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ESTABLISHMENT, MORPHOLOGY AND FUNCTIONAL CHARACTERISTICS OF THE OVIDUCT EPITHELIAL CELLS IN CULTURE

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In this study mechanical and enzymatic methods for harvesting and establishment of the laboratory animals' oviduct epithelial cells culture were compared. Obtained cells population was described and tested as feeder culture system aimed to investigate the application of cells monolayer for embryo development *in vitro*. The results strongly suggest the monolayer of the oviduct epithelial cells provokes blastocysts expansion but doesn't promote blastocysts hatching.

Key words: oviduct epithelial cells culture, embryo.

The main role of the oviduct epithelial cells (OECs) is providing the optimal environment for the various events that take a place in the oviduct lumen and production of the oviduct fluid. It is generated by transudation into the oviduct lumen of the specific substances synthesized in epithelial cells [1]. The epithelial cell layer covering the luminal surface of the mammalian oviducts consists mainly of two different cell types, ciliated and secretory cells [2]. The main function of ciliated cells 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 [1, 4]. The growth factors synthesized by epithelial cells are important modulators in many reproductive processes that work in an autocrine or paracrine manner [5].

Bovine oviduct epithelial cells are widely used in co-culture experiments to improve embryonic development of in vitro – cultivated and micromanipulated embryos in Embryo Transfer programmes but their molecular nature isn't studied completely [3, 6]. 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 and, finally, a presence mesenchimal stem cells in the cell population obtained from pushed away uterine mucus suggests that monolayer of mammalian oviduct epithelial cells possesses new unknown properties [7].

The aim of this study was to compare different techniques that allow getting a pure population of the oviduct epithelial cells and to test them as feeder matrix for pre-implantational embryos at different stages of the development.

The reproductive organs were removed from CD-1 mice and Wistar rat females on 3.5 days after natural mating and rinsed in the sterile phosphate balanced salt solution (PBS) containing penicillin/streptomycin. After they were moved to the dry Petri dish and uteruses were flushed by HEPES-buffered M2 medium with penicillin/streptomycin to confirming the blastocysts are there and stage of embryo development was determined correctly. The oviducts were cut off from tops of the horns and ovums were detached as well.The mechanical and enzymatic methods were applied for establishment of the oviduct epithelial cells culture.

Mechanical method. Isthmuses and ampoules of the oviducts from 6 mice were used as source of the epithelial cells (Fig.1). The organs from few females were squeezed by a pair of the

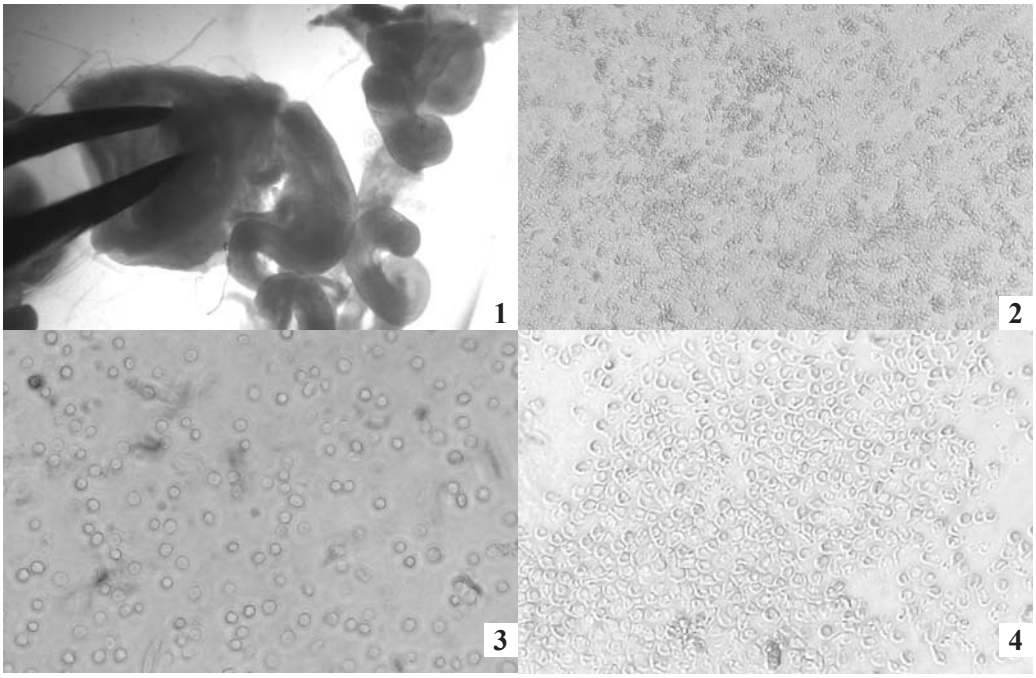

Fig. 1. Tips of the eye-forceps show the oviduct that is free-opened into a lumen of a uterine horn in mice. This is a more perspective part of the oviduct providing secretory epithelial cells.

Fig. 2. Concentrated suspension of mouse isolated oviduct epithelial cells that was obtained by mechanical method, x 150.

Fig. 3. The solitary spindle shape mouse oviduct epithelial cells lost cilia in culture. Their shape changed to round due to trypsin treatment, x 250.

Fig. 4. The mice oviduct epithelial cells settled down on the bottom of Petri dish and started to create a cell monolayer.

sharp eye-forceps as though a yellow cream and placed into small Petri dish containing 3 ml M2 medium (Sigma) with penicillin/streptomycin but without fetal calf serum (FCS). Concentrated cell suspension was pipette throughout 200 µl tip 10 times and squeezed throughout 27 g surgical needle 25 times until almost single cell suspension was reached. Single cells and not numerous cell clusters were allowed to settle down during 40 min in the wet chamber of the incubator at 37ºC and 5% CO_2. The medium covered cells were removed and DMEM medium with 10% FCS, 0.1 mg/ml α-glutamine, 2.5 µl/ml fungizone and 0.05 mg/l gentamicyne sulphate were given. Cells were incubated 40 min to settle down again and then the medium was removed. After third settle down and washing cycle the cells finally suspended in fresh DMEM medium with the same ingredients and cellular content was controlled and distributed into small (d=25 mm) Petri dishes with 1 ml suspension per each (6-8 x 105 cells per dish).

Enzymatic method. Trypsin was used to establish of the rat oviduct epithelial cells. Oviducts from 2 females were flushed with M2 medium, 1 ml per animal, for embryo harvesting. Subsequently, 0.25% trypsin with 0.02% EDTA (Sigma) was diluted two times to avoid enzymatic cellular destruction. The left over (just in this method) ovum served as a seal for the cranial end. A small serafine was put on the caudal thick end were an oviduct had been finished by horn. The trypsin was injected by surgical needle. The oviducts sealed in this way were cultivated for 3

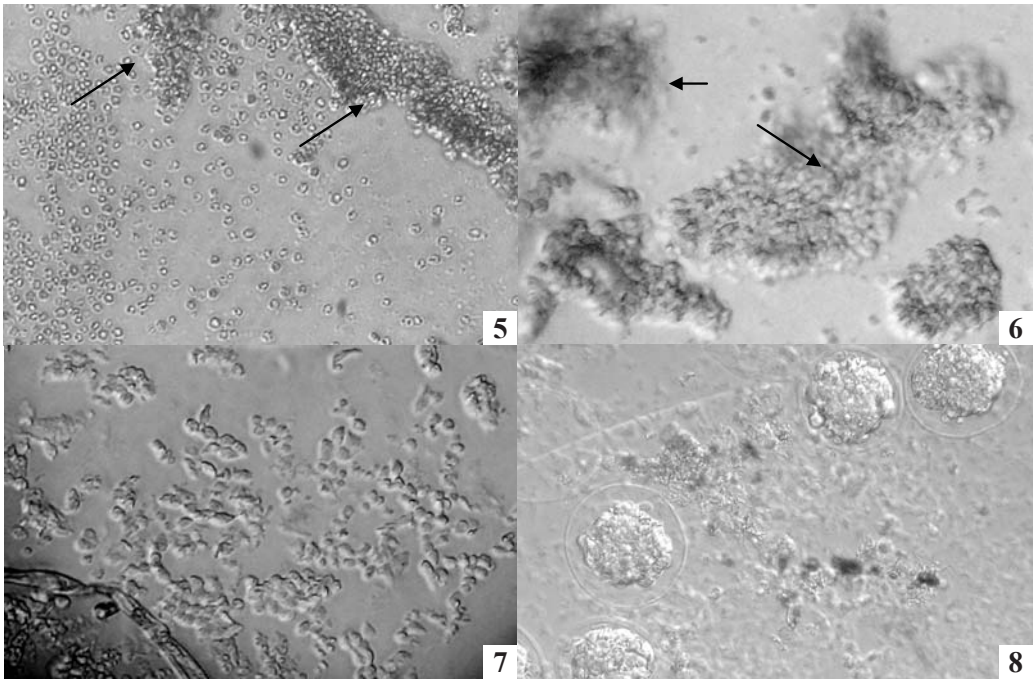

Fig. 5 and 6. The highly adherent features of the oviduct epithelial cells were expressed by a possession of them to create free-floating cell conglomerates, x 280.

Fig. 7. The suspension of the oviduct epithelial cells obtained by mechanically and used to improve embryo development *in vitro*.

Fig. 8. The well-expanded blastocysts collapsed with a visible stop of primary differentiation in them on fourth day from embryo seeding.

min on the wet surface of a Petri dish in the incubator. The trypsin was inactivated by flushing of the organs with 0.2 ml DMEM +10% FCS. After the cells have settled down the population was washed three times in the DMEM medium with the same ingredients for 40 min in the incubator, the same as in the previous method.

Modificated mechanical method. Some modification of the mechanical method was applied to reach a pure epithelial cell population from rat oviducts. The cells were mechanically isolated as described above and cell suspension was incubated after first washing cycle for 10 min at 37ºC and 5% CO_2 in 0.05% trypsin with EDTA (0.25% Trypsin, Sigma, was diluted 1:4 in DMEM). After trypsin inactivation the cells were spindled down at 1000rpm for 5 min and pellet was resuspended in 3 ml DMEM with ingredients to culture the in vitro and primary culture establishment. The culture medium was changed on the second day. Cellular growth was controlled by phase contrast microscopy, Axiovert 2000, x 280.

Embryo cultivation on the Mouse Oviduct Epithelial Cells Culturec(MOECs). The suspension of the single epithelial cells obtained from mouse oviducts was tested as feeder substrate to improve embryo development at different stages, for morulae as well as blastocysts. After 4 days in culture the embryos were moved to fresh DMEM but without feeder cells in order to take control of their morphological changes.

Results and discussions. The suspensions of isolated oviduct epithelial cells from mice and rat females were morphologically assessed before the placing the embryos for in vitro deve-

lopment (Fig. 2). Trypsin treatment and mechanical isolation of the oviduct epithelial cells caused perfect dissociation of the cells in rats (Fig. 3). Trypsin flushing of oviducts in rat provided lower quantity of solitary cells together with larger fragments of the epithelium that consisted of ciliated and nonciliated cells. However, both techniques caused some debris that could have been deleted by empiric well-matched time bar for cultivation and trypsin concentration. Obviously, lower trypsin concentrations or shorter incubational period would help to find the solution for the problem. Mechanical method, delicate pipette throughout surgical needle and three cycles of cell washing and settling down provided the cell suspension that consisted from single cells and small (3–4 cells) clusters. The following observations confirmed the perspective of this method.

Phase-contrast microscopy showed that ciliate activity was still remaining in suspension of MOEC preferably in small cell clusters after 3-days culture period. In general, the cilia activity was lost by single cells and most of them showed a tendency to forming of oblong shape aggregates at four to five hours after a last washing cycle (Fig. 5). The cell morphology changed also from spindle-like to round shape. After 24 h these aggregates transformed to vesicles and after 3–4-th days in culture the vesicles begun to adhere to the bottom of the culture dish (Fig. 6). Next 3 days the cells proliferated and reached confluent on 7-th day after seeding (Fig. 4). Not all oviduct epithelial cells that have settled down adhered to the bottom, nearly 60% from them remained in the suspension as free-floating clusters. The method of cell production that was arranged didn't affect the described observations. The behavior of the MOEC in culture was identical to the mechanical squeezing as well as to the trypsin treatment in the both cases.

Mechanically produced mice oviduct epithelial cells suspension was employed to improve embryo development *in vitro* (Fig. 7). Total of 76 good expanded, 27 early with middle-size cavities blastocysts and 33 compacted morulae were flushed from 9 CD-1 mice, an average 17.5 embryos per donor, that were mated with males after hormonal treatment. The well-known scheme was used: 5IU of the PMSG (Pregnant Mare Serum Gonadotropine) at 2.00 pm, 5IU of the hCG (human Chorionic Gonadotropine) 48 h later with immediately mating and checking the copulative plugs early next morning.

In the each of three attempts the embryos were split on four Petri dishes that meant four different groups. The expanded blastocysts were divided on two groups but the early blastocysts and morulae created their own groups. The dish 1 was filled by DMEM medium with ingredients but without the oviduct epithelial cells and served as a control group for expanded blastocysts cultivation. The dish 2 contained 1ml MOEC suspension obtained as described above and also the expanded blastocysts. The early blastocysts were cultivated on the monolayer of epithelial cells in the dish 3 and the morulae created fourth group in the dish 4 where they have been cultivated at the same conditions. After first 24 h all compacted morulae formed blastocysts with different size cavities but early blastocysts developed to well-expanded embryos. Expanded blastocysts of the control group were hatched or half-hatched despite the fact that just 5 from 38 expanded blastocysts in the dish 2 reached hatching. The rest of the blastocysts in the group 2 didn't show any visible changes. Attachment of the hatched blastocysts to the bottom of the Petri dish was noticed at 48 h after the embryo seeding in the control group. All other dishes (2, 3 and 4 groups) were containing well-expanded blastocysts that developed from the earlier stages on the epithelial feeder cells in spite of the fact that the expanded blastocysts into dish 2 remained without changes.

The embryos were staying in the medium without disturbs during 4 days to avoid infringements of the molecular balance in the cell-embryo system. On fourth day after embryo seeding the well-expanded blastocysts collapsed with a visible stop of primary differentiation in them (Fig.8) but they recovered to cavity form after transfer them to fresh DMEM medium without feeder cells.

To confirm their viability 20 blastocysts from dish/group 2 were transferred to 2 foster mothers, 10 embryos per each recipient. One female became pregnant and 3 live offspring were born.

All methods were found suitable to provide sufficient amounts of isolated epithelial cells for primary cell culture. Mechanical cell isolation was the most reliable method. It was easily performed, inexpensive, and possible cellular damage during treatment with enzymes was excluded. The amount of mechanically isolated cells was sufficient for cell monolayer creating although it was realized that only a part of the epithelial layer was removed. The highly adherent features of the oviduct epithelial cells were expressed by a possession of them to create free-floating cell conglomerates. They are supposed actively to transport substances like specific proteins/growth factors from their interior to outside culture medium and in this way to influence to cell-embryo processes. Thus, the actively secreting oviduct epithelial cells are main demand for embryo co-cultivation on the feeder monolayer.

Differentiation between oviduct epithelial cells *in situ* and *in vitro* has been noted by I. Walter, 1995 [7]. Culture oviduct epithelial cells contained ample glycogen which was not present in oviduct epithelium *in situ*. These glycogen deposits caused dedifferentiation of the embryonic blastomeres or increased amounts of glucose in the culture medium which interprets as signs of dedifferentiation as well. Because of oestrogen receptors in secretory oviduct epithelial cells and in stromal fibroblasts also in smooth muscle cells but not in ciliated epithelial cells [2], further experiments with creating of organ-like three-dimensional culture can substitute for mixed population better than pure epithelial cells using.

Thus, the conclusions can be made that the oviduct epithelial cells are remaining in the culture medium as suspension but show different morphology connected with the time of cultivation. All methods are suitable to provide oviduct epithelial cells as monolayer for embryo culture, however mechanical method revealed the maintenance of original cell physiology and morphology. Activity of the oviduct epithelial cells influences to embryo primary differentiation at co-culture: it provokes blastocysts expansion but doesn't promote blastocysts hatching.

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МОРФОФУНКЦІОНАЛЬНІ ОЗНАКИ СТАНОВЛЕННЯ КУЛЬТУРИ ЕПІТЕЛІАЛЬНИХ КЛІТИН ЯЙЦЕПРОВОДІВ ЛАБОРАТОРНИХ МИШЕЙ І ЩУРІВ

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Представлено результати досліджень тривалого періоду культивування епітеліальних клітин яйцепроводів, при цьому методом фазово-контрастної мікроскопії виявлено, що клітини суттєво змінюють свою морфологію. Описано різні методи отримання первинних культур, що можуть бути використані для забезпечення ефективного отримання моношару з епітеліальних клітин яйцепроводів і застосовуватися для культивування ембріонів. Показано, що активність епітеліальних клітин яйцепроводів впливає на процеси первинної ембріональної диференціації впродовж культивування: стимулює утворення бластопорожнини і процесів експандації, однак не забезпечує процеси вилуплення бластоцист із прозорої оболонки.

Ключові слова: культура епітеліальних клітин яйцепроводу, ембріон.

МОРФОФУНКЦИОНАЛЬНЫЕ ОСОБЕННОСТИ ДИФФЕРЕНЦИАЦИИ КУЛЬТУРЫ ЭПИТЕЛИАЛЬНЫХ КЛЕТОК ЯЙЦЕПРОВОДОВ ЛАБОРАТОРНЫХ МЫШЕЙ И КРЫС

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Представлены результаты исследований длительного периода культивирования эпителиальных клеток яйцепроводов мышей и крыс, при этом методом фазово-контрастной микроскопии выявлено, что клетки активно меняют свою морфологию. Описаны разные методы получения первичных культур, которые могут быть использованы для получения монослоя из эпителиальных клеток яйцепроводов и могут применяться для культивирования эмбрионов. Показано, что активность эпителиальных клеток яйцепроводов влияет на процессы первичной эмбриональной дифференциации во время культивирования: стимулирует образование бластополости и процессы экспандации, однако не обеспечивает при этом вылупление бластоцист из прозрачной оболочки.

Ключевые слова: культура эпителиальных клеток яйцепровода, эмбрион.