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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.***

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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).

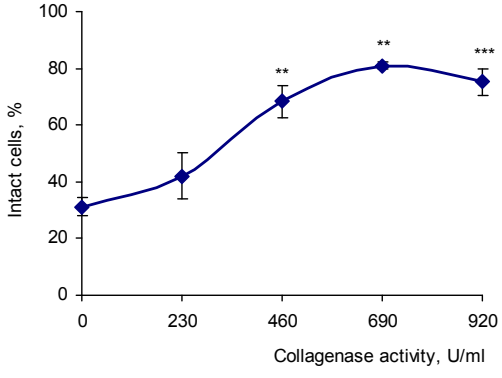


**Fig. 1. Dependence of the amount of lacrimal gland secretory cells with integral plasma membrane on concentration of collagenase for the second incubation:**
[NaCl] = 119 mM, [KCl] = 6 mM, [$MgCl_2$] = 1.2 mM, [glucose] = 10 mM, [$NaHCO_3$] = 25 mM, [HEPES] = 10 mM, [$CaCl_2$] = 1 mM, lidase activity – 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*).

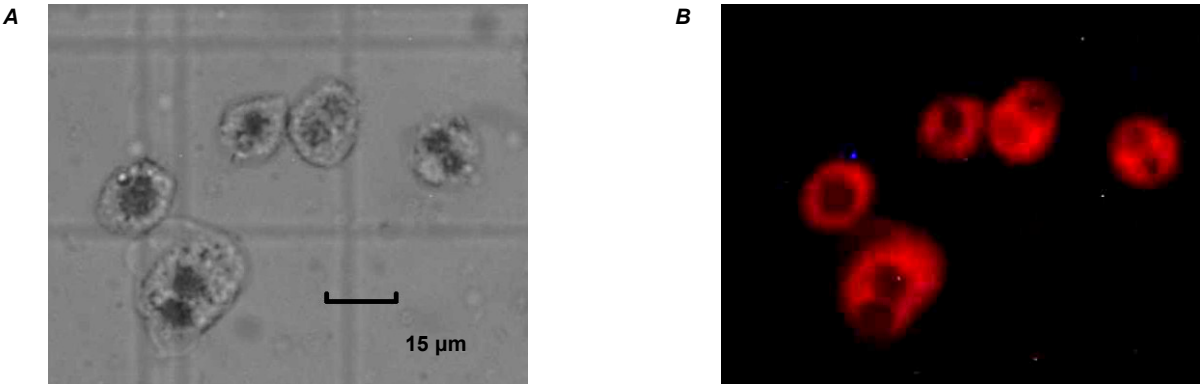


**Fig. 2. Permeabilized secretory cells of the rat exorbital lacrimal gland (light microscope Nicon optiphot 2, Japan):**
A – light microscopy; B – fluorescence registration (Filter Block G-2A, 590 nm); [KCl] = 140 mM, $[Ca^{2+}] = 10^{-7}$ M, $[MgCl_2]$ = 1.5 mM, [HEPES] = 10 mM, [trypan blue] = 0,1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

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*).

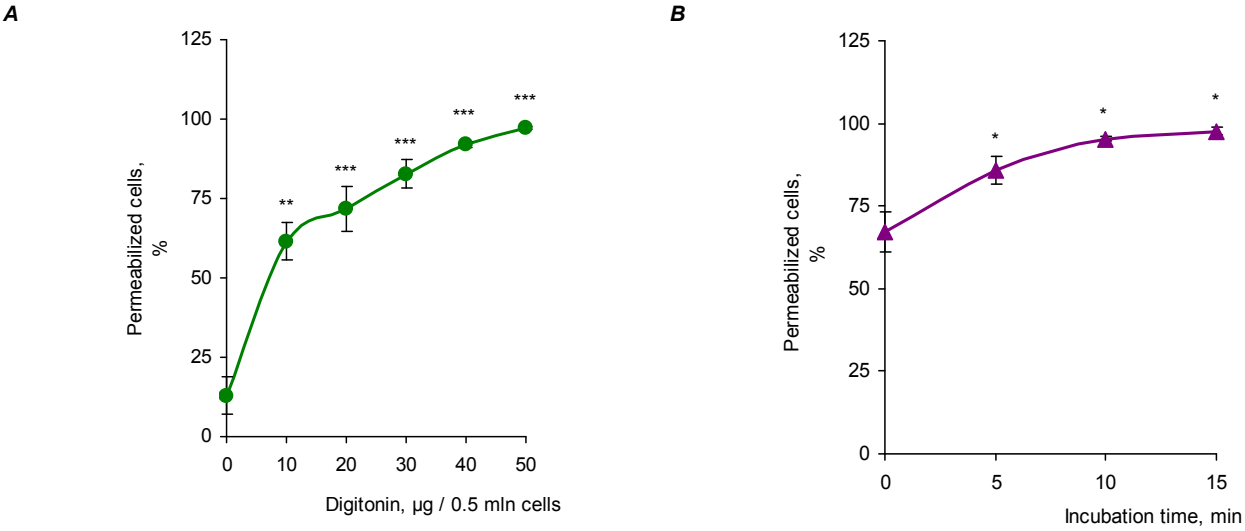


**Fig. 3. Dependence of permeabilized cells of exorbital lacrimal gland of the rat on digitonin concentration and incubation time with it:**
A – dependence of the percentage of permeabilized cells on digitonin concentration in the medium, incubation time – 10 min;
B – dependence of the percentage of 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 $37^0$C 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 $37^0$C 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.

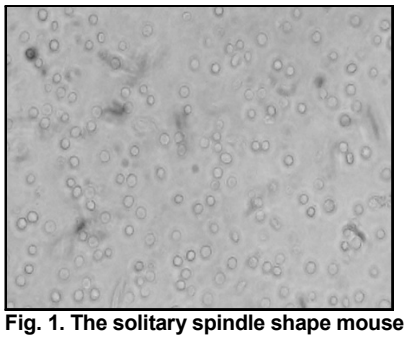

**Fig. 1. The solitary spindle shape mouse oviduct cells lost cilia in culture. Their shape changed to round due to trypsin treatment, x 250.**

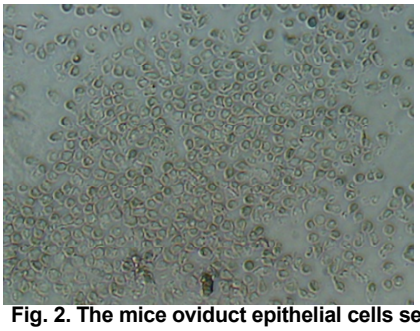

**Fig. 2. The mice oviduct epithelial cells settled down on the bottom of Petri dish and started to create a cell monolayer.**