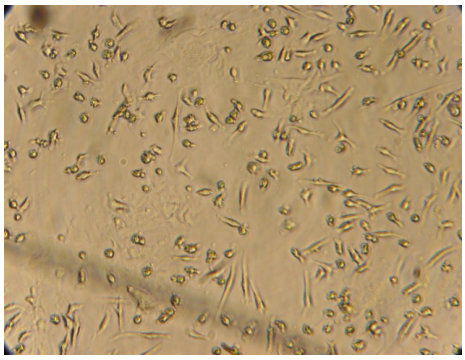

Figure. 1. Mouse MSCs cultured in culture medium DMEM, 4-day cultivation

Violation of genetically rooted combination of bone marrow cells alters cell-cell interactions circuit and causes the change of behavior of cells, which strongly depends on the combination of cells has been flagged for centrifugation in density gradients of different sizes. Over such a mechanism is out of dormantnoho MSC status in primary culture. This may be an explanation of our results with very high deviation between groups of experiments (Table 2).

Revealed that the parameter density gradient centrifugation at 1,074 is most appropriate for a population of cells with the highest proliferative activity, namely the 9 days of culturing the resulting cell population was already formed a continuous monolayer (Fig. 2).

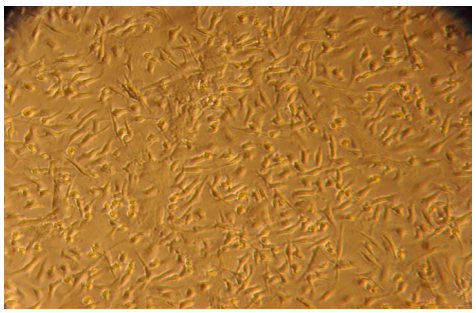

Figure. 2. Mouse MSCs cultured in DMEM culture medium by centrifugation of initial parameter in the fikol density gradient material – 1,074, 9-day cultivation

Cell samples from density gradient 1,076 covered only the surface of the culture dishes by 45 and 60% (Figure 3, Figure 4). After a 9-day cultivation experiments in samples from primary material parameters centrifugation in density gradient fikolu 1,078 and 1,080 received by us forming monolayer cells within 40-50%.

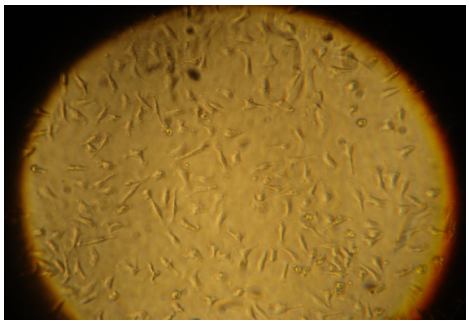

Figure. 3. Mouse MSCs Mouse MSCs cultured in culture medium RPMI, centrifugation parameter for the primary material of fikol gradient density – 1,076, 9-day cultivation

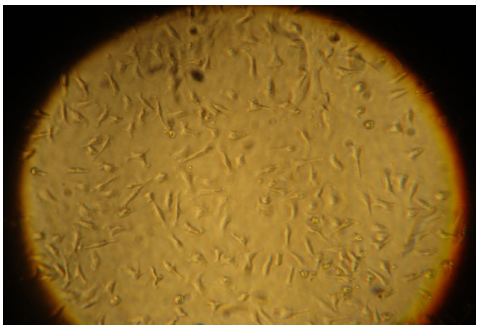

Figure. 4. Mouse MSCs cultured in culture medium DMEM, centrifugation parameter for the primary material of fikol gradient density 1,076, 9-day cultivation

Conclusions. Thus, the parameters centrifugation suspension mouse bone marrow cells in fikol density gradient 1, 074 with centrifugal force of 300 g provide a fraction of cells, the most enriched for MSCs, and a mixture of bone marrow cells obtained by us in these parameters, the most promotes proliferative activity of mouse MSCs. Obviously, under these conditions in the resultant fraction is more MSCs.

References

1. Bianco P. Bone marrow stromal stem cells: nature, biology, and potential applications / P. Bianco, M. Riminucci, S. Gronthos et.al. // Stem cells. – 2001. – № 19. – P.180–192.

2. Кухарчук А.Л. Стволовые клетки: эксперимент, теория, клиника. Эмбриональные, мезенхимальные, нейральные и гемопоетические

стволовые клетки / Кухарчук А.Л., Радченко В.В., Сирман В.М. – Черновцы: Золоті Литоври, 2004. – 505 с.

3. Мушкамбаров Н.Н. Молекулярная биология/ Н.Н. Мушкамбаров, С.Л. Кузнєцов. – М.: Медицинское информационное агенство, 2003. – 535 с.

4. Мушкамбаров Н.Н. Молекулярная биология/ Н.Н. Мушкамбаров, С.Л. Кузнєцов. – М.: Медицинское информационное агенство, 2003. – 535 с.

5. Hart P.H. Mononuclear cell factors, that inhibit fibroblast collagen synthesis. *In vitro* conditions determinity their production and Expression./ P.H. Hart, L.W. Powell, W.G.E. Cooksley [et al.]//Scand. J. Immunol. – 1985. – Vol.18. – P.51–58.

6. Metzyer Z. Suppression of fibroblast proliferation by activated macrophages: Involvment of H_2O_2 and a non-prostaglandin E product of the cyclooxygenase pattway / Z. Metzyer, J.T. Hoffeld, J.J. Oppenheim // Cell. Immmunol. –1986. –Vol. 100. – № 2. – P.501–514.

7. Чупикова Н.И. Получение и биологическая характеристика мультипотентных мезенхимных стромальных клеток костного мозга человека и создание на их основе костных структур: дис. канд. биол. наук : 03.00.23/ Чупикова Наталия Игоревна. – М., 2007. – 133 с.

8. Фриденштейн А.Я. Пролиферативные и дифференцировочные потенции костномозговых колониеобразующих клеток / А.Я. Фриденштейн, Р.К. Чайлахян, Ю.В. Герасимов // Цитология. – 1986. – №28(3). – С.341–349.

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ОПТИМАЛЬНІ УМОВИ ВИДІЛЕННЯ ТА КУЛЬТИВУВАННЯ АДГЕЗИВНОЇ ФРАКЦІЇ МОНОНУКЛЕАРНИХ КЛІТИН КІСТКОВОГО МОЗКУ МИШІ

Встановлено, що параметри центрифугування клітин кісткового мозку миші в градієнті щільності фікол-верографіну 1,074, з відцентровою силою 300 g забезпечують отримання адгезивної фракції клітин, найбільш збагаченої на мезенхімальні стовбурові клітини. Визначено умови виділення клітин кісткового мозку миші, за яких їх суміш найбільш сприяє проліферативній активності мезенхімальних стовбурових клітин.

Ключові слова: мезенхімальні стовбурові клітини, фікол-верографін.

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ОПТИМАЛЬНЫЕ УСЛОВИЯ ВЫДЕЛЕНИЯ И КУЛЬТИВИРОВАНИЯ АДГЕЗИВНОЙ ФРАКЦИИ МОНОНУКЛЕАРНЫХ КЛЕТОК КОСТНОГО МОЗГА МЫШИ

Установлено, что параметры центрифугирования клеток костного мозга мышей в градиенте плотности фикол-верографина 1,074, с отцентровой силой 300 g обеспечивают получение адгезивной фракции клеток, наиболее обогащенной мезенхимальными стволовими клетками. Установлены условия выделения клеток костного мозга мыши, при которых смесь последних наиболее способствует пролиферативной активности мезенхимальных стволовых клеток.

Ключевые слова: мезенхимальные стволовые клетки, фикол-верографин.

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EFFECT OF VEGF ON NO-PRODUCTION BY ENDOTHELIAL CELLS

It was established that VEGF ma anti-VEGF have an opposite effect on endothelium cells. VEGF stimulates production of NO and inhibits apoptosis, and anti-VEGD slightly decrease NO production rate and induce apoptosis.

Key words: endothelium cell., apoptosis., VEGF.

Introduction. One of intracellular mediators of angiogenesis is an reactive nitrogen species in particular NO [1]. It was shown that NO has a direct influence on the proliferation and migration of endothelial cells, protein synthesis and increasing of vascular permeability. These are very important processes for the angiogenesis initiation. Upon the blocking of NO synthesis, the proliferation and differentiation of endothelial cells are inhibited. The differention cease appeares by the violation of precapillary structures formation as well as loosing of the endothelial cells ability to migrate in certain direction. It is able both to increase lipid peroxidation in cell membranes and serum lipoproteins. Their inhibition may cause vasodilation, induce apoptosis and show a protective effect against the introduction of other agents [2]. Recent *in vitro* studies have established that the VEGF (vascular endothelial growth factor) stimulates endothelial cells to produce nitric oxide [3]. Vascular endothelial growth factor (VEGF) is a potent endothelial cell-specific mitogen that promotes angiogenesis, vascular hyperpermeability, and vasodilation by autocrine mechanisms involving NO [5]. NO of both endogenous and exogenous origin inhibits apoptosis in a variety of cells and tissues. NO is a small membrane permeable gas that serves as a mediator of many physiological events. It is produced by the oxidation of L-arginine by a family of isoenzymes-NOS (NO-synthase) [4] Today we can surely claim that one of the factors, which stimulates the increasing of NO content in the culture medium, is a factor of endothelial cells (VEGF). It interacts with eNOS in caveolaes in normal endothelial cells, regulates their activity and thereby promotes the production of NO and prostacyclin, as well as through the activation of cytosolic phospholipase A_2 [6]. There is another possible mechanism of VEGF-dependent activation of NO-production connected with involving of the heat shock protein 90. Activation of NO-synthase and increasing of vascular permeability in endothelial cells are instated by interaction of VEGF with the VEGF2 receptor. The feedback was shown. In a certain concentration range NO causes the synthesis of VEGF by HIF-1 (Hypoxia-inducible factor 1)mediated way [7].

Aim. The aim of this study was the comparing of the NO production level in the culture medium of endothelial cells upon the implementation of VEGF and anti-VEGF.

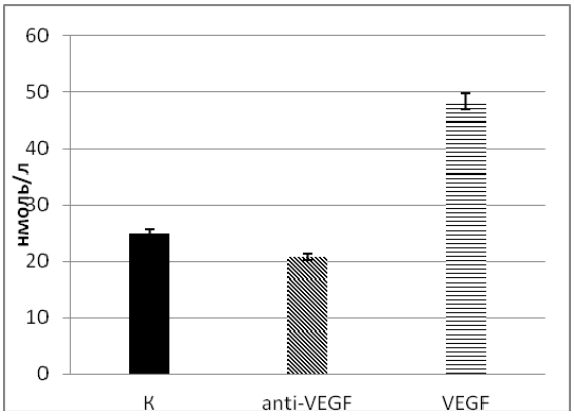
Materials and methods. Mouse endothelial cells (MAEC) [8]., cultivated under standard conditions in DMEM with addition of 10% FBS, were used in this study. In case of long-term cultivation unfed culture model was used, which allows estimating cell's functions under both paracrine and autocrine regulation with intra- and extracellular factors. Effectiveness of angiogenesis was estimated using light microscopy, cytofluorometric analysis and MTT-test. VEGF was used as a specific angiogenesis-inducing factor, while its antagonist – anti-VEGF – as a downregulator.)

The measurement of NO was performed in culture medium using a colorimetric method with Griss reagent (wave length 540 nm) [9] The results of measurements in units of optical absorption were extrapolated to a standard curve. VEGF was obtained from the conditioned medium of tumor cells of Lewis lung carcinoma and purified by the two-stage chromatography, identity was established and proved by the Western blot analysis [10]. Polyclonal anti-VEGF were gotten by 5-fold rabbits immunizing supplemented with Freund's adjuvant. The biological activity of the obtained polypeptide and antibodies was identified in the cross analysis using commercially available analogues.

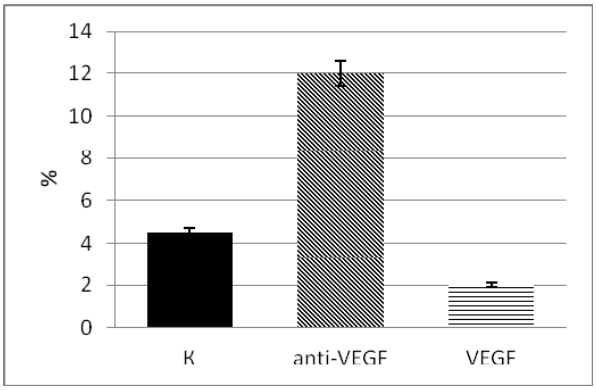
Determination of apoptosis rate in endothelial cells cultured was evaluated with VEGF and anti-VEGF at low concentrations. MAEC cells (5n x 104/ml) were seeded in 60 mm Petri dishes in a volume of 6 ml of complete culture medium and incubated with VEGF and anti-VEGF for 2 day at the standard conditions. Then the cell nuclei were isolated, stained with propidium iodide and DNA content was analyzed on flow cytofluorimeter (Becton Dickinson, USA) equipped with argon laser, at the exitation wave's length of 488 nm. The number of apoptotic cells per 10^4 cells was counted using standard program.

Results. Anti-VEGF inhibited proliferation of endothelial cells *in vitro*. In contrary, adding VEGF resulted in intensification of their proliferative activity. Under conditions of long-termed cultivation, VEGF also induced differentiation of endothelial cells, which resulted in formation of capillary-like structures.Intensity of this process was estimated from the cells' migration area and formation rate of "chainlets". Adding anti-VEGF caused inhibition of endotheliocytes' proliferation and destruction of capillary-like structures, previously formed under the effect of VEGF.

Due to derived results it was shown that VEGF can affect production of NO by endothelial cells. The production of NO increased by 93,2 ± 5,8%($p<0,05$) after endothelial cells being treated with exogenous VEGF in comparison with control. Accordingly, the production of NO by endothelial cells treated with anti-VEGF decreased by 17 ± 3%($p<0,05$).



Also a capability of NO to inhibit apoptosis was shown. The decrement of apoptosis level was observed in presence of VEGF in cultivation medium. The level of apoptosis was 4,5%±1,2%, 2%±0,7% and 12,1%±1,4% in control sample, in presence of VEGF and in presence of anti-VEGF respectively.



It was demonstrated that VEGF stimulates proliferation of endothelial cells, and differenciation if cultivate for a long time.

Conclusion: As result it was ascertained that VEGF increases the rate of NO synthesis by endothelial cells and anti-VEGF has an adverse effect. Also an anti-apoptotic effect of this molecule as demonstrated.

References

1. Folkman J(1971)Tumor angiogenesis: therapeutic implications. N Engl J Med. 1 Nov 18;285(21):1182-6.

2. Southan GJ, Szab C. (February 1996). Selective pharmacological inhibition of distinct nitric oxide synthase isoforms. Biochem. Pharmacol. 51 (4): 383.

3. Papapetropoulos A, Garcia-Cardena G, Madri JA, Sessa WC. Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells. J Clin Invest. 1997; 100: 3131–3139.

4. Tsurumi Y, Murohara T, Krasinski K, Chen D, Witzenbichler B, Kearney M, Couffinhal T, Isner JM. (1997) Reciprocal relation between VEGF and NO in the regulation of endothelial integrity. Nat Med.; 3: 879–86.

5. Matsunaga T, Weihrauch DW, Moniz MC, et al. Angiostatin inhibits coronary angiogenesis during impaired production of nitric oxide. 2002; 105: 2185–2191.2).

6. : Ulibarri JA, Mozdziak PE, Schultz E, Cook C, Best TM. Nitric oxide donors, sodium nitroprusside and S-nitroso-N-acetylpencillamine, stimulate myoblast proliferation in vitro. In Vitro Cell Dev Biol Anim 1999;35:215–8. 3).

7. Tsurumi Y, Murohara T, Krasinski K, Chen D, Witzenbichler B, Kearney M, Couffinhal T, Isner JM. (1997) Reciprocal relation between VEGF and NO in the regulation of endothelial integrity. Nat Med.; 3: 879–86.

8. Bastaki M., Nelli E.E., Dell'Era P. et al. Basic fibroblast growth factor-induced angiogenis phenotyre in mouse endothelium. A study of aortic and microvascular endothelial cell lines. Arterioscler Thromb Vasc Biol. – 1997. – Vol. 17. – P. 454-64.

9. О. М. Перепелиціна, Л. В. Гарманчук, Є. А. Шевченко, М. В. Сидоренко, Л. І. Остапченко Порівняння рівня продукції NO та проліферації клітин раку молочної залози MCF-7 за модифікації умов мікрооточення, Укр. біохім. журн., 2010, т. 82, № 4.

10. Гарманчук Л., Пясковська О., Соляник Г. Вплив проангіогенних цитокінівна проліферативну активність та виживання ендотеліальних клітин. Biopolymers and Cell. – 2010. – Vol. 26. – N 2. – P. 1-6.

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ВПЛИВ *VEGF* НА *NO* ПРОДУКЦІЮ ЕНДОТЕЛІАЛЬНИМИ КЛІТИНАМИ

Було встановлено, що VEGF та анти-VEGF мати протилежний ефект на ендотеліальних клітинах. VEGF стимулює вироблення NO та інгібує апоптоз, а анти-VEGD зменшуэ вироблення NO і індукують апоптоз.

Ключові слова: ендотеліальні клітини, апоптоз, VEGF.

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ВЛИЯНИЕ *VEGF* НА *NO* ПРОДУКЦИЮ ЭНДОТЕЛИАЛЬНЫМИ КЛЕТКАМИ

Было установлено, что VEGF та анти-VEGF иметь противоположный эффект на эндотелиальные клетки. VEGF стимулирует выработку NO и ингибирует апоптоз, а анти-VEGD уменьшает выработку продукцию NO и индуцируют апоптоз

Ключевые слова: эндотелиальные клетки, апоптоз, VEGF.

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CLONING AND MOLECULAR-GENETIC ANALYSIS DESCHAMPSIA ANTARCTICA MITICHONDRIAL SEQUENCES

First mitochondrial DNA was isolated from Deschampsia antarctica.Mitochondrial DNA sequences cloned into the pUC19 vector. Based on this vector a of recombinant clones pDac was created. Nucleotide fragments of the D. antarctica mitochondrial genome were sequenced and analyzed. The presence of mitochondrial DNA genes D. Antarctica: first and fifth NADH dehydrogenase subunits, 26S ribosomal RNA was found. Bioinformatic analysis of mitochondrial DNA sequences of D. Antarctica was made. The high (90%) homology with the mitochondrial genomes of representatives of the family Gramineae (T. aestivum, O. sativa, B. oldhamii etc.) was showed.

Key words: Deschampsia antarctica, mitochondrial genome, Antarctica, sequesce.

Introduction: Subkingdom Embryophyte is represented in Antarctica flora only by two species: *Deschampsia antarctica* (*Poacea*) and *Colobanthus quitensis* (*Caryophyllaceae*). The science interest conditionaled due to their adaptive abilities to survive in the hard climate conditions (low temperatures, high insensitive sunlight during the polar day etc).

Searching thought National Centre of National Center for Biotechnology Information databases showed, that nowadays is known 228 nucleotides sequences (without any mitochondrial DNA), only 86 proteins, 4 references on markers and genome mapping. The biomedical literature database showed only 33 articles about *D. antarctica* and references in 19 books. Therefore, *D. antarctica* isn`t investigated enough.

Studies of physiological and biochemical mechanisms of plants stress adaptation to low temperature has the great theoretical and practical significance. Especially with regard to overcoming low temperature limits for farmed crops. Even a slight growing in frost resistance could cause a significant effect. Thus increasing hardiness of winter wheat by only 2°C could extend its production in the area that is used for spring wheat, which yields 25-40% lower.

It is very difficult to increase the adaptive capacity of agricultural plants using traditional breeding methods. Further enhance frost resistance required purposeful change in the genetic apparatus of plants: expression of certain genes, increasing their copies, including cryoprotective genes into the plant genome etc. This in way is impossible without knowledge of the biochemical mechanisms of plant frost resistance.

The aim of this study was molecular genetic analysis of *Deschampsia antarctica* mitochondrial DNA, search and evaluation of the degree of homology with other members of the family Gramineae, identify possible mechanisms of adaptation to survive in climate of Antarctic region.

Materials and methods: Plants samples were obtained from the 15-th Ukrainian Antarctic expedition to the experimental station "Akademik Vernadsky", located on Cape Galindez Island Marina, 7 km from the western coast of the Antarctic Peninsula.

The plants were put into sterile culture by surface sterilization and cultivated for a long time in vitro [1].

Plant material in the amount of 10 g of fresh leaves of *D. antarctica*, was homogenized in a buffer solution that carried 0.33 M sorbitol, 50 mM Tris, 25 mM EDTA, β-mercaptoethanol 1%. Mitochondrias were isolated in stepwise sucrose density gradient (52% and 30% respectively) by conventional method [2]. Organelles were lysed in a buffer solution that included 50 mM Tris, 25 mM EDTA, β-mercaptoethanol 1%. The isolated DNA was deproteinased. Then it was hydrolyzed by endonuclease HindIII (Thermo Scientific) according to the manufacturer's recommendations. The resulting DNA fragments were cloned into plasmid vector pUC19. After transformation of recombinant plasmids in *Escherichia coli* strain XL-1 Blue, performed the selection of individual colonies and separation of plasmid DNA. These clones are studied through the

analysis of restriction enzymes HindIII, EcoRI, PstI and SalI. Restriction products were separated by electrophoresis in agarose gels. Selected clones were sequenced with primer M13REV. Comparative analysis of fragments already known nucleotide sequences Genetic Bank (GenBank) [3] was carried out using the Basic Local Alignment Search Tool (BLAST) on NCBI.

Results and discussion. As a result of cloning a library of recombinant clones pDac was created (Table 1). Size of cloned sequences ranged from 200 to 4000 bp This occurred the same length fragments, which were separated by mixes of three different restriction endonucleases (Fig. 1). In the first mixture were used enzymes HindIII, EcoRI, PstI, in the second – HindIII, EcoRI, SalI, the third – HindIII, EcoRI, PstI, SalI [4].

On the next step was sequenced the longest fragments of selected clones using primer M13REV. Sequencing was performed at the Institute of Molecular Biology and Genetics, NAS of Ukraine Department biosynthesis of nucleic acids. Six clones have already sequenced. Two of the six sequenced sequences (clones pDac15 and pDac22) gave unsuccessful results in sequencing, which can be explained by the possible contamination of foreign DNA or insufficient cleaning of mixing. Characterization sequenced sequences presented in Table 2.

Assessing the degree of homology was used by resource leveling Blast NCBI. The data is presented in Table 3.

***Tab*. 1 The assignment of the recombinant clones pDac using electrophoresis in agarose gel after processing by restrictions mixes**

Clone pDac*	HindIII	HindIII, EcoRI, PstI	HindIII, EcoRI, SalI	HindIII, EcoRI, PstI, SalI
252.6	4000, 100	4000, 100	4000, 100	4000, 100
270.9	1700	–	–	–
270.10	1500	1500	1100, 400	1100, 400
295.3	1500	1000	1000	1000
293.7	1350	1000, 350	1350	1000, 350
305	1200, 100	–	–	–
252.1	1200	700, 500	700, 500	700, 500
252.7	1200	700, 500	700, 500	700, 500
252.10	1200	700, 500	700, 500	700, 500
283.5	1000	1000	1000	1000
285.7	1000	1000	1000	1000
307	1000	1000	900, 100	900, 100
205.3	800, 700	–	–	–
270.4	800	600, 200	600, 200	600, 200
290.3	300	–	–	–
290.1	200	–	–	–

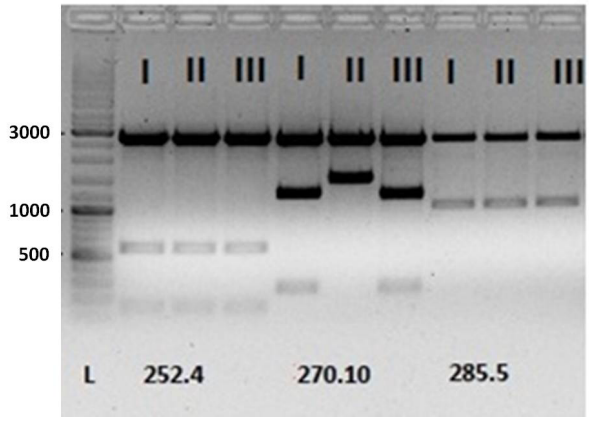

Fig. 1.Restriction analysis of pDac clones L Ladder Mix; arabic numerals show clone number; roman numerals show restriction mix: I HindIII, EcoRI, PstI; II HindIII, EcoRI, SalI; III HindIII, EcoRI, PstI, SalI

During the searching for nucleotide database for clone pDac.270.4 were found 122 adjustment with E-value from 0.00 to 2 × 10-23. To characterize the plan it is important to consider the most similar matches – almost identical sequences. Thus, there are 122 sequence homologs for research in the database with E-number ≤ 10-10. These sequences can be considered close family. Clone pDac.270.10 showed 125 matches of E-number from 0.00 to 4.9. For clone pDac.283.5 were found 125 matches with E-value from 0.00 to 4.9. There are13 sequences with E-value ≤ 10-10. These sequences can be considered as close family. To clone pDac.295.3 found 193 similar sequences in the database. They all belong to the genomic sequences of four plants: bamboo, sweet flag, sorghum and corn.

***Tab*. 2. The results of recombinant clones pDac sequencing**

Clone pDac	Sequence size (nucleotides)	Gene	The highest homology
3	–	–	–
22	–	–	–
270.4	948	1-st та 5-th subunits of the NADH dehydrogenase	*T. aestivum* (98%)
270.10	944	ribosomal RNA 26S and Open Reading Frame	*O. sativa* (91%)
283.5	935	1-st subunit of the NADH dehydrogenase	*B. oldhamii* (98%), *T. aestivum* (97%)
295.3	936	1-st та 5-th subunits of the NADH dehydrogenase	*B. oldhamii* (99%), *T. aestivum* (98%)

Comparative analysis of mithochondrial sequences indicated the presence of the following genes: 26S ribosomal RNA, 1-st, 2-nd and 5-th subunits of NADH dehydrogenase. It was also found a sequence that similar to the open reading frame.

Analyzing the data sequence homology showed close relationships between *D. antarctica* and species from the family Gramineae (Poaceae). The highest homology was observed to areas of mitochondrial genomes of wheat (*T. aestivum*), rice (*O. sativa*), bamboo (*B. oldhamii*). Also observed affinity to the species *Sorghum bicolor, Zea mays* and others.

***Tab*. 3. Results alignment using nucleotide database**

Clone pDac	Plant Specie	Overlap (%)	Number of bases convergence (%)	E-value	Alignment weight
270.10	*Sorghum bicolor*	95	89	0.0	1117
	Zea mais	76	81	0.0	708
	Oryza sativa	77	87	0.0	1845
	Sorghum bicolor	95	89	0.0	1117
283.5	*Bambusa arnhemica*	96	97	0.0	1537
	Ferrocalamus rimosivaginus	96	97	0.0	1531
	(*Sorghum bicolor*	72	94	0.0	2072
	Zea mais	68	98	0.0	1084
295.3	*Bambusa arnhemica*	97	98	0.0	1605
	Oryza sativa	97	98	0.0	3069
	Triticum aestivum	97	98	0.0	1589
	Ferrocalamus rimosivaginus	97	98	0.0	1596

Conclusions: The high degree of similarity of mitochondrial gene sequences between *D. antarctica* and plant crops indicates the close relitionsships of these species. However, the presence of variability in the sequences suggest that these variable regions may be responsible for the unique properties of *D. antarctica*.

Further analysis of mitochondrial DNA *D. Antarctica* will provide more reliable data on the molecular mechanisms of adaptation to cold, high levels of ultraviolet radiation, will shed light on the factors that induce flowering higher plants and vegetation in coastal Antarctica. It also will determine affinity *D. antarctica* from different plant species, especially from *Poaceae* family.

References

1. Матвеева Н.А., Белокурова В.Б., Рудас В.А., Тыщенко О.В., Кучук Н.В. Сохранение и микроразмножение *in vitro* растений Антарктики // Біотехнологія. – 2010. – №3.

2. Albert J. Wilson, Prem S. Chourey A rapid inexpensive method for the isolation of restrictable mitochondrial DNA from various plant sources //Plant Cell Reports December 1984, Volume 3, Issue 6, pp 237-239.

3. Benson D. A., Karsch-Mizrachi I., Clark K., Lipman D. J., Ostell J., Sayers E. W. GenBank // Nucleic Acids Research November 2011 Volume 40, Issue D1 Pp. 48-53.

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КЛОНУВАННЯ І МОЛЕКУЛЯРНО-ГЕНЕТИЧНИЙ АНАЛІЗ ПОСЛІДОВНОСТЕЙ МІТОХОНДРІАЛЬНОЇ ДНК ЩУЧНИКА АНТАРКТИЧНОГО

Вперше було виділено мітохондріальну ДНК щучника антарктичного. Послідовності мітохондріальної ДНК клоновано у вектор pUC19 і на його основі було створено бібліотеку рекомбінантних клонів pDac. Секвеновано і проведено аналіз нуклеотидних фрагментів мітохондріального геному Deschampsia antarctica. Встановлено наявність в мітохондріальній ДНК щучника антарктичного генів 1-ої та 5-ої субодиниць NADH дегідрогенази, 26S рибосомальної РНК. Проведено біоінформатичний аналіз послідовностей мітохондріальної ДНК D. antarctica. Показана висока (понад 90%) гомологія з мітохондріальними геномами представників родини Злакових (T. aestivum, O. sativa, B. oldhamii та ін.)

Ключові слова: Антарктида, Deschampsia antarctica, мітохондріальний геном, секвенуванння.

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КЛОНИРОВАНИЕ И МОЛЕКУЛЯРНО-ГЕНЕТИЧЕСКИЙ АНАЛИЗ ПОСЛЕДОВАТЕЛЬНОСТЕЙ МИТОХОНДРЕАЛЬНОЙ ДНК ЩУЧНИКА АНТАРКТИЧЕСКОГО

Впервые было выделено митохондриальную ДНК щучника антарктического. Последовательности митохондриальной ДНК клонировано в вектор pUC19 и на его основе роздано библиотеку рекомбинантніх клонов pDac. Секвенировано и проведено аналіз нуклеотидных фрагментов митохондриального генома Deschampsia antarctica. Установлено наличие в митохондриальной ДНК щучника антарктического генов 1-ой и 5-ой субъединиц NADH-дегидрогеназы, 26Sрибосомальной РНК. Проведено биоинформатический анализ последовательностей митохондриальной ДНК D. antarctica. Показана высокая гомология с митохондриальными геномами представителей семейства Злаковых (T. aestivum, O. sativa, B. oldhamii та ін.)

Ключевые слова: Антарктида, Deschampsia antarctica, митохондриальный геном,секвенирование.

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EXPRESSION OF HUMAN BETA-DEFENSINS-1-4 MRNA IN NAMALWA AND JURKAT CELLS

The expression profile of human beta-defensins-1-4 in human Burkitt lymphoma Namalwa cell line and Jurkat leukemic T-cell line has been analyzed. It has been shown that in Namalwa and Jurkat cells, hBD-1, hBD-2 and hBD-3 genes are expressed, while hBD-4 expression is absent. Treatment of Namalwa and Jurkat cells with 0.1-1000 nM of recombinant hBD-2 results in significant concentration-dependent suppression of viability of these cell lines.

Keywords: human beta-defensins, expression, lymphoma cell line, leukemic cell line.

Introduction. Human beta-defensins (hBDs), cationic peptide antibiotics and important components of innate immunity system in humans, are mainly expressed in epithelial cells of different origin and provide first-line defense of epithelial surfaces from microbial challenge. The family of hBDs includes more than 30 members, however, up to date, the most studied members of beta-defensin family are hBD-1 and hBD-2, and to the lesser extent – hBD-3 and hBD-4. hBD-1 is supposed to be constitutively expressed in epithelial cells, while three other hBDs – hBD-2,-3, and -4 are peptides with inducible character of expression. All these beta-defensins demonstrate potent antimicrobial activity against a variety of bacterial pathogens, that's why direct microbial killing is supposed to be their main function. However, experimental data suggest that defensins are multifunctional molecules with a wide spectrum of important biological activities and could be involved not only in local immediate antimicrobial response but also in chemotaxis, modulation of inflammatory response, angiogenesis, and wound healing [1, 2]. In a number of studies there have been documented abilities of hBDs to influence many vital cell processes – cell proliferation, viability, differentiation, and apoptosis, and it has been shown that such effects of hBDs are concentration-dependent and could be exerted against many cell types [3-5]. In a similar manner hBDs may affect growth patterns of tumor cells and may play a role in promotion or suppression of human cancer cell growth. According to our data [6], recombinant hBD-2 (rec-hBD-2) causes significant suppression of lung cancer cell growth *in vitro* via cell cycle regulation. It is of interest to analyze whether hBD-2 could exert its growth suppressing effects on growth patterns of human malignant white blood cells. In this work we have analyzed for the first time the expression patterns of hBD-1-4 mRNA in human Burkitt lymphoma Namalwa cell line and Jurkat leukemic T-cell line and influence of rec-hBD-2 on viability of mentioned malignant cells.

Materials and methods. Human Burkitt lymphoma Namalwa cell line and Jurkat leukemic T-cell line were obtained from the Bank of Cell Lines from Human and Animal Tissues, R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, NAS of Ukraine (Kyiv, Ukraine). The cells were cultured *in vitro* in DMEM culture medium supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin G sodium, 100 μg/mL streptomycin sulphate in 5% CO_2 atmosphere at 37^oC.

To study the effect of exogenous defensin on cell growth, we have used the preparation of rec-hBD-2 expressed in bacterial cells as GST-hBD-2 fusion protein and purified by standard two-step procedure as described earlier [7]. Protein concentration was determined by UV absorbance at 280 nm using spectrophotometer Nanodrop-1000 (USA).

To evaluate the effect of rec-hBD-2 on cell viability, MTT-test has been applied [8]. Shortly, Namalwa and Jurkat cells were seeded into 96-well plates and incubated with rec-hBD-2 for 48 h in serum-free medium. Then the cells were treated with MTT (3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide), and colorimetric reaction was evaluated with the use of ELISA reader (Awareness Technology Inc, USA) at the wave lengths λ_{min} =545.

To evaluate the patterns of hBD-1-4 mRNA expression, semiquantative RT-PCR was used. Total RNA was isolated from Namalwa and Jurkat cells by the method of Chomzynski and Sacchi [9]. For detection of hBDs expression, semiquantitative RT-PCR analysis was performed using specific primers (see Table 1).

***Table* 1. Primers for the genes of interest**

Gene	Primers
DEFB1 (hBD-1)	F:-5′-TGTTGCCTGCCAGTCGCCATGAG R: 5′-TCACTTGCAGCACTTGGCCTTCCC
DEFB4 (hBD-2)	F: – 5′-GAAGCTCCCAGCCATCAGCC R: – 5′-GTCGCACGTCTCTGATGAGGGA
DEFB103 (hBD3)	F: CCTGTTTTTGGTGCCTGTTCC R: CTTTCTTCGGCAGCATTTTCG
DEFB104 (hBD-4)	F: 5′-GAAGCTCCCAGCCATCAGCC R: 5′-GTCGCACGTCTCTGATGAGGGA
Beta-actin	F: 5′-CTGGAACGGTGAAGGTGACA R: 5′-AAGGGACTTCTTGTAACAATGCA

The expression level of beta-actin (the housekeeping gene) served as a loading control. The products of RT-PCR were routinely analyzed by electrophoresis in agarose gel.

The data are reported as the mean ± SD. The statistical significance of differences between mean values was assessed by the Student's *t*-test. Values $p < 0.05$ were considered as statistically significant.

Results and discussion. The results of semiquantative RT-PCR analysis have demonstrated that human Burkitt lymphoma Namalwa cells and Jurkat leukemic T-cells express hBD-1, -2 and -3 mRNA, while expression of hBD-4 mRNA in these cells is not detected (Fig. 1).

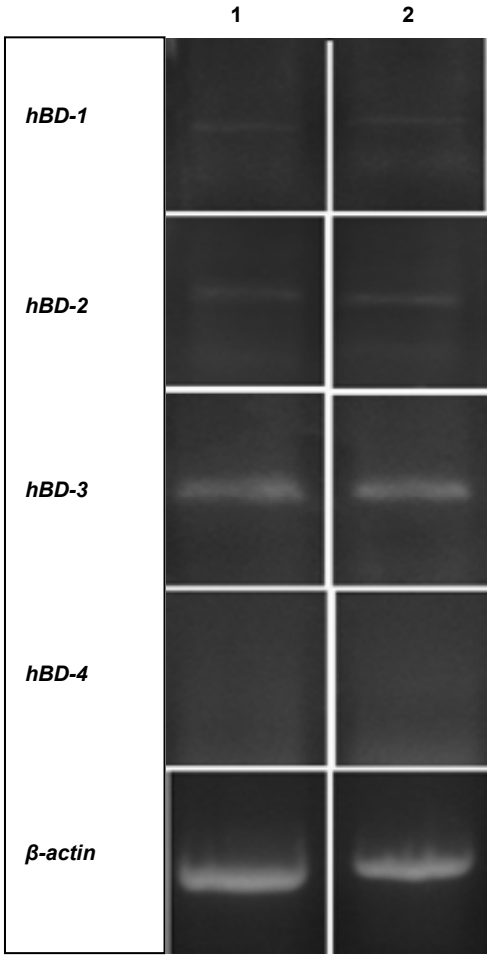

Fig. 1. Semiquantative RT-PCR analysis of hBD-1-4 mRNA expresssion in Jurkat (3, 5, 7, 9) and Namalwa (4, 6, 8, 10) cells. β-actin is used as a house-keeping gene

Up-to-date this is the first observation of beta-defensin mRNA expression in mentioned human lymphoma and leukemic cells, and so far the functional role of beta-defensin expression in malignant white blood cells remains unknown.

Next, we have analyzed an influence of rec-hBD-2 on viability of cultured Jurkat and Namalwa cells and have determined that the defensin causes a concentration-dependent effect on the numbers of viable cells (Fig. 2).

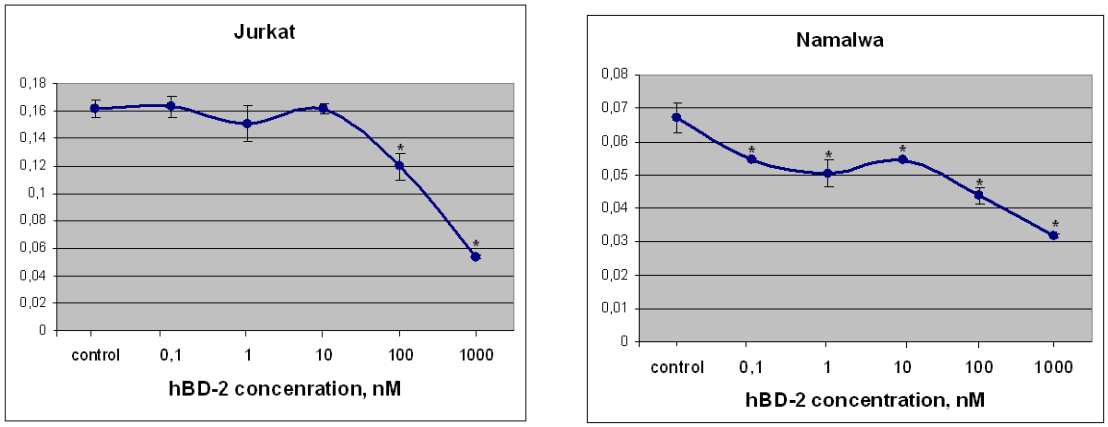

Fig. 2. Effect of exogenous rec-hBD-2 on the viability of cultured Jurkat and Namalwa cells (MTT analysis). The data of three independent experiments are presented as the mean ± SD. *The difference is significant as compared to control ($p < 0.05$)

In concentration range of 0.1-10 nM rec-hBD-2 has no significant effect on viability of Jurkat cells, but its action in higher concentrations (10-1000 nM) results in drastic suppression of Jurkat cell viability. Viabity of Namalwa cells treated with 0.1-1000 nM rec-hBD-2 was significantly lower ($p<0.05$) than that of control cells, so one may conclude that these lymphoma cells are more sensitive to the growth suppressive activity of hBD-2 than leukemic cells.

These data are in agreement with our previously published data on involvement of hBD-2 into the growth regula-

tion of human cancer A431 and A549 cell lines and a concentration-dependent effect of this defensin on proliferation and viability of human carcinoma cells. The most important observation is the fact that beta-defensins 1-3 are expressed by malignant white blood cells and that rec-hBD-2 could suppress Jurkat and Namalwa cell viability in nanomolar concentration range. Such concentrations are close to reported physiologic serum concentrations of hBD-2 registered in healthy individuals and in the cases of some human pathologies (psoriasis, atopic dermatitis, rheumatoid arthritis) [10]. So, it's of interest to further explore *in vivo* patterns of hBD-2 expression in leukemia and lymphoma cells; the knowledge on mechanisms of beta-defensin gene induction in human lymphoma and leukemia cells and biologic effects of human peptide antibiotics toward cancer cells will help to understand the involvement of defensins in tumorigenesis and their potential use in treatment of human pathologies.

Conclusions. Human Namalwa and Jurkat cells express hBD-1, hBD-2 and hBD-3 genes, and respond on growth suppressive activity of rec-hBD-2 in a concentration-dependent fashion.

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References

1. Auvynet C., Rosenstein Y. Multifunctional host defense peptides: antimicrobial peptides, the small yet big players in innate and adaptive immunity // FEBS. J. – 2009. – V. 276. – P. 6497-508.

2. Kohlgraf K.G., Pingel L.C., Dietrich D.E., Brogden K.A. Defensins as anti-inflammatory compounds and mucosal adjuvants// Future Microbiol. – 2010. – V.5. – P. 99-113.

3. Baroni A., Donnarumma G., Paoletti I., et al. Antimicrobial human beta-defensin-2 stimulates migration, proliferation and tube formation of human umbilical vein endothelial cells // Peptides. – 2009. – V.30. – P. 267-72.

4. Winter J., Pantelis A., Reich R., et al. Human beta-defensin-1, -2, and -3 exhibit opposite effects on oral squamous cell carcinoma cell proliferation // Cancer Invest. – 2011. – V.29. – P. 196-201.

5. Bullard R.S., Gibson W., Bose S.K., et al. Functional analysis of the host defense peptide human beta defensin-1: new insight into its potential role in cancer // Mol. Immunol. – 2008. – V. 45. – P. 839-48.

6. Zhuravel E., Shestakova T., Efanova O., et al. Human beta-defensin-2 controls cell cycle in malignant epithelial cells: in vitro study // Exp. Oncol. – 2011. – V. 33. – P. 114-20.

7. Lisovskiy I.L., Markeeva N.V., Shnitsar V.M., et al. Production of recombinant of hBD-2 – human antimicrobial peptide expressed in cervical and vulval cancer // Exp. Oncol. – 2003. – V.25. – P. 36-9.

8. Mosman T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays // Immunol. Methods. – 1983. – V. 65. – P. 55-63.

9. Chomczynski P., Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction // Anal. Biochem. – 1987. – V. 162. – P. 156-9.

10. Jansen P.A., Rodijk-Olthuis D., Hollox E.J., et al. Beta-defensin-2 protein is a serum biomarker for disease activity in psoriasis and reaches biologically relevant concentrations in lesional skin // PLoS One. – 2009. – V.4. – e4725.

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ЕКСПРЕСІЯ МРНК БЕТА-ДЕФЕНСИНІВ ЛЮДИНИ 1-4 В КЛІТИНАХ ЛІНІЙ *NAMALWA* ТА *JURKAT*

Досліджено профіль експресії генів бета-дефенсинів людини 1-4 в клітинах Namalwa (лімфома Беркітта) та Jurkat (Т-клітинна лейкемія). Встановлено, що в клітинах Namalwa та Jurkat експресовано гени hBD-1, hBD-2 та hBD-3, але ген hBD-4 не експресовано. Інкубація клітин Namalwa та Jurkat з рекомбінантним hBD-2 в концентрації 0.1-1000 нМ мала наслідком значуще концентраційно-залежне пригнічення життєздатності вказаних клітин.

Ключові слова: бета-дефенсини людини, експресія, лінія клітин лімфоми, лінія клітин лейкемії.

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ЭКСПРЕССИЯ МРНК БЕТА-ДЕФЕНСИНОВЧЕЛОВЕКА 1-4 В КЛЕТКАХ ЛИНИЙ *NAMALWA* И *JURKAT*

Проанализирован профиль экспрессии генов бета-дефенсинов человека 1-4 в клетках Namalwa (лимфома Беркитта) и Jurkat (Т-клеточная лейкемия). Установлено, что в клетках Namalwa и Jurkat экспрессированы гены hBD-1, hBD-2 и hBD-3, но не экспрессирован ген hBD-4. Инкубация клеток Namalwa и Jurkat с рекомбинантным hBD-2 в концентрации 0.1-1000 нМ приводила к значительному концентрационно-зависимому угнетению жизнеспособности указанных клеточных линий.

Ключевые слова: бета-дефенсины человека, экспрессия, линия клеток лимфомы, линия клеток лейкемии.

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THE INFLUENCE OF MALEIMIDE DERIVATIVE AND 5-FLUORACIL ON MORPHOLOGICAL STATE OF THE SMALL INTESTINE OF RATS WITH CHEMICAL-INDUCED COLON CANCER

The influence of maleimide derivative (1-(4-Cl-benzyl)-3-Cl-4-(CF_3-phenylamino)-1H-pyrrole-2,5-dione, MI-1) and 5-fluorouracil (5FU) for 6 weeks on the small intestine mucosa of rats with 1,2-dimethylhydrazine-induced colon cancer. Found that MI-1 and 5FU reduces the rat small intestine mucosa damage caused by the exposure of DMH. The positive effect of MI-1 is more pronounced than 5FU.

Keywords: maleimide derivative, 5-fluorouracil, 1,2-dimethylhydrazine, colon cancer, small intestine.

Introduction. Despite the significant development of modern science the problem of oncologic diseases remains unresolved for medicine and biology. Researchers at many laboratories work to develop new anticancer medications. The promising substance is maleimide derivative 1-(4-Cl-benzyl)-3-Cl-4-(CF_3-phenylamino)-1H-pyrrole-2,5-dione (MI-1). This substance was created by *insilico* design as an inhibitor of ATP-binding site of proteinkinases and was synthesized in Chemical Faculty of Taras Shevchenko National University of Kyiv [1-3]. Most anticancer drugs except antineoplastic effects have also high cytotoxicity. Therefore it is important to study the influence of potential antitumor substances for the state not only affected organs, but also healthy, especially those through which exogenous compounds come and metabolize [3, 4]. Thus the aim of our research is to study the morphological state of the small intestine of rats with 1,2-dimethylhydrazine (DMH)-induced colon cancer under the influence of a potential

antineoplastic compounds MI-1 compared with existing anticancer drug 5-fluorouracil (5FU).

Materials and methods

All experimental procedures were conducted in the manner prescribed by the approval principles of bioethics, legislation and regulations of the "European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes" (Strasbourg, 1986) and "General ethical principles of animal experiments" approved in First National Congress of Bioethics (Kyiv, 2001). Forty white nonlinear male rats weighing from 170 to 200 g were used. The animals were kept under standard vivarium conditions at natural light. Rats were divided into four groups: I – control, III-V – 20 weeks DMH (20 mg/kg) [5] and the next 6 weeks: III – received nothing, IV – MI-1 in dose 2.7 mg/kg (on average 10^{-4}mol/l in blood) in 0.1 ml of sunflower oil intragastrically daily, V – intraperitoneal 5-FU injections (45 mg/kg).

Animals were anesthetized and decapitated on the 27 week of the experiment. The middle segments of the rat small intestine were taken for histology investigation. The tissue samples were fixed and treated by conventional histological methods for making paraffin section [6]. The sections were stained with hematoxylin and eosin. The morphometric study of the intestinal mucosa was accomplished using a digital system connected to an optic microscope. Morphometrics included the total thickness of intestinal mucosa, villi height, crypt depth, diameter of villi and their stroma, absorption cell height (μm) and cross-sectional area of their nuclei (μm^2), number of goblet cells per villus and their cross-section area (μm^2). Statistical analysis of the experimental results was performed using Microsoft Excel for PC.

Results. The small intestinal mucosa of control rats has a typical structure. The thickness of the mucosa is 643.8 ± 43.8 μm. Villi have the right form. The villi height is 412.5 ± 55.3 μm. Villous stroma has blood and lymph capillaries. Diameter of the villi is 107.6 ± 1.6 μm and its stroma – 37.8 ± 2.4 μm. The largest populations in intestinal epithelial cells is absorptive cells that cover the villi. The absorptive cells height is 31.1 ± 1.9 μm, the cross-sectional area of their nuclei – 27.1 ± 2.0 μm^2. Between absorptive cells are goblet cells that produce mucus, number of this cells per villus is 22.8 ± 1.2 pcs and their cross-section area is 90.5 ± 0.8 μm^2. The intestinal epithelium forms tubular invagination around the ***villi*** – it is intestinal crypts. The crypts depth is 203.6 ± 9.7 μm in control.

There are significant morphological changes in the rats small intestine mucosa after 6 weeks after last DMH injection. Carcinogen causes swelling of the villi, epithelial desquamation, stagnation in the vessels of the villi. ***There is a tendency*** the thickness of the mucosa to increase (700.4 ± 42.4 μm). The villi height nonsignificant increase – 498.0 ± 39.7 μm (fig.1)

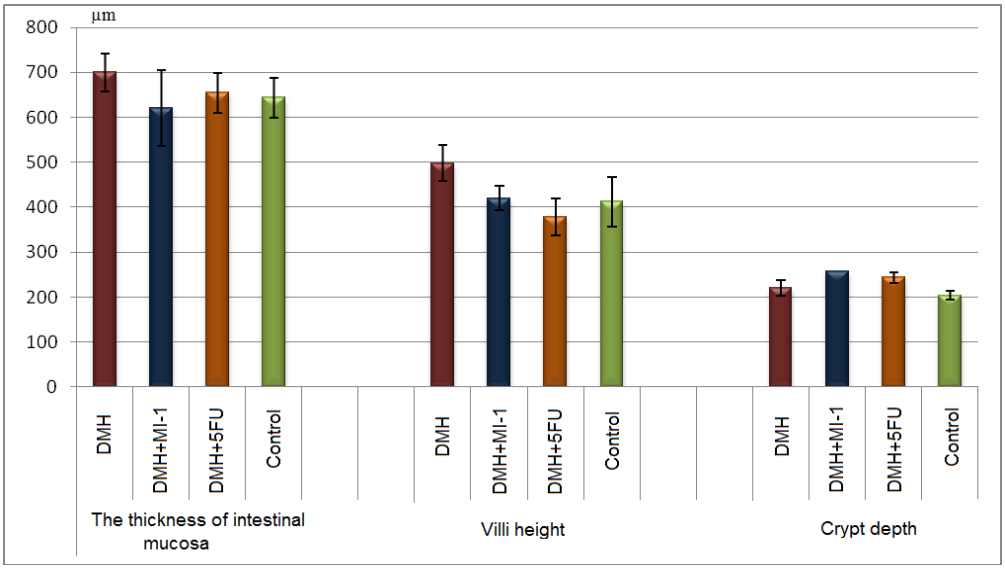

Fig. 1. The thickness of intestinal mucosa, villi height and crypt depth under the influence of the MI-1 and 5FU on the background of DMH -induced colon cancer

The diameter of villi increases by 9% compared with the control (117.5 ± 3.5 μm) (fig.2) . The diameter of villi stroma is 52.5 ± 6.8 μm (fig.2). The absorption cells height is 37.3 ± 1.2 μm which is 20% more than in the control (fig.2). The cross-sectional area of the absorption cells nuclei significantly increases compared with control (27.5%) and amounts to 34.6 ± 2.1 μm^2 (fig.3). Number of goblet cells is 27.3 ± 2.9 pcs on the villus, which is 20% higher than control values. At the same time the cross-sectional area of goblet cells increases – 105.5 ± 11.9 μm^2 (fig.3). The crypts depth is 220.1 ± 17.3 μm (fig.1).

The action of MI-1 for 6 weeks at a dose of 2.7 mg/kg after 20 weeks injection of DMH restores the rat small intestine mucosa. The mucous thickness is 620.4 ± 84.9 μm (fig.1). The form of villi is right. The villi height is 420.3 ± 27.4 μm and has no significantly different with control (fig.1). The villi diameter is also in the range of control values (114,3 ± 9,8 μm). The villi stroma does not change, its diameter is 45.9 ± 4.9 μm (fig.2). The height of the absorption cells is 29.9 ± 2.2 μm, the cross-sectional area of their nuclei is 34.4 ± 1.8 μm^2 (fig. 2,3). Number of goblet cells on the villous is near the control (21.3 ± 1.4 pcs), but the cross section area of the goblet cells significantly increases – 113.1 ± 4.2 μm^2 (25% compared with control) (fig.3). The crypts histoarchitectonics doesn't change, but there is a tendency their depth to increase (by 26% compared to control – 257.3 ± 0.3 μm) (fig.1).

The are some morphological changes in the rat small intestinal mucosa under the 5-fluorouracil action at a dose of 45 mg/kg for 6 weeks after a 20-week injection of DMH. The mucous thickness is in the range of control values – 654.7 ± 44.3 μm (fig.1). The villi height is 379.2 ± 41.1 μm (fig.1). There is a tendency villi diameter (101.8 ± 2.1 μm) to reduce relative to control (fig.2).

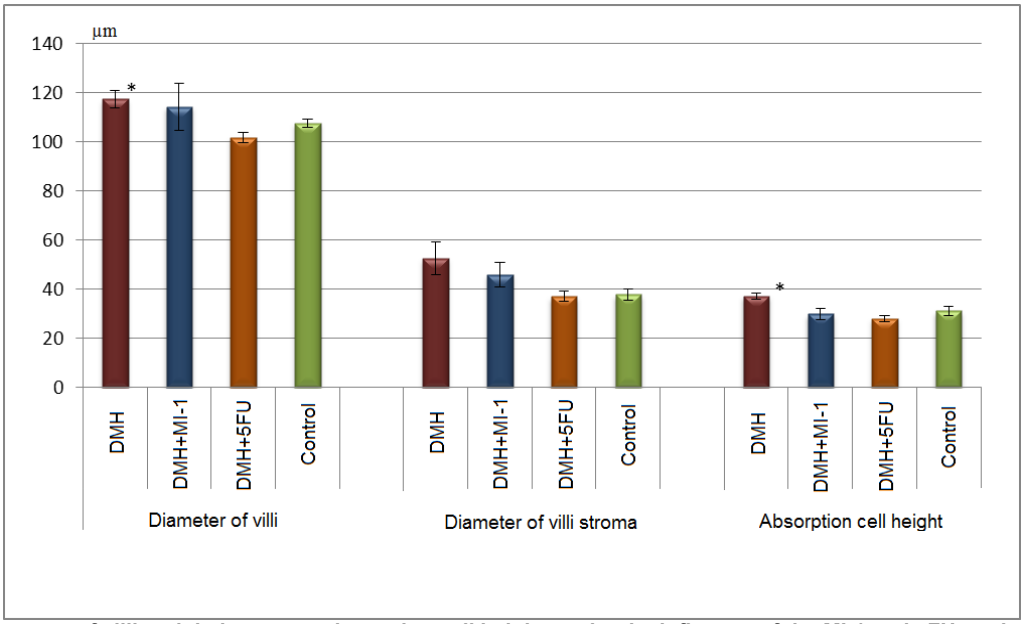

Fig. 2. The diameter of villi and their stroma, absorption cell height under the influence of the MI-1 and 5FU on the background of DMH-induced colon cancer. Significant differences compared to the corresponding control: * – p ≤ 0,05

There is swelling of the villi but its significant reduction compared with DMH. The villous stroma diameter is smaller compared to the DMH and it is equal to 37.0 ± 2.2 µm (fig.2). The height of rat small intestine mucosa absorption cells has no significantly different with control and is 28,1 ± 1,2 µm (fig.2). The cross-sectional area of absorption cells nuclei is 27.7 ± 2.2 µm^2 (fig.3).

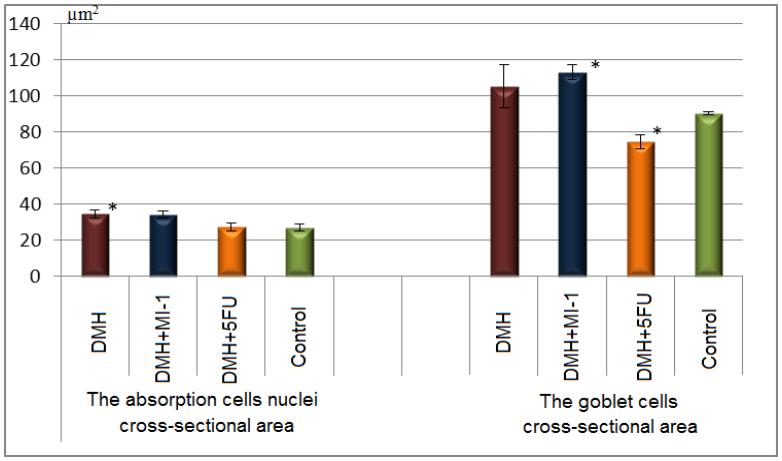

Fig. 3. The cross-sectional area of the absorption cell nuclei and goblet cells under the influence of the MI-1 and 5FU on the background of DMH -induced colon cancer.. Significant differences compared to the corresponding control: * – p ≤ 0,05

However, the number of goblet cells is a significant increased compared with control (19%) and is 27.2 ± 1.0 pcs. At the same time the cross-sectional area of goblet cells is a significant decreased (17%) and equals 77.7 ± 3.9 µm^2 (fig.3). Crypts has normal structure, the depth is not considerably different from control and is 243.9 ± 12.1 µm (fig.1).

Conclusion. Maleimide derivative MI-1 reduces swelling of the villi, epithelial desquamation, stagnation in the vessels of the villi that cause DMH. The changes in the crypts depth and goblet cells area indicating improvement of levels of the epithelium physiological regeneration and activation of adaptive mechanisms under the influence of MI-1. 5-Fluorouracil reduced swelling of the villi and villi diameter compared with the administration of the DMH. The increasing number of goblet cells and reducing their area, indicating the activation of protective processes in rat small intestine mucose under 5FU action. Thus, MI-1 and 5FU reduces the rat small intestine mucosa damage caused by the exposure of DMH. Positive effect of MI-1 is more pronounced than 5FU. Maleimide derivative MI-1 is a promising compound for further research.

References

1. Yablonska S. Antiproliferative properties and low hepatotoxicity of new cytostatic maleimide derivate / S. Yablonska, O. Filinska, G. Ostrovska [et al.] // Biochemistry of cell regulation: 33rd FEBS Congress and 11th IUBMB Conference. – Athens, Greece, 2008. – P. 348.

2. Pat. 22204 (UA). Compoundof 1,4-disubstituted 5-amino-1,2-dihydropyrrole-3-one havinganticanceractivity: G.G. Dubinina, Yu. M.Volovenko – 21.02.2006. Appl. U200601855. 25.04.2007.

3. Rybalchenko V., Kharchuk I., Kyrychenko V., Lynchak O. Effect of the maleimide derivative MI-1, a novel antitumor agent, on the rat myocardium and cardiomyocytes // The Winnipeg Heart International Conference, October 13-16, 2011 // Experimental and Clinical Cardiology. – 2011. – Vol.16, Suppl. A. – P. 25A.

4. Lynchak O. Morphological changes in rat liver of under the influence of maleimide derivative on the background of chemo-induced colon cancer // Studia Biologica. – 2013. – Vol. 7, №1. – P. 41-54.

5. Perse M., Cerar A. The dimethylhydrazine induced colorectal tumours in rat – experimental colorectal carcinogenesis. Radiol. Oncol., 2005; 39 (1): 61-70.

6. Goralskiy L, Khomych V., Kononskyy A. Fundamentals of histological techniques and morphological methods for research in normal and pathological conditions. – Zhitomir: Polissja, 2005. – 288 p.

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ВПЛИВ ПОХІДНОГО МАЛЕІМІДУ І 5-ФТОРУРАЦИЛУ НА МОРФОЛОГІЧНИЙ СТАН ТОНКОЇ КИШКИ ЩУРІВ З ХІМІЧНО-ІНДУКОВАНИМ РАКОМ ТОВСТОЇ КИШКИ

Досліджено профіль експресії генів бета-дефенсинів людини 1-4 в клітинах Namalwa (лімфома Беркітта) та Jurkat (Т-клітинна лейкемія). Встановлено, що в клітинах Namalwa та Jurkat експресовано гени hBD-1, hBD-2 та hBD-3, але ген hBD-4 не експресовано. Інкубація клітин Namalwa та Jurkat з рекомбінантним hBD-2 в концентрації 0.1-1000 нМ мала наслідком значуще концентраційно-залежне пригнічення життєздатності вказаних клітин.

Ключові слова: бета-дефенсини людини, експресія, лінія клітин лімфоми, лінія клітин лейкемії.

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ВЛИЯНИЕ ПРОИЗВОДНОГО МАЛЕИМИДА И 5-ФТОРУРАЦИЛА НА МОРФОЛОГИЧЕСКОЕ СОСТОЯНИЕ ТОНКОЙ КИШКИ КРЫС С ХИМИЧЕСКИ-ИНДУЦИРОВАННЫМ РАКОМ ТОЛСТОЙ КИШКИ

Проанализирован профиль экспрессии генов бета-дефенсинов человека 1-4 в клетках Namalwa (лимфома Беркитта) и Jurkat (Т-клеточная лейкемия). Установлено, что в клетках Namalwa и Jurkat экспрессированы гены hBD-1, hBD-2 и hBD-3, но не экспрессирован ген hBD-4. Инкубация клеток Namalwa и Jurkat с рекомбинантным hBD-2 в концентрации 0.1-1000 нМ приводила к значительному концентрационно-зависимому угнетению жизнеспособности указанных клеточных линий.

Ключевые слова: бета-дефенсины человека, экспрессия, линия клеток лимфомы, линия клеток лейкемии.

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IN VITRO TESTING FOR PREDICTION OF TOXITY AND FOR SCREENING OF POTENTIAL CARCINOGENS AND ANTI–CANCER DRUGS

Cell-based technologies can be used for screening of potential carcinogens and evaluation of stem cell quality for clinical applications. 3D spheroids can serve as intermediate in vitro/ in vivo model for evaluation of anti-carcinogenic effects of therapeutic substances.

Key words: mesenchymal stem cells, MCF7 cell line, cell transformation, cell differentiation, spheroids, transformed foci.

Introduction. Cell-based technologies have broad experimental applications including pre-clinical studies in drug discovery and in regenerative medicine. Multi-potent stem cells have many clinical applications due to their capacity to be expanded *in vitro,* differentiate into several lineages, and participate in tissue regeneration. Clinical application of stem cells requires quality control to avoid potential carcinogenic effects of non-differentiated cells. Cell transformation (foci formation) and cell differentiation assays can provide information about stem cell quality and also can be used to evaluate drug effects.

Standard two-dimensional cell cultures for testing effects of anticancer agents are simple and convenient. However, they have significant limitations in reproducing the complexity and pathophysiology of *in vivo* tumor tissue [31; 33; 34; 37; 38; 39; 46]. Three-dimensional culture systems are of increasing interest in cancer research since tissue architecture and the extracellular matrix (ECM) significantly influence tumor cell responses to micro–environmental signals [43].

Vinci et al. [45] discovered differential sensitivity to targeted agents between two-dimensional and three-dimensional cultures, and also demonstrated enhanced potency of some agents against cell migration and invasion compared to cell proliferation. 2D and 3D *in vitro* functional assays can enhance the biological relevance of early preclinical cancer studies. These assays will increase the translational predictive value of *in vitro* drug evaluation studies and reduce the need for *in vivo* studies by more effective triaging of compounds.

Methods. Cells: Mouse mesenchymal stem cell line was obtained from the Tulane University Center for Gene Therapy under Material Transfer Agreement. These cells were originally derived from femurs and tibiae of C57BL mice. Cells were grown in IMDM (Invitrogen/GIBCO, CA) that was supplemented with 10% Premium Select FBS (Atlanta Biologicals, GA), 10% HS (Hyclone, UT), 100 U/mL penicillin, 100 µg/mL streptomycin (Invitrogen, CA). Colony formation and differentiation assays were performed periodically with iron oxide loaded and unloaded cells according to the instructions provided by the cell supplier. Briefly, cells were grown to 70-90% confluency in 6-well tissue culture plates, then incubated for 3 weeks in osteogenic or adipogenic differentiation medium, with medium change twice a week, and then stained for 10 min with 2 ml of 40 mM Alizarin red (pH 4.1; Sigma) for mineral deposits [36] or Oil Red-O (Sigma) for fat globules [18, 32], respectively. 3D transformed foci were comprised of cells that lost contact inhibition. We determined transformation frequency as ratio of foci number and the number of plated cells.

For tumor spheroid assay, we used MCF7 breast cancer cell line, kindly provided by Dr. I.Gut. We used modified standard method for multi-cellular spheroid generation [11]. Confluent cells were trypsinized, and single-cell suspension was seeded on low-adhesive substrate with density 1.0x105 cells/ml in growth medium with 0.24% carboxymethyl-cellulose (CMC). We generated spheroids in 6-wells test-plates (Nunclon, Belgium) and Petry dishes. Dishes with cells were placed on shaker with low rotation (150 rpm) for one hour. The number of live cells was determined in wells using MTT-colorymetric test. Cell count was performed after 48 hours incubation using a Trypan Blue dye [25]. Distribution of cells between different phases of the cell cycle and apoptotic index were assessed by flow cytometry [26]. Glucose absorption and LDG-

activity were tested by typical methods. Pro- and anti-carcinogenic effects evaluation was performed using cell proliferative index, spheroid size, and cell distribution between spheroid and adhesive fractions. Two types of culture media were used: complete (with 10 % FBS), and incomplete (without serum). As pro-carcinogenic factor, we used mitogen EGF. Herceptin (monoclonal antibody to HER2/neu receptor) was used as an anti-carcinogen.

Transfection:

MSC were transfected by electroporation with a recombinant plasmid pEF1□-GFP-IRES-CD4. To construct plasmid, the EGFP coding sequence was cloned into the pEF-1□ Myc/His vector along with an internal ribosome entry site (IRES) sequence coupled to the coding sequence of a truncated human CD4 (□CD4) receptor giving: pEF-1□-GFP-IRES-□CD4. Transfected cells were labeled with superparamagnetic MACS CD4-MicroBeads and isolated on MACSelect magnetic column. Stable pEF-1□-GFP-IRES-□CD4 expressing MSC clones were prepared in selection media with G418.□□CD4 receptor expression was evaluated immunocytochemically and by FACS using □CD4-PE antibodies [29].

Results and Discussion. In this study, we used: a) foci formation assay to compare frequency of transformation in control (non-transfected or transfected with buffer without DNA) and transfected mesenchymal stem cells and b) three-dimensional tumor spheroid assay for dynamic quantitative evaluation of pro- and anti-carcinogenic drug effects.

We evaluated frequency of transformation in culture of bone marrow derived murine mesenchymal stem cell line C57BL. Plasmid transfection resulted in stable and efficient GFP fluorescence; however, expression levels of CD4 were neither efficient nor stable possibly due to limited functionality of the IRES construct [29]. We also discovered that transfected MSC in long-term culture spontaneously formed foci of transformation with higher frequency than control cells (Figure 1C). Figure 1D,E demonstrates origination of transformed focus from GFP-expressing transfected cells. Subpopulations of genetically modified stem cells (transfected with the reporter gene) also changed their differentiation ability (Figure 2).

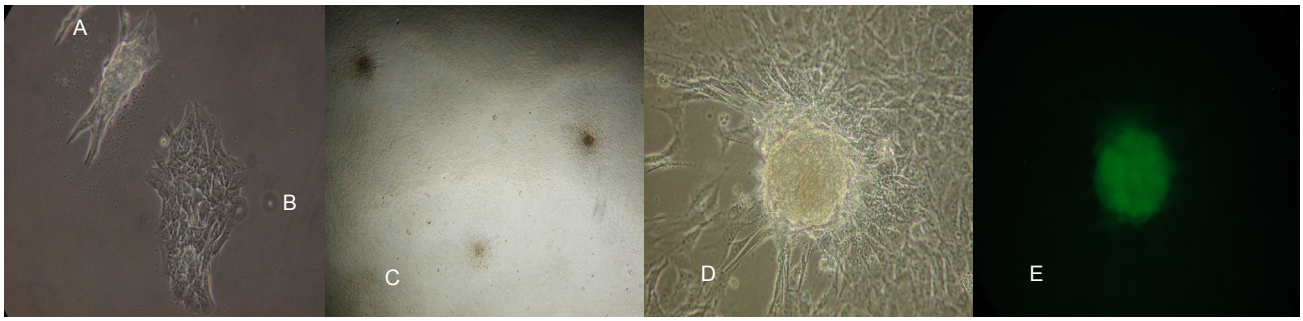
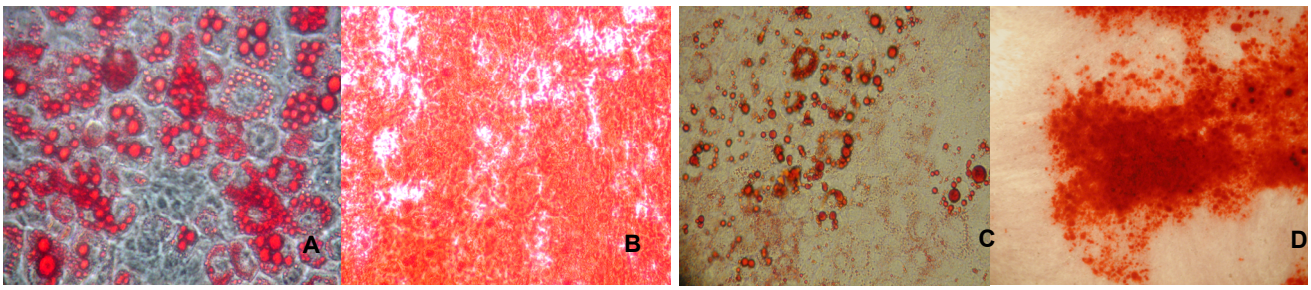


Figure 1. Spontaneous transformation in transfected murine MSC: A – transformed focus and B-normal growth; C – cell monolayer with three transformed foci; D, E – transformed focus on monolayer of MSC transfected with reporter gene: D – white light; E – green fluorescence of GFP



Passage 5; control **Passage 14; transfected cells**

Figure 2. Differentiation assay of mouse MSC in adipogenic (A,C) or osteogenic (B,D) medium: A, C – Oil Red-O staining for fat globules; B, D – Alizarin Red staining for mineral deposits

Thus, we demonstrated that genetically engineered stem cells in long-term culture are unstable: they may change their behavior and transform at higher frequency. Our findings indicate importance of bio-safety studies for long-term cell imaging and for efficient stem cell clinical applications [27; 28]

Foci formation assay is also applicable to drug screening for evaluation of drug carcinogenic potential and to discover drugs that prevent initiation of transformation. By identifying the potential for *in vivo* toxicity early, this assay helps to minimize the likelihood of late stage drug failure and allows drug development resources to be allocated toward advancing those candidates with the best chances for success.

To study pro- and anti-carcinogenic effects, we used 3D MCF7 cell culture. Many studies have highlighted significant differences between two-dimensional and three-dimensional cultures [9, 48]. 3D spheroids better reflect the *in vivo* tumor microenvironment in terms of cellular heterogeneity, nutrient and oxygen gradients, cell- cell interactions, matrix deposition and gene expression profiles [4; 7; 8; 9; 12; 48].

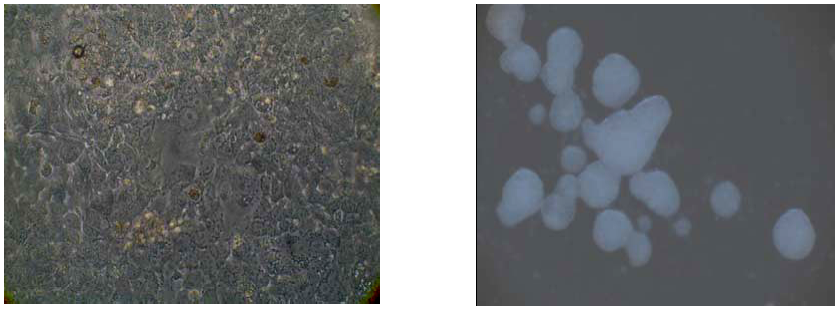
To model solid tumors more effectively, several three-dimensional (3D) culture systems have been established: whole perfused organs, tissue explants,

scaffold/microcarrier-based cultures, hollow-fiber bioreactors, organotypic cultures (multicellular spheroids and cellular multilayers) and gel/matrix-based cultures [2; 14; 19; 24; 30]. Of these, the multicellular tumor spheroid model is the best characterized and most widely used.

Tumor spheroids are heterogeneous cellular aggregates that, when greater than 500 μm diameter, are frequently characterized by hypoxic regions and necrotic centers [15; 41]. Three-dimensional spheroids are therefore considered valid models to recapitulate features of tumor microregions, neovascular domains or micrometastases [9; 20].

Sutherland et al. first applied this technique in cancer research [41]. Since then, several methods have been used to generate tumor spheroids: spontaneous aggregation [5], spinner flasks [47], rotary cell culture systems [24; 44], poly-2-hydroxyethyl methacrylate (poly-Hema)-coated plates [17; 51], hanging drops [6], liquid overlay on agar [10; 22; 49], low binding plates [42; 50], gel/matrix-based culture [21], polymeric scaffolds [8], or micropatterned plates [13; 23]. Each method has advantages and limitations [19; 24], and simple, standardized and rapid protocols appropriate for routine preclinical drug development studies within academic or pharmaceutical labs are lacking. Vinci et al. screened and classified a diverse collection of human tumor cell lines for their ability to form spheroids [45]. Spheroid formation depends on the cell type, cell density at seeding, rotation speed, type of culture medium, FTS concentration, and incubation time.

In this study, we used MCF7 cell line representing highly malignant human breast tumors and characterized by an optimal three-dimensional structure (Figure 3).



A **B**

Figure 3. 2D (A) and 3D (B) growth of MCF7 cells *in vitro*

Breast cancer is very invasive and highly metastatic tumor. The breast carcinomas development involves a set of complex phenotypic alterations in breast epithelial cells and in surrounding tissues [3]. MCF7 is a well known and widely used breast cancer cell line, which can be propagated as monolayer and as spheroid system. Architecture of three-dimensionally propagated MCF-7 cells is very similar to avascular tumor areas. The gradient of diffusion in cell aggregates leads to reduced proliferation rates and increased drug resistance.

Gene expression profiles of cells in spheroids are more similar to natural tumors, than profiles of the same cells in monolayer culture. Spheroid morphology is often resembles real tumor morphology.

We used 3D model of tumor growth to study pro- and anti- carcinogenic effects of serum deprivation. Figure 4 illustrates those effects. We evaluated spheroids' number, size, and shape, ratio of adhesive and suspension fractions, proliferation rate, number of apoptotic and necrotic cells, and cell cycle progression. Some of those parameters are summarized in Table 1. This system also allows for evaluation of drug effects on biochemical parameters reporting metabolic status of cells during spheroid growth under different conditions. Comparison of those parameters in 2D (monolayer) and 3D (spheroids) culture of MCF7 cells under serum starvation conditions demonstrates differential reactivity of those systems. 2D and 3D MCF7 cell cultures reacted differently to serum deprivation (Table 1).

***Table* 1. Serum starvation effects on cells in spheroid (3D) and monolayer (2D) culture.**

Apoptotic cells %		Necrotic cells %		Cells in G_0/G_1 (%)		LDG-activity		Glucose absorption	
2D	3D	2D	3D	2D	3D	2D	3D	2D	3D
Complete culture media (with 10 % FBS)									
19.0±1.3	14.2±1.7	11.4±1.7	17.0±2.5	56.3±1.5	41.0±1.0	0.18±0.01	0.12±0.01	0.19±0.01	0.07±0.01
Serum free medium (without FBS)									
39.2±7.3	22.4±3.9	33.5±2.8	20.4±2.2	54.1±1.4	67.5±1.7	0.27±0.02	0.32±0.03	0.29±0.01	0.23±0.01

LDG-activity showed in μKat/1000 cells; glucose absorption in μM/1000 cells.

Cells in 2D system are more sensitive to serum starvation than in 3D cultures. Cell viability is significantly higher in 3D system. Levels of necrotic and apoptotic cells in 2D culture are twice higher in comparison to 3D culture. Serum-free medium has no detectible effects on morphology of 3D cultured cells. The LDG-activity is increased in both culture systems under serum-free conditions. In serum-free medium, Glucose absorption level increases more significantly in 3D cultures. All parameters demonstrate increased cell survivability in spheroid system.

EGF has anti-apoptotic effect on cells in both systems in contrary to Herceptin that has pro-apoptotic effect (Figure 4). EGF increases proliferative pool in MCF7 cells. Herceptin has the opposite effect on MCF7 cell proliferation. Spheroid number and size in standard field of view was also different in the medium containing both agents (EGF and Herceptin) in comparison to those parameters in control (Table 2).

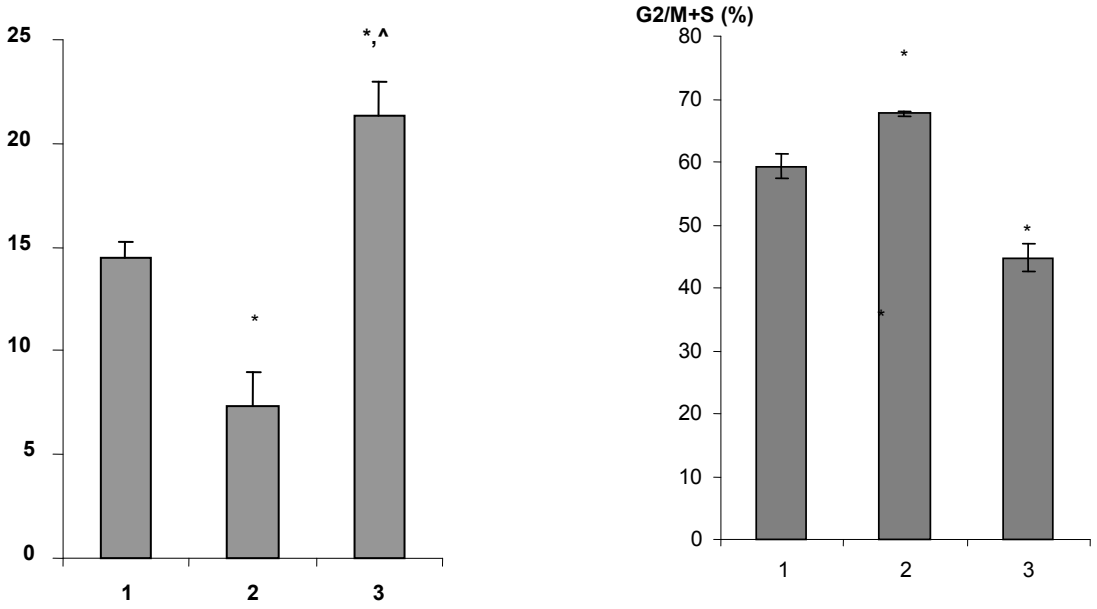
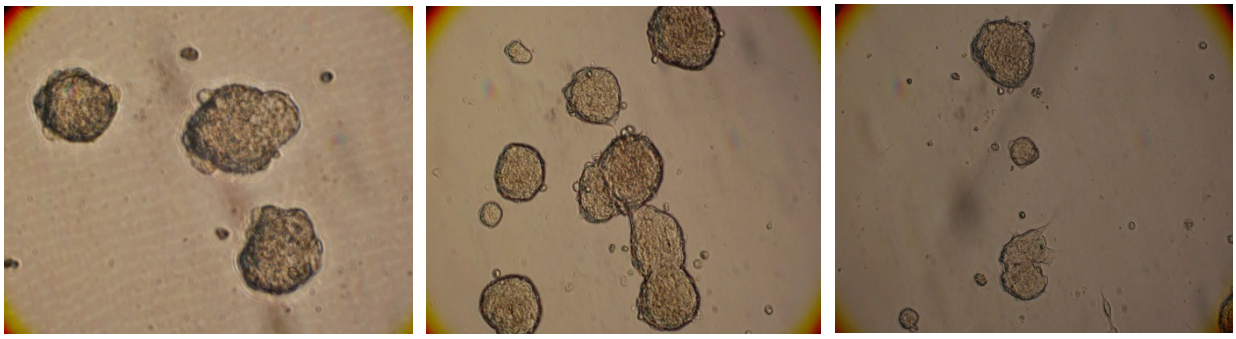


Figure 4. Effect of EGF and Herceptin on cell cycle and apoptotic index: 1 – control; 2 – EGF; 3 – Herceptin

***Table* 2. Effgect of EGF and Herceptin on the spheroid parameters**

MCF7 3D growth	Area of spheroids (мкм^{2}x10^3)	Spheroid number in the standard field of view	Cell distribution between adhesive and spheroid fraction (%)
control	3,3±1,7	5,1±1,3	15:85
EGF	5,7±0,8*	9,9±2,9*	24:76
Herceptin	2,7±1,4	4,1±1,1	33:67



Control **Pro-carcinogenic effect (EGF)** **Anti-carcinogenic effect (Herceptin)**

Figure 5. Pro- and anti-carcinogenic effects of EGF and Herceptin

Conclusions. *In vitro* testing on primary cells and cell lines can allow investigators to preview *in vivo* responses, thus facilitating design of better dosing strategies and optimization of animal and Phase I clinical studies. Cell transformation assay and spheroid model provide useful tools for evaluation of potential carcinogenic risk and early toxicity and for screening of anti-carcinogenic drugs. Expansion of preclinical testing to incorporate assays that better predict desired effects or potential toxicities earlier in the drug development process has obvious advantages for the selection of successful lead candidates.

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References

1. Abbott A. Cell culture: biology's new dimension. Nature 2003, 424:870-872.

2. Agudelo-Garcia PA, De Jesus JK, Williams SP,et al. Glioblastoma cell migration on three-dimensional nanofiber scaffolds is regulated by substrate topography and abolished by inhibition of STAT3 signaling. Neoplasia 2011, 13:831-840.

3. De Jong JS, van Diest PJ, van der Valk P, et al. 1998, Expression of growth factors, growth-inhibiting factors, and their receptors in invasive breast cancer. II: Correlations with proliferation and angiogenesis. .J Pathol. 1998 Jan;184(1):53-7.

4. De Witt Hamer PC, Van Tilborg AA, Eijk PP, et al.: The genomic profile of human malignant glioblastoma is altered early in primary cell culture and preserved in spheroids. Oncogene 2008, 27:2091-2096.

5. Deisboeck TS, Berens ME, Kansal AR, et al.: Pattern of self-organization in tumour systems: complex growth dynamics in a novel brain tumour spheroid model. Cell Prolif., 2001, 34:115-134.

6. Del Duca D, Werbowetski T, Del Maestro RF: Spheroid preparation from hanging drops: characterization of a model of brain tumor invasion. J Neurooncol 2004, 67:295-303.

7. Ernst A, Hofmann S, Ahmadi R, et al. Genomic and expression profiling of glioblastoma stem cell-like spheroid cultures identifies novel tumor-relevant genes associated with survival. Clin Cancer Res 2009, 15:6541-6550.

8. Fischbach C, Chen R, Matsumoto T, et al.: Engineering tumors with 3D scaffolds. Nat Methods 2007, 4:855-860.

9. Friedrich J, Ebner R, Kunz-Schughart LA: Experimental anti-tumor therapy in 3-D: spheroids–old hat or new challenge? Int J Radiat Biol 2007, 83:849-871.

10. Friedrich J, Seidel C, Ebner R,et al.: Spheroid-based drug screen: considerations and practical approach. Nat Protoc 2009, 4:309-324.

11. Garmanchouk LV, Perepelytsina OM, Sidorenko MV, et al. Growth Kinetics of 2- and 3-D Cell Models as Influenced by the Microenviroment// Cytology and genetics, 2009, Vol.43, No.5, P 305-309.
12. Ghosh S, Spagnoli GC, Martin I, et al.: Three-dimensional culture of melanoma cells profoundly affects gene expression profile: a high density oligonucleotide array study. J Cell Physiol 2005, 204:522-531.
13. Hardelauf H, Frimat JP, Stewart JD, et al.: Microarrays for the scalable production of metabolically relevant tumour spheroids: a tool for modulating chemosensitivity traits. Lab Chip 2011, 11:419-428.
14. Haycock JW: 3D cell culture: a review of current approaches and techniques. Methods Mol Biol 2011, 695:1-15.
15. Hirschhaeuser F, Menne H, Dittfeld C, et al.: Multicellular tumor spheroids: an underestimated tool is catching up again. J Biotechnol 2010, 148:3-15.
16. Hutchinson L, Kirk R: High drug attrition rates–where are we going wrong? Nat Rev Clin Oncol 2011, 8:189-190.
17. Ivascu A, Kubbies M: Diversity of cell-mediated adhesions in breast cancer spheroids. Int J Oncol 2007, 31:1403-1413.
18. Kelly KA and Gimble JM, (1998). 1,25-dihydroxy vitamin D3 inhibits adipocyte differentiation and gene expression in murine bone marrow stromal cell clones and primary cultures. Endocrinology. 139, 2622-8.
19. Kim JB, Stein R, O'Hare MJ: Three-dimensional in vitro tissue culture models of breast cancer-a review. Breast Cancer Res Treat 2004, 85:281-291.
20. Kunz-Schughart LA, Freyer JP, Hofstaedter F, et al.: The use of 3-D cultures for high-throughput screening: the multicellular spheroid model. J Biomol Screen 2004, 9:273-285.
21. Lee GY, Kenny PA, Lee EH,et al.: Three-dimensional culture models of normal and malignant breast epithelial cells. Nat Methods 2007, 4:359-365.
22. Li Q, Chen C, Kapadia A, et al.: 3D models of epithelial-mesenchymal transition in breast cancer metastasis: high-throughput screening assay development, validation, and pilot screen. J Biomol Screen 2011,16:141-154.
23. Matsuda Y, Ishiwata T, Kawamoto Y, et al.:Morphological and cytoskeletal changes of pancreatic cancer cells in three-dimensional spheroidal culture. Med Mol Morphol. 2010,43:211-217.
24. Mazzoleni G, Di Lorenzo D, Steimberg N: Modelling tissues in 3D: the next future of pharmaco-toxicology and food research? Genes Nutr 2009, 4:13-22.
25. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxic assayas // J Immunol Methods – 1983. – 65, N 1-2. – P. 55-63.
26. Nicoletti I. Migliorati G., Pagliacci M.C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry // J. Immunol. Methods. -1991. -139, N 2. -P. 271-80.
27. Ostrovska L. Strategies for non-invasive monitoring of mesenchymal stem cells. Proceedings, The 5th Annual Maryland Stem Cell Research Symposium, Annapolis, MD, October, 2012.
28. Ostrovska L. Two approaches to stem cell Magnetic Resonance Imaging: advantages and limitations. Proceedings ESMRMB Congress, Lisbon, PT, p. 126 , October 4-6, 2012.
29. Ostrovskaya, L., Winnard Jr, P.T., Botlagunta,B.,et al. Challenges with Mesenchymal Stem Cells Imaging in Tumor Vasculature. Joint Molecular Imaging Conference, Providence, RI, September 8-11, 2007.
30. Pampaloni F, Reynaud EG, Stelzer EH: The third dimension bridges the gap between cell culture and live tissue. Nat Rev Mol Cell Biol 2007, 8: 839-845.
31. Parent-Massin D, Hymery N, Sibiril Y. Stem cells in myelotoxicity. Toxicology 267: 112-117, 2012.
32. Peister A, Mellad JA, Larson BL, et al. (2004). Adult stem cells from bone marrow (MSCs) isolated from different strains of inbred mice vary in surface epitopes, rates of proliferation, and differentiation potential. Blood. 2004 103, 1662-8.
33. Pessina A, Albella B, Bayo M, et al. Application of the CFU-GM Assay to Predict Acute Drug-Induced Neutropenia: An International Blind Trial to Validate a Prediction Model for the Maximum Tolerated Dose (MTD) of Myelosuppressive Xenobiotics. Toxicology Sciences 75: 355367, 2003.
34. Pessina A, Parent-Massin S, Albella B, et al. Application of human CFU-Mk assay to predict potential thrombocytotoxicity of drugs. Toxicology in Vitro, 2008. Doi:10.1016/j.tiv.2008.11.006.
35. Sivaraman A, Leach JK, Townsend S, et al.: A microscale in vitro physiological model of the liver: predictive screens for drug metabolism and enzyme induction. Curr Drug Metab 2005, 6:569-591.
36. Stanford CM, Jacobson PA, Eanes ED,et al.: Rapidly forming apatitic mineral in an osteoblastic cell line (UMR 106-01 BSP). J Biol Chem.1995, 270, 9420-8.
37. Stem Cells in Predictive Toxicology. California Institute for Regenerative Medicine (CIRM) Workshop Report: July 2008.
38. Stevens JL, Baker TK. The future of drug safety testing: expanding the view and narrowing the focus. Drug Discovery Today 14:0162-167, 2009.
39. Stevens JL. Future of Toxicology – Mechanisms of Toxicity and Drug Safety: Where Do We Go from Here? Chemical Research in Toxicology 19: 1393-1401, 2006.
40. Sutherland RM, McCredie JA, Inch WR: Growth of multicell spheroids in tissue culture as a model of nodular carcinomas. J Natl Cancer Inst 1971, 46:113-120.
41. Sutherland RM, Sordat B, Bamat J,et al.: Oxygenation and differentiation in multicellular spheroids of human colon carcinoma. Cancer Res 1986, 46:5320-5329.
42. Takaishi S, Okumura T, Tu S, et al.: Identification of gastric cancer stem cells using the cell surface marker CD44. Stem Cells 2009, 27:1006-1020.
43. Timmins NE, Nielsen LK. Generation of multicellular tumor spheroids by the hanging-drop method.// Methods Mol. Med. 2007;140:141-51.
44. Unsworth BR, Lelkes PI: Growing tissues in microgravity. Nat Med 1998, 4:901-907.
45. Vinci M, Gowan S, Boxall F, et al.: Advances in establishment and analysis of three-dimensional tumor spheroid-based functional assays for target validation and drug evaluation, BMC Biology 2012, 10:29.
46. Volpe DA, Warren MK. Myeloid clonogenic assays for comparison of the in vitro toxicity of alkylating agents. Toxicology in Vitro 17: 271-277, 2003.
47. Wartenberg M, Donmez F, Ling FC, et al.: Tumor induced angiogenesis studied in confrontation cultures of multicellular tumor spheroids and embryoid bodies grown from pluripotent embryonic stem cells. FASEB J 2001, 15:995-1005.
48. Weaver VM, Petersen OW, Wang F,et al.: Reversion of the malignant phenotype of human breast cells in three-dimensional culture and in vivo by integrin blocking antibodies. J Cell Biol 1997, 137:231-245.
49. Weiswald LB, Guinebretiere JM, Richon S, et al: In situ protein expression in tumour spheres: development of an immunostaining protocol for confocal microscopy. BMC Cancer 2010, 10:106.
50. Wright MH, Calcagno AM, Salcido CD,et al.: Brca1 breast tumors contain distinct CD44+/CD24- and CD133+ cells with cancer stem cell characteristics. Breast Cancer Res 2008, 10:R10.
51. Xiang X, Phung Y, Feng M,et al.: The development and characterization of a human mesothelioma in vitro 3D model to investigate immunotoxin therapy. PLoS One 2011, 6:e14640.

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ПРОГНОСТИЧНЕ ТЕСТУВАННЯ ТОКСИКОЛОГІЧНОГО, ПРО- ТА АНТИКАНЦЕРОГЕННОГО ВПЛИВУ В СИСТЕМІ *IN VITRO*

Досліджено вплив похідного малеіміду (1-(4-Cl-бензил)-3-Cl-4-(CF_3-феніламіно)-1H-пірол-2,5-діону; MI-1) і 5-фторурацилу (5FU) протягом 6 тижнів на слизову оболонку тонкої кишки щурів на тлі 1,2-диметилгідразин-індукованого раку товстої кишки. Встановлено, що MI-1 і 5FU зменшують пошкодження слизової оболонки тонкої кишки, викликаних впливом канцерогену. Позитивний ефект MI-1 є більш вираженим, ніж 5FU.

Ключові слова: похідне малеіміду, 5-фторурацил, 1,2-диметилгідразин, рак товстої кишки, тонка кишка.

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ПРОГНОСТИЧЕСКОЕ ТЕСТИРОВАНИЕ ТОКСИЧЕСКОГО ПРО-И АНТИКАНЦЕРОГЕННОГО ВЛИЯНИЯ В СИСТЕМЕ *IN VITRO*

Исследовано влияние производного малеимида (1-(4-Cl-бензил)-3-Cl-4-(CF_3-фениламино)-1H-пиррол-2,5-дион, MI-1) и 5-фторурацила (5FU) в течении 6 недель на слизистую оболочку тонкой кишки крыс с 1,2-диметилгидразин-индуцированным раком толстой кишки. Установлено, что MI-1 и 5-фторурацил уменьшают повреждения слизистой оболочки тонкой кишки, вызванные воздействием канцерогена. Положительный эффект от MI-1 является более выраженным, чем 5FU.

Ключевые слова: производное малеимида, 5-фторурацил, 1,2-диметилгидразин, рак толстой кишки, тонкая кишка.

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MOLECULAR CLONING OF HUMAN BRAIN-TYPE CREATINE KINASE GENE INTO BACTERIA EXPRESSION VECTORS PET-17B, PET-14B AND FLAG TAGGED MAMMALIAN EXPRESSION VECTOR PCMV

Due to a vast distribution of creatine kinase in the human tissue this enzyme plays a paramount role in the synthesis of high energy source – ATP (adenosine triphosphate) molecule. Creatine kinase enzyme has three major forms, CKMM – muscle-type creatine kinase; CKBB – brain-type creatine kinase; CKMB – mitochondrial-type creatine kinase. It is shown, that particularly brain isoform of the enzyme is involved in the development of neurodegenerative dieses. We have cloned the gene of CKBB using bacteria and mammalian expression vectors. These constructs should be suitable for protein expression of the protein.

Key word: brain-type creatine kinase, cloning, sequencing, neurodegenerativedieses.

Introduction. Human brain creatine kinase (CKB; monomer, CKBB; dimer) is a cytosolic homodimer protein with molecular weight of the subunit of about 42 kDa. The enzyme is mainly expressed in brain tissue, where it catalyzes the reversible transfer of the γ-phosphoryl group of ATP molecule to creatine, resulting in the formation of phosphocreatine and ADP, which plays a role of a 'temporal energy buffer' according to [1, 2]. The overall structure of CKBB was suggested to consist of an N-terminal helical domain (residue 1-100) and a C-terminal α/β domain (residue 125-381) between which located a long linker region (101-124) [3]. Chemical modification studies have shown that muscle creatine kinase (CKM), which shares ~90% of amino acid sequence identity with CKBB, possess one reactive Cys282 per monomer. Therefore, was concluded that modification of the Cys282 would led to complete abolition of CK activity. Additionally, were provided clear evidence that the pK_a of 5.4 observed for wild-type CK belongs to Cys282 and, for the optimal binding of creatine, the cysteine should be in the form of thiolate anion [4]. By employed computational methodology of Naor and Jensen [5] was predicted that the hydrogen bond between Cys282 and Ser285 also contributed to the lowered pK_a value of CK.

The folding process of creatine kinase involves several important intermediates: a partially active dimeric, an inactive dimeric, a compact monomeric and a partially-folded monomeric intermediate, among which the inactive dimeric intermediates are prone to aggregate [6, 7,8]. The stability of CK has been investigated with various stresses, such as urea [9], guanidine hydrochloride [10], and temperature [11]. The study [12, 13] indicated that the molecular chaperone GroEL was able to bind with dimeric state of urea inactivated CK intermediate, which resembles the molten globule state and aggregation, was efficiently suppressed by the action of GroEL chaperone. The thermal denaturation of CK was shown to satisfy the validity criteria for the two-state irreversible model proposed by Kurganov et al [14]. Although, was additionally suggested [15] that the CKBB undergoes sequential structural changes when subjected to thermal stress. A stable monomeric CKBB could be identified during CKBB thermal inactivation, and the reversibility at temperatures below 55 ºC was found to correlate closely to the reassociation of the monomers into native dimeric enzyme [16].

It has been reported that CKBB is a major target of oxidative stress and the activity level of enzyme are gradually reduced in brain affected by the neurodegenerative diseases [17, 18, 19]. Although, by the same authors, was suggested that a posttranslational modification may cause a decrease of CKBB activity in Alzheimer's disease. Therefore, impaired CKBB activity levels may greatly facilitate as an accurate biomarker to improve early detection of neurodegenerative diseases [20]. Based on these facts of the undeniable value of brain-type creatine kinase, we have aimed to clone the gene of CKBB into bacterial and mammalian plasmids to further allow molecular and protein manipulations with this enzyme.

Material and methods. The gene of human brain creatine kinase was amplified according to the sequence (GenBank accession number CR542268.1). The primerset was CKBB-F, 5'-CATATGCCCTTCTCCAACAG-3' (underline indicates the cleavage site for *Nde* I); CKBB-R, 5'-GGATCCTCATTTCTGGGCAG-3' (underline indicates the cleavage site for *Bam* HI). The resulting PCR products were cloned into pET17b, and pET14b vectors for the expression of non- or His_6-tagged CKBB proteins. For the expression of FLAG-tagged protein pFLAG-CMV vector was used with the following primers set: CKBB-F, 5'-AAGCTTATGCCCTTCTCCAAC-3' (underline indicates the cleavage site for *Hind* III); CKBB -R, 5'-GGATCCTCATTTCTGGGCAG-3' (underline indicates the cleavage site for *Bam* HI). PCR conditions: 30-35 cycles were applied with a melting temperature of 94°C for 30 seconds, an annealing temperature of 55°C for 30 seconds and an extending temperature of 72°Cfor 30 seconds, followed by a final extension at 72°C for 7 minutes. To purify PCR products the volume of 50 μL were taken in 1 to 5 ratios with purification buffer. Following procedures were done according to the manufacturer manual (GeneAllExpin PCR SV). Purified products were ligated into pGEMT-T easy vector system (Promega) using T4 ligase at 4°C overnight.Nucleotide sequencing was carried out to ensure the absence of PCR-induced mutations *Escherichia coli* strain DH5α was used for plasmid amplification of the CKBB protein.The nucleotide sequence comparison was conducted using free software BioEdit.

Results and discussion. Creatine kinase BB isoform is widely distributed in the brain and is located exclusively intracellular in both neurons and astrocytes and represents about 80-95% of total CK activity [21].The enzyme is involved in production of high-energy phosphate that is used for ATP synthesis. Several studies have reported CKBB as one of the specific targets of protein oxidation in Alzheimer's disease (AD) [19]. Therefore, it is used as an indicator of various physiological abnormalities such as the determination of neurodegenerative processes in the CNS [21].

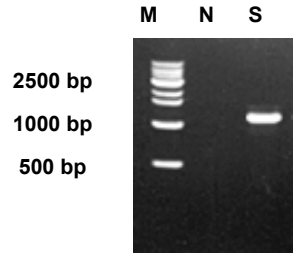

Figure 1.PCR product of amplified CKBB gene
M-DNA ladder; N-negative control; S-amplified product

To isolate the creatine kinase gene, we screened a HeLa genomic library with the set of primers amplifying the CKBB gene. Primers were designed corresponding to different restriction sites of used bacterial and mammalian expression plasmid. The product of interest was generated by applying polymerase chain reaction with correctly set temperature regimes indicated in the materials and methods. The obtained fragments were run on DNA 1 % agarose gel electrophoresis and stained with ethidium bromide. In the result of electrophoresis, we have identified amplified product with 1146 bp what matched with the original CKBB gene length (GeneBankCR542268.1) (Fig. 1).

The CKBB product was purified using Expin PCR SV purification columns. Prior to the transformation we thawed competent *E.coli*cells on the ice for 5-10 min. Ligated products of CKBB gene in pGEMT-easy vector were transformed into *E.coli* DH5-alpha host cells and streaked over an agar plate. We could judge by the number of grown bacterial colonies that the transformation was successful. Picked positive transformants were confirmed on the presence of CKBB insert by PCR and restriction enzyme digestion (Fig. 2).

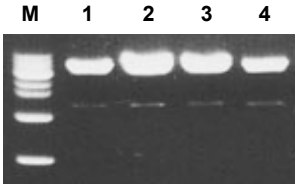

Figure 2. Double restriction enzyme digestion with Hind3/BamH1 of CKBB gene from pGEMT-Easy vector
1, 2, 3, 4 – randomly picked colonies with transformed vehicles

The gene of CK was further subcloned into pET-14b vector (4.6 kb), what carries an N-terminal His-Tag sequence followed by a thrombin site and three cloning sites. We chose pET-14b expression system to further simplify the purification process of His-Tagged CKBB protein using Ni^{2+}-nitrilotriacetic acid (Ni-NTA) affinity chromatography with NTA chelating agarose CL-6B. The pET system is the most powerful system yet developed for the cloning and expression of recombinant proteins in *E.coli*. Based on the T7 promoter-driven system was originally developed by Studier and colleagues [22, 23].So, we employed pET-17b (3.3 kb) bacterial expression system to overexpress the CK gene using LB growing medium at the temperature of 37°C.The results of the sequence alignment and double restriction enzyme digestionfrom both vectors were identical and are fully consistent with the sequence of the native molecule (Fig. 3).

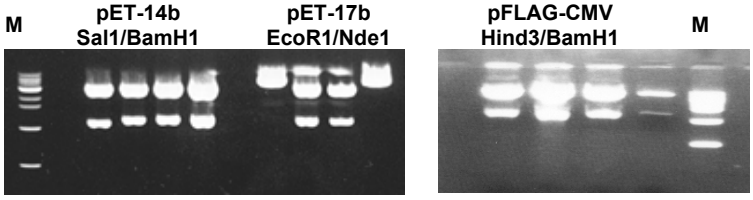

Figure 3. Double restriction enzyme digestion of CKBB gene from pET-14b, pET-17b and pFLAG-CMV vectors

For expression of the protein in mammalian cells we have turned to the pFLAG-CMV expression vector (4.7 kb). This transient system is suitable for intracellular expression of N-terminal FLAG fusion proteins. We also determined the DNA sequence of the cDNA-derived gene encoding the human brain CK isozyme from the pFLAG-CMV/CKBB construct. Again, the DNA sequence that we obtained from this construct was identical and consistent with the original one.

The correct nucleotide sequence of all subcloned brain creatine kinases is shown in Fig. 4. Considering the high sequence similarity of all creatine kinase types, it is possible to apply the same amplifying strategy what we used to clonethe gene of brain creatine kinase.

Figure 4. Multiple sequence alignments of forward reading frame of CKBB gene cloned into different expression vectors

Conclusion. In this study, we report the cloning, sequence analysis, and expression in DH5-alpha strain of *Escherichia coli*of brain-type creatine kinase. We demonstrate a successful primer design for CKBB gene sequence amplification. Therefore, these cloning methods may be applied for expression and purification of the enzyme what catalyzes a reversible transfer of the phosphoryl group of ATP molecule to creatine.

References

1. Wallimann T., Wyss M., Brdiczka D., Nicolay K., and Eppenberger H.M. (1992) Intracellular compartmentation, structure and function of creatine kinase isoenzymes in tissues with high and fluctuating energy demands: the 'phosphocreatine circuit' for cellular energy homeostasis. Biochem. J. 281, 21-40.

2. McLeish M.J., and Kenyon G.L. (2005) Relating structures to mechanism in creatine kinase Crit. Rev. Biochem. Mol. Biol. 40, 1-20.

3. Bong S.M., Moon J.H., Nam K.H., Lee K.S., Chi Y.M., and Hwang K.Y. (2008) Structural studies of human brain-type creatine kinase complexes with the ADP-Mg^{2+}-NO_3 creatine transition-state analogue complex. FEBS Lett.582, 3959-3965.

4. Wang P.F., McLeish M.J., Kneen M.M., Lee G., and Kenyon G.L. (2001) An unusual low pK(a) for Cys282 in the active site of human muscle creatine kinase. Biochemistry 40, 11698-11705.

5. Naor M.M., and Jensen J.H. (2004) Determinant of cysteine pKa values in creatine kinase and alpha1-antitrypsin. Proteins 57, 799-803.

6. Gross M., Lustig A., Wallimann T., and Furter R. (1995) Multiple-state equilibrium unfolding of guanidino kinases.Biochemistry 34, 10350-10357.

7. Webb T.I., and Morris G.E. (2001) Structure of an intermediate in the unfolding of creatine kinase. Protein 42, 269-278.

8. Zhao T.J., Ou W.B., Xie Q., Liu Y., Yan Y.B., and Zhou H.M. (2005) Catalysis of creatine kinase refolding by protein disulfide isomerase involves disulfide cross-link and dimer to tetramer switch. J. Biol. Chem. 280, 13470-13476.

9. Zhou H.M., and Tsou C.L. (1986) Comparison of activity and conformation changes during refolding of urea-denatured creatine kinase. Biochim.Biophys.Acta.869, 69-74.

10. Yao Q.Z., Hou L.X., Zhou H.M., and Zho C.G. (1982) Conformational changes of creatine kinase during guanidine denaturation. Sci. Sin. B. 25, 1186-1193.

11. Lyubarev A.E., Kurganov B.I., Orlov V.N., and Zhou H.M. (1999) Two-state irreversible thermal denaturation of muscle creatine kinase Biophys. Chem. 28, 199-204.

12.Li S., Bai J.H., Park Y.D., and Zhou H.M. (2001) Aggregation of creatine kinase during refolding and chaperonin-mediated folding of creatine kinase. Int. J. Biochem. Cell.Biol. 33, 279-286.

13. Hahn H.S., Park Y.D., Lee J.R., Park K.H., Kim T.J., Yang J.M., and Hahn M.J. (2003) Aggregation and folding of recombinant human creatine kinase. J. Protein. Chem. 22, 563-570.

14. Kurganov B.I., Lyubarev A.E., Sanchez-Ruiz J.M., and Shnyrov V.L. (1997) Analysis of differential scanning calorimetry data for proteins. Criteria of validity of one-step mechanism of irreversible protein denaturation.Biophys.Chem. 69, 125-135.

15. Gao Y.S., Su J.T., and Yan Y.B. (2010) Sequential events in the irreversible thermal denaturation of human brain-type creatine kinase by spectroscopic methods.Int. J. Mol. Sci. 11, 2584-2596.

16. Gao Y.S., Zhao T.J., Chen Z., Li C., Wang Y., Yan Y.B., and Zhou H.M. (2010) Isoenzyme-specific thermostability of human cytosolic creatine kinase. Int. J. Biol. Macromol. 47, 27-32.

17. Aksenov M. Aksenova M., Butterfield D.A., and Markesbery W.R. (2000) Oxidative modification of creatine kinase BB in Alzheimer's disease brain. J. Neurochem. 74, 2520-2527.

18. Aksenov M.Y., Aksenova M.V., Butterfield D.A., Geddes J.W., and Markesbery W.R. (2001) Protein oxidation in the brain in Alzheimer's disease. Neuroscience 103, 373-383.

19. David S., Shoemaker M., and Haley B.E. (1998) Abnormal properties of creatine kinase in Alzheimer's disease brain: correlation of reduced enzyme activity and active site photolabeling with aberrant cytosol-membrane partitioning.Brain Res. Mol. Brain Res. 54, 276-287.

20. Kim J., Amante D.J., Moody J.P., Edgerly C.K., Bordiuk O.L., Smith K., Matson S.A., Matson W.R., Scherzer C.R., Rosas H.D., Hersch S.M., and Ferrante R.J. (2010) Reduced creatine kinase as a central and peripheral biomarker in Huntington's disease. Biochim.BiophysActa. 1802, 673-681.

21. Vazquez M.D., Sanchez-Rodriquez F., Osuna E., Diaz J., Cox D.E., Perez-Carceles M.D., Martinez P., Luna A., and Pounder D.J. (1995) Creatine kinase BB and neuron-specific enolase in cerebrospinal fluid in the diagnosis of brain insult. Am. J. Forensic Med. Pathol.16, 210-214.

22. Studier, F.W., Rosenberg, A.H., Dunn, J.J., and Dubendorff, J.W. (1990) Use of T7 RNA polymerase to direct expression of cloned genes.Methods Enzymol.185, 60-89.

23. Dubendorff, J.W. and Studier, F.W. (1991) Controlling basal expression in an inducible T7 expression system by blocking the target T7 promoter with lac repressor.J. Mol. Biol.219, 45-59.

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КЛОНУВАННЯ ГЕНУ КРЕАТИНКІНАЗИ ГОЛОВНОГО МОЗКУ ЛЮДИНИ З ВИКОРИСТАННЯМПЛАЗМІД БАКТЕРІЙ *PET-17B,PET-14B* ТА РЕКОМБІНАНТНОЇ ПЛАЗМІНИ *PCMV-FLAG*

У даній роботі нами було проведено клонування гена креатінкінзи головного мозку. Цей фермент є ключовим ферментом каталізуючим синтез високоенергетичного з'єднання креатинфосфату з молекулами АТФ і креатину.

Клюсові слова: креатинкіназа головного мозку людини, клонування, ампліфікація.

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КЛОНИРОВАНИЕ ГЕНА КРЕАТИНКИНАЗЫ ГОЛОВНОГО МЕЗГА ЧЕЛОВЕКА С ИСПОЛЬЗОВАНИЕМ ПЛАЗМИД БАКТЕРИЙ *PET-17B, PET-14B* И РЕКОМБИНАНТНОЙ ПЛАЗМИДЫ *PCMV-FLAG*

В данной работе нами было проведено клонирование гена креатинкинзы главного мозга. Этот фермент является ключевым ферментом катализирующим синтез высокоэнергетического соединения креатинфосфата с молекулы АТФ и креатина.

Ключевые слова: кретинкиназа головного мозга человека, клонирование, амплификация.

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