The new sheep ovarian cell culture and the prospect its application in virology

T V Galnbek, A F Shulyak*, A A Pronina and E A Zhuravleva

Federal State Budget Scientific Institution “Federal Scientific Centre VIEV”, Russian Academy of Science, 24 bld 1, Razanskiy ave., Moscow, 109428, Russia

*E-mail: anfeshu@yandex.ru

Abstract. The new sheep ovarian cell culture named as SO was derived and its reproduction has been successful carried out using available growth mediums such as mixture of modified Eagle's medium, lactalbumin hydrolysate and bovine serum. The monolayer was formed at a seeding concentration of 100-120 thousand cells/ml in 3-5 days. The proliferation index was 2.81-3.24. Fibroblast-like cells predominated at early generations of the culture. During high passages, domination of epithelial-like cells was observed. While passaging, the modal class of 54 chromosomes was retained but the number of chromosome dispersion increased and the number of cells with a diploid set decreased. The cell viability was 82-75% during long-term storage in liquid nitrogen. The monolayer was retained for at least two weeks without changing the medium at 37°C. The culture produced estradiol and progesterone. Cell culture was highly sensitive to bovine herpesvirus 1, parainfluenza-3, nodular dermatitis and pustular stomatitis (Orf) viruses, but had low sensitivity to Orthopneumovirus. It didn’t allow reproduction of pestivirus – causative agent of bovine viral diarrhea – mucosal disease.

1. Introduction

Cell of different origins are widely used as models in many branches of experimental biology, diagnostics of human and animal diseases, production and testing of biological and pharmacological preparations, etc. To a large extent, success is determined by the presence of diverse and characteristic cell cultures capable of stably reproducing and providing reproduction of biological agents. Important part of cell biotechnology is the screening of existing and searching for new cell culture sensitive to viruses.

Ovaries are a prospective source of cell for culturing in vitro. This mammalian organ consists of follicles in which the oocytes are surrounded by somatic granulosa cells and a basal membrane. The pool of follicles is developing during the embryonic period. To the time of birth mammalian ovaries contain a lifetime supply of primary follicles. Ovarian cells have a pronounced ability to grow and reproduce in vitro. Depending on needs, several methods have been developed for obtaining ovarian cell: from whole organs, from intact follicles, or from primary follicles.

There are some reports on deriving cultures from ovaries of different animals such as cattle, sheep, and, that have been mainly used for studying of ovarian development, pathological state, the influence of drugs, chemicals, hormone production [1, 2, 3, 4]. Ovarian cell cultures have been successfully used for the cultivation of foot and mouth disease, african swine fever, sheep pox virus, suid herpes, classical swine fever virus [5, 6]. The cell cultures of goat gonads (CG-91), goat ovaries (YADK-04), Chinese
hamster ovaries (CHO -K1, DHB-11), scoop and silkworm ovaries (Sf-9; Bm ), and gonads of rainbow trout (RTG-2, OMG) have been presented in the collections of FSC VIEV, the Gamaleya National Center of epidemiology and microbiology, Institute of Cytology, ATCC, ECACC, etc. At the same time, cell cultures of the same origin, like clones of the same culture, can differ significantly in their properties. These conditions demonstrate the importance of deriving new cultures with selected properties.

The aim of our investigation was to obtain a sheep ovarian cell culture, to study the conditions of its cultivation, cultural and morphological characteristics, hormonal activities and sensitivity to viruses.

2. Methods, materials

The cells were isolated from the ovaries of a sheep at the age of 8 months. The ovaries were chopped into 1-2 mm size pieces, rinsed from erythrocytes with Hank’s solution (HBSS) with antibiotics. Trypsin solution (0.25%) was used to disaggregate the tissue. Disaggregation was carried out fractionally. Every 10-15 minutes of stirring, the suspended cells were poured into centrifuge flasks, 3-5% of bovine serum was added to inactivate trypsin, and a fresh portion of ferment was poured into the flask. The suspension was centrifuged at 1000 rpm for 10 minutes. The supernatant was discarded and the sediment was resuspended in a small amount of growth medium.

After counting the cells, the suspension was diluted to a concentration of 200-300 thousand cells/ml with a mixture of lactalbumin hydrolysate (GLA) and Eagle MEM medium in a 1:1 ratio supplemented with 10% bovine serum. Antibiotics (penicillin 100 U / ml, streptomycin 100 μg / ml, enrofloxacin 5-10 μg / ml) were added. The cells were seeded in the culture flasks and were incubated at 36.5-37.5°C.

After monolayer formation, for subculturing, a mixture of 0.02% of ethylenediaminetetraacetic acid (EDTA) and 0.25% trypsin solution in a 9:1 ratio was used. Cell viability was determined on an NS 100 analyzer and with using a 0.5% trypan blue solution.

For the preparation of chromosome slides the 48 hour-old cell culture was processed by 1 μg/ml colchicine for 3-4 hours. Then cells were dispersed, centrifuged and exposed to a hypotonic solution consisting of HBSS and distilled water (1:3). The cells were pelleted by centrifugation. Freshly prepared fixing mixture of ethyl alcohol and glacial acetic acid (3:1) was dripped into the sediment. This procedure was repeated three times. The cell suspension was then dripped onto wetted slides, dried and stained with azure-eosin according to Romanovsky-Giemsa method.

The cultures of various generations were preserved by cryopreservation. For this purpose, monolayer was dispersed with an EDTA (0.02%) and trypsin (0.25%) mixture of solutions in a ratio of 9:1. The cell suspension of Bovine serum (3-4%) was added to. Cells were pelleted by centrifugation at 1000 rpm for 10-15 minutes and then resuspended in a cryoprotective medium consisted of 50% Eagle MEM, 40% bovine serum and 10% dimethylsulfoxide (DMSO). The suspension was poured into cryo vials, kept at 4°C temperature for 2-3 hours then was gradually frozen to -70°C and placed in liquid nitrogen.

For recovery the cells were derived from the nitrogen and warmed to 37°C and cultured as described above. The medium was changed after 24 hours.

The ability of the culture to produce estradiol, progesterone and 17-OH-progesterone was determined in a solid-phase competitive enzyme-linked immunosorbent assay by commercial kits manufactured by "Vector-Best" and "Hema" companies, according to their instructions.

The cultures of different generations were infected with DNA and RNA viruses such as infectious bovine rhinotracheitis (BHV-1), parainfluenza-3 (PI-3), bovine viral diarrhea-mucosal disease (Pestivirus A), nodular dermatitis, contagious pustular stomatitis of sheep and goats (Orf), and bovine Orthopneumovirus (RSV). The virus species were determined in real-time PCR using “Vet-factor” kits. One to four strains of each virus were used in experiments. The infectious dose was 0.1 TCD50/cell. The infected and control cell cultures were incubated at 37°C. The changes were observed visually by microscopy and photography.

The yield of viruses was determined by titration in 96-well culture plates. The ability of cell culture to stably provide reproduction of viruses was studied by conducting 5-7 consecutive passages.
3. Results

Primary sheep ovaries cell culture formed a confluent monolayer on 3-5 days of cultivation with changing medium after 24-48 hours of seeding. The monolayer consisted of fibroblast-like and epithelial-like cells with a predomination of fibroblast-like cells (figure 1). The epithelium-like cells were polygonal types with oblong or roundish nuclei. Some cells contained two nuclei. There are contained 2 to 3 nucleoli in the nuclei. Some epithelium-like cells are larger than others. Fibroblast-like cells are two types: elongated and oblong resembling a spindle and elongated, but shorter. The nuclei are oval in shape, contain 2-3 nucleoli.

The monolayer was formed in 3-5 days of cultivation in 1-3 passages, at a seeding concentration of 100-120 thousand cells/ml. The index of proliferation was 2.81-3.24. In subsequent passages (10-15), the cell population changed, the polygonal epithelium-like cells started dominating. The number of fibroblast-like cells decreased and most of them had a shortened shape. At a seeding concentration of 90-110 thousand cells/ml, a monolayer was formed in 3-4 days. The ratio of reseeding composed 1: 2-1: 3.

![Figure 1. Primary culture SO. Azur-eosin staining, x400 magnification.](image)

The culture at passage 10 was represented by two types of cells, but epithelium-like cells were in greater numbers in contrast to the primary culture and are located in separate areas. The culture at 15-20 passages mainly consisted of uniform polygonal cells. Cell walls were tightly adjoined to each other and the nucleus was rounded in shape with 3-4 nucleoli. Some cells contained two nuclei. The monolayer was formed after 3-4 days at a seeding density of 100 thousand cells/ml. The monolayer lasted up to 15 days in the growth medium and to 8 days in the serum-free medium.

The viability of generation 0 cells after storage in liquid nitrogen for 2 years was 82%, generations 20-32 were 75%.

Dispersion of the number of chromosomes in chromosomal plates was within 40 to 54. In the second passage the number of chromosomal plates with a diploid set 2n = 54 was 54%. The range of variability fluctuated from 50 to 54. In the culture of the 16th passage, number of chromosomal plates containing a diploid set decreased to 23%. The wider spread of chromosomes were 42-54, but with the preservation of the modal class 54.

The SO cell culture produced sex hormones. The results are presented in the table 1.
As the passage level of culture increased the progesterone production decreased and estradiol production tended to increase. This is probably a consequence of changes in the cellular composition of the population in distant generations: a decrease in the number of descendants of stromal cells and the predominance of descendants of somatic granulosa cells.

The cell culture was sensitive to BHV-1. The dynamics of reproduction, the timing and nature of the CPE and the yield of the virus didn’t depend from the cell generation. The CPE was noticed a day later, complete destruction was observed at 48 hours after viral inoculation. CPE was characterized by cell rounding and in contrast to other sensitive cultures, without pronounced cluster formation (figure 2). The yield of virus was $10^6-10^7$ TCD$_{50}$ / ml, depending on the strain. These parameters didn’t differ significantly for six consecutive passages.

The PI-3 virus was reproduced identically in the passages 6-11 and 22-24 of the cultures. CPE appeared within a day and complete destruction was observed after 72 hours of infection. Cell rounding was the dominant feature. Symplast formation and cell elongation were less pronounced (figure 3). The viral yield, on average, was $10^6$ TCD$_{50}$ / ml or $10^7$ by hemadsorption. These characteristics hadn’t variability for five consecutive passages.

The culture SO was highly sensitive to the Orf virus. The CPE manifested itself less than a day after infection. The rounded hypertrophied cells were located throughout the whole monolayer (figure 4). During cultivation, their number increased, symplasts appeared, and complete destruction of the monolayer occurred within 72 hours. The maximum yield of the virus reached $10^6$ TCD$_{50}$ / ml, which is comparable to the standard cell models. Stable reproduction of the virus was observed during serial passaging starting from the primary infection and without adaptation.

**Table 1.** The dynamics of hormone production in SO cell culture. (n = 3).

| Hormones | Days | Passage 2  | Passage 15 | Passage 29 | Passage 32 | Control |
|----------|------|------------|------------|------------|------------|---------|
| Estradiol (pg / mol) | 4 | 104.21±7.51 a | 92.81±7.25 | 121.75±7.50 | 106.41±7.31 | 39.54 ±/4 b |
| Estradiol (pg / mol) | 7 | 62.85±6.32 | 120.4±6.4 | 61.36±3.61 | 132.80±8.50 | 31.68±6.02 |
| Estradiol (pg / mol) | 10 | 41.16±3.11 | 138.7±7.07 | 42.96±3.66 | 111.85±7.25 | 39.02±2.05 |
| Progesterone (nmol / L) | 4 | 92.22±1.47 | 27.72±0.98 | 0.88±0.3 | 3.32±0.92 | 6.576/0.401 |
| Progesterone (nmol / L) | 7 | 119.29±2.31 | 73.11±1.05 | 1.09±0.92 | 2.12±0.91 | 12.42±0.32 |
| Progesterone (nmol / L) | 10 | 107.46±1.61 | 92.61±1.5 | 0.03±0.03 | 3.21±1.01 | 58.57±0.91 |
| 17-OH Progesterone (nmol / L) | 4 | 0.41±0.17 | 0.356±0.91 | 0.386±0.21 | 0.339±0.13 | 0.02/0.03 |
| 17-OH Progesterone (nmol / L) | 7 | 0.604±0.21 | 0.429±0.15 | 0.226±0.25 | 0.303±0.15 | 0.49±0.19 |
| 17-OH Progesterone (nmol / L) | 10 | 0.503±0.15 | 0.386±0.82 | 0.122±0.14 | 0.315±0.12 | 0.361±0.12 |

a Indicators in serum medium.

b Indicators in medium without serum.

c Serum control.

d Control of medium with serum.
Infection with the virus of nodular dermatitis led to the development of CPE (figure 5). One day after infection, cells acquired a strongly elongated shape, then vacuoles and inclusions appeared in the cytoplasm, and a small number of symplasts formed. The cells were detached from the surface of the flasks to 96 hours after inoculation. The yield reached $10^6\ TCD_{50}\/\text{ml}$, which is fairly high.

RSV propagated in the culture SO causing CPE in the form of single small symplasts and rounded cells. Passage didn’t increase the reproduction level and viral titer was $10^{-2}\ TCD_{50}\/\text{ml}$.

The culture didn’t provide the reproduction of the Pestivirus A which was confirmed by the absence of CPE, negative PCR, and unsuccessful adaptation during blind passages.

4. Conclusion
A new cell culture SO has been derived from sheep ovaries. It is capable of being reproduced for a long time on available growth media with a tendency to form a continuous line. The culture retained its steroidogenic activity during passaging and was susceptible to DNA and RNA viruses from various taxonomic affiliations.

The sheep ovarian cell culture SO has been deposited to the Specialized collection of continuous somatic cell cultures of farm and fur-bearing animals (VIEV) and can be used in various branches of biology.

References
[1] Zanotelli M R, Henningsen J D, Hopkins P M, Dederich A P, Herman T, Puccinelli T J and Salih S M 2016 An ovarian bioreactor for in vitro culture of the whole bovine ovary: A preliminary
report *J. of Ovarian Res* **9** 47

[2] Peng X, Yang M, Wang L, Tong C and Guo Z 2010 In vitro culture of sheep lamb ovarian cortical tissue in a sequential culture medium *J. of Assisted Reproduction and Genetics* **27** 247-57

[3] Higuchi C M, Maeda Y, Horiuchi T and Yamazaki Y 2015 A Simplified Method for Three-Dimensional (3-D) Ovarian Tissue Culture Yielding Oocytes Competent to Produce Full-Term Offspring in Mice *PloS* **11**-7

[4] Morgan S, Campbell L, Allison V, Murray A and Spears N 2015 Culture and Co-Culture of Mouse Ovaries and Ovarian Follicles *J of Visualized Experiments*

[5] Gerasimov V N, Gerasimova N I, D'jakonov L P, Gruzdev K N, Zakharov V M and Manin B L 2008 *Ovaries cell line of goat Capra Hircus L. YDK-04 for reproduction of animal viruses Patent No 2335537*

[6] Zaerko V I, Gevlich O A, Fominova I O, Shulyak A F, Galnbek TV and Velichko G N 2018 Comparative value of cell culture sensitivity to Poxviruses of animals *Veterinaria* **10** 27-33