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STORAGE OF PIGLET KIDNEYS AT SUBNORMAL TEMPERATURES FOR PRIMARY TRYPSINIZED CELL MONOLAYER PREPARATION

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SUMMARY

Optimization of the technology for preparation of the primary cells suitable for virological studies requires long-term storage of internal organs and tissues. Results of tests of organs stored at subnormal temperatures under laboratory conditions for the purpose of further preparation of complete monolayer of primary cells for their preservation are presented. Method for 6-day storage of kidneys aseptically taken from 3–5 month-old piglets was proposed. Serum-free medium 199 supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin, 100 U/ml of gentamycin was used as a stabilizing substance. The organ was placed in a wide-neck vessel at medium/air phase ratio of 1:1 and the vessel was covered by a Petri dish to optimize medium chemical homeostasis and stored at temperature of –1 to +4 °C up to 6 days avoiding medium and organ crystallization. It was concluded that any freezing with crystallization and without cryoprotector (of –2 to –4 °C) had a negative effect on cell and organ tissue survivability. Trypsinization and complete monolayer preparation were carried out upon the kidney storage period completion. All variants of prepared cell materials used for virological studies were suitable for testing semi-preparations for their avirulence as well as for virus neutralization tests and foot-and-mouth disease virus isolation. Proposed method for porcine kidney storage allows more effective use of donor organs for primary culture preparation for virological studies. This method of organ storage could be recommended for preservation and transportation of organs derived from domestic and wild animals under field conditions.

Key words: piglet kidney cell cultures, medium 199, subnormal temperatures, trypsinization, seeding concentration, foot-and-mouth disease virus.

INTRODUCTION

Methods of preserving human organs at subnormal temperatures (0... +4 °C) have been used in transplant surgery for several decades [8]. Owing to fundamental experiments on animal organs, it was found that parenchymal organs can be transplanted within a day after removal from the donor, the cornea of the eye – within 3 days of storage [5, 8, 9]. At the end of this period irreversible biochemical and morphological changes occur in tissues, leading to organ rejection by the recipient [1, 7]. In most cases irreversible changes occur in the vascular systems (blood clotting) and in the stromal tissues ensuring organ survival.

Despite the recommended 2–4 hours of storage of internal organs and tissues at 0...+4 °C [4], preservation for more than 2 days has also a scientific and applied value in veterinary virology. The first reason for the need for long-term kidney storage is the rationalization of the technological process of obtaining primary cells, when the trypsinization period needs to be extended to produce fresh, not overgrown cultures suitable for virological studies. The second reason is the study of the preservation of organs at subnormal temperatures in the laboratory for further use of these data in the field with the aim of obtaining a full-fledged monolayer of primary cells from the stored organs. When obtaining a complete monolayer of primary cells from trypsinization organs, it is also necessary to study their suitability for virological studies.

MATERIALS AND METHODS

Primary trypsinized culture was prepared from kidneys of 3–5 week-old piglets using standard techniques of primary cell culture preparation [5, 6]. The isolated kidneys were stored at +4 °C in serum-free medium 199 supplemented with 50 μg/ml of gentamicin. Kidney trypsinization was performed after 1–6 days of storage.

In the course of the experiment the adhesive ability of individual cells and their aggregations, the morphology of the sedimented cells, the timing of the monolayer formation and sensitivity to the FMDV were studied [2, 3]. The

Table 1
The results of determining the FMD virus sensitivity of PK cell culture prepared from the stored suspension

Virus Type	Cell-cultured FMD virus titer (lg TCD_{50}/0.1 cm^3)	
	PK cell suspension, native	PK cell suspension stored for 48 hours at +4–6 °C
A	2.00	2.00
	2.50	2.75
	2.75	2.50
O	1.75	0.75
	2.0	1.25
Asia-1	2.00	1.75
	2.00	1.00
$M \pm m$	2.11 ± 0.15	1.71 ± 0.29

Eagle's medium with 0.25% GLA (Serva) and fetal bovine serum (Bioclot) were used for the cultivation of primary porcine kidney cells (PK). Cell cultivation was carried out in penicillin vials and plastic culture vessels with a growth area of 25 cm^2.

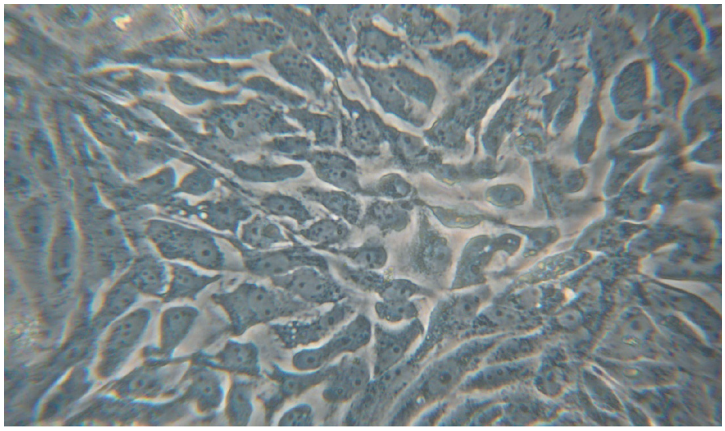
Cell and monolayer morphological analysis was performed using Olympus CKX41 Phase Contrast Microscope. The productivity of primary cells was also calculated after the formation of a monolayer on days 5–7.

The sensitivity of the primary PK cell culture was assessed based on the results of titration of the virus working dose using virus neutralization assay. The FMD culture virus at the same dilution was titrated on the monolayer of the primary culture of PK cells grown immediately after trypsinization. On days 2–3 the infectivity control of the virus grown in the PK cell culture after storage of cell suspension or whole porcine kidney cells for 2–6 days at 0...+4 °C was repeated.

RESULTS AND DISCUSSIONS

The trypsinized cell suspension was used for virological purposes to obtain the required amount of additional primary cell culture which was stored for 48 hours at +4 °C. The cell monolayer was prepared within 5 days when the inoculation density was 200,000 cells per ml, but the study of FMD virus reproduction showed that the cells were low sensitive to the virus (Table 1).

Fig. 1. PK primary cell monolayer with extracellular matrix



The results presented in Table 1 confirm the fact that the storage of the trypsinized cell suspension at +4 °C had a negative effect on the sensitivity of PK cells to the virus. It should also be noted that a 2-day-storage of the suspension of primarily trypsinized PK cells resulted in formation of a monolayer with numerous aggregates and stromal fibroblastic cells. A high percentage of non-specific monolayer degeneration was observed in the process of the neutralization assay.

During the morphological analysis of the monolayer the intercellular space was found to contain a large amount of extracellular matrix - a protein that is likely to partially isolate cells from viruses (Fig. 1) and thereby reduce the sensitivity of cells to FMD virus. Therefore, it was decided to conduct tests on trypsinization of kidneys stored in a nutrient medium at a temperature near 0 °C.

The kidneys were taken from 4-week-old piglets and placed in a wide-neck vessel with a nutrient medium 199 supplemented with 50 μg/ml gentamicin. The vessel was covered with a sterile Petri dish, placed in a refrigerator and kept at a temperature near 0 °C. The vessel with the organ was not sealed with a stopper to provide passive gas exchange with the ambient air. This storage condition prevents acidification of the medium, since glycolysis occurs even in cooled organs [5]. When piglet kidney was stored in 100 ml of medium 199 at the temperature near 0 °C for 96 hours, the pH of the medium increased from 7.0 to 7.1–7.2. The pH in a vial containing medium 199, where the organ was not placed, increased to 7.6. The stabilization of the medium pH where the kidney was placed indicates that the tissues breathe, utilizing glucose and releasing carbon dioxide and metabolites.

The next stage of the experiment was to study the effect of organ freezing at a temperature of –2…–4 °C on the quality of the cell cultures prepared. For cell preparation a piglet kidney was trypsinized after the crystalline phase of the "medium + kidney" system was thawed (that is, the kidney was covered with ice and was probably partially frozen). Then the cell suspension was seeded into penicillin vials and plastic flasks with a growth area of 25 cm^2. The cells not stained with trypan blue (concentration 200,000 cells/ml) did not adhere during seeding and did not form a monolayer. Disaggregated cells were at the stage of apoptosis. It was concluded that any freezing with crystallization without cryoprotectants adversely affects the survival of cells and tissues of organs containing about 90% of water [1, 9].

The piglet kidneys stored at +2 °C for 2–6 days did not change their texture and color. The cortical layer was crushed using steel scissors and dispersed with warm trypsin solution using a standard method. Up to 30 million cells not stained with trypan blue were prepared from 1 g of the kidney tissue. As it was found in previous studies, trypsinized cells were polymorphic and belonged to different histotypic tissues, not all cells were able to form colonies [2, 4].

In the course of the experiment the cells prepared from kidneys stored at +2 °C for 2–6 days were also polymorphic and later demonstrated the standard dynamics of sedimentation, adhesion and proliferation. The first indicator of cell integrity after trypsinization is the number of viable cells prepared from 1 g of kidney tissue and tested by tryp-

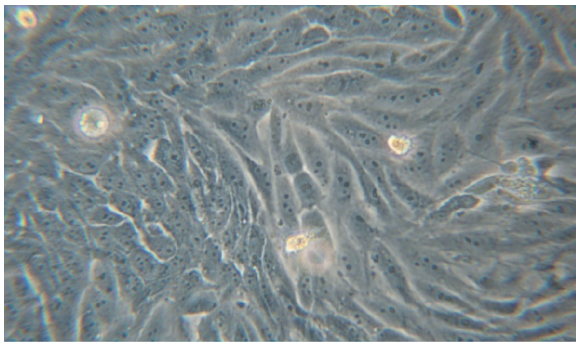


Fig. 2. PK cell culture prepared from kidney that was not previously stored

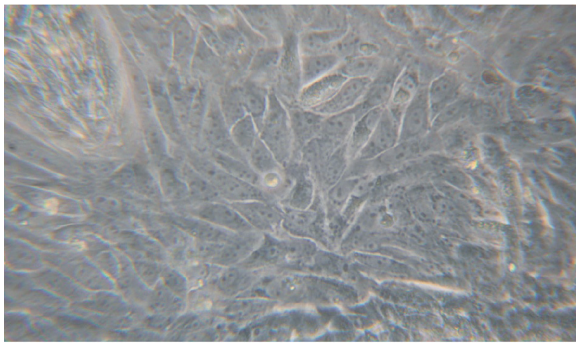


Fig. 3. PK cell culture prepared from kidney that was stored for 72 hours

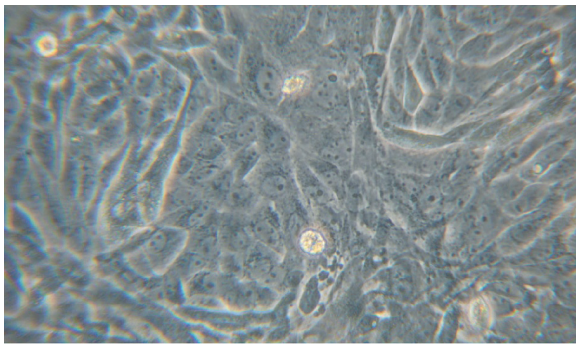


Fig. 4. PK cell culture prepared from kidney that was stored for 96 hours

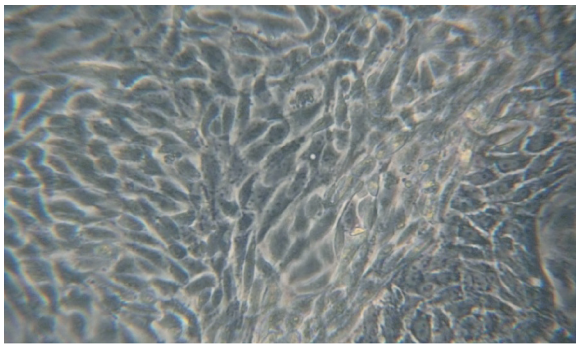


Fig. 5. PK cell culture prepared from kidney that was stored for 144 hours

an blue staining. The number of live cells prepared from 1 g of kidney tissue in all cases was on average 15–20 million.

In the course of experiments on storage of kidneys at temperatures near 0 °C the morphological characteristics of the primary cells were studied based on the age of the monolayer (Fig. 2–5).

As it is shown in Figures 2–5, in all cases the formed monolayer is represented by polymorphic cells with a minimal amount of extracellular matrix. When kidneys are stored at temperatures near 0 °C for 0–3 days and the cortical layer tissues are subsequently trypsinized (at the optimal cell seeding concentration), the monolayer is formed within 5 days; when stored for 4 to 5 days – a complete monolayer is formed in 6 days. When the kidneys were stored for 6 days, a monolayer was formed within 7 days with a predominance of normal polymorphic cells having no extracellular matrix (Table 2).

When the piglet kidneys were visually observed at storage temperatures close to 0 °C, they did not change color, had no obvious signs of necrosis, dispersion by trypsin occurred in a standard mode. However after 5-day storage of the kidneys, the proliferative activity of the cells that survived after trypsinization was 15–20% lower. The dynamics of the monolayer formation was delayed by an average of 1 day. No contamination with common microflora was observed during the experiments and the sterile primary culture was prepared.

When carrying out virological studies, all variants of the prepared cell material were suitable for determining the avirulence of semifinished products, performing the neutralization assay and FMDV isolation (Table 3).

Table 2
Characteristics of the prepared primary cell monolayer depending on the storage period of piglet kidneys at +2 °C

Kidney storage period at +2 °C, hours	Seeding concentration, cells/ml	Monolayer formation duration, days	Monolayer quality	Sensitivity to FMD virus
0	150,000	5	standard polymorphic	standard
24	120,000	5	standard polymorphic	standard
48	120,000	5	standard polymorphic	standard
72	150,000	5	standard polymorphic	standard
96	150,000	6	standard polymorphic	standard
120	170,000	6	standard polymorphic	standard
144	180,000	7	standard polymorphic	standard

Table 3
The effect of storage of piglet kidneys on the sensitivity of primarily trypsinized cell culture monolayer to FMD virus

Virus Type	Cell-cultured FMD virus titer (lg TCD_{50}/0.1 cm^3)	
	PK cell suspension, native	PK cell suspension stored for 48 hours at 0…+4 °C
A	2.25	2.00
	2.50	3.00
	2.00	2.75
	2.50	2.75
	3.00	2.75
	2.00	1.75
	2.50	2.00
	2.50	3.00
O	2.00	2.75
	2.00	2.50
Asia-1	2.25	2.00
	2.00	2.25
$M \pm m$	2.29 ± 0.09	2.46 ± 0.13

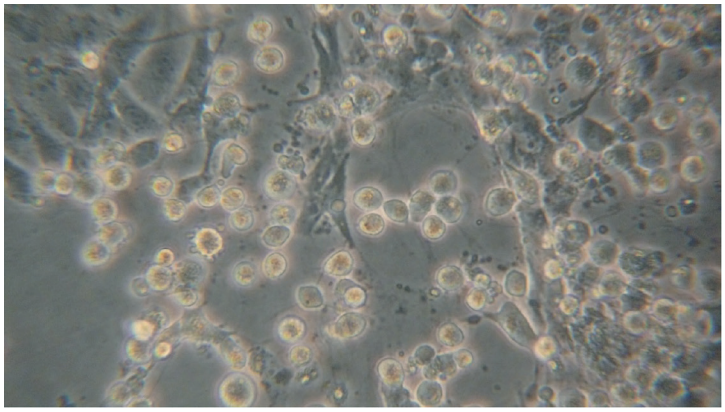


Fig. 6. Degeneration of primary PK cell monolayer when interacting with FMD virus

The sensitivity of cells to FMDV was compared with controls. The FMDV cytopathic effect on the cells prepared after the organ was stored for up to 6 days was typical and intense (Fig. 6).

The dynamics and specificity of the cytopathic effect of the FMDV virus on primary cells were tested in virological experiments. It was observed that the epithelial monolayer cells are the first to be affected. They become spherical, the process of de-adhesiveness is accompanied by appearance of numerous cytoplasmic outgrowths. After a while the surface of the cells becomes smooth, then grainy and subsequently fragmentation, i. e. the destruction of the monolayer to detritus occurs. It is assumed that epithelial-like cells of the grown monolayer are precursors of epithelial tissues lining the surface of bowman capsules that are the first to contact with viruses circulating in the body of a piglet. The descendants of stromal cells of blood vessels and organs (fibroblasts) are the last to be affected by a virus infection.

CONCLUSION

No data regarding animal kidney storage conditions for the production of primary cell cultures were found in the literature. The conducted studies suggest a possibility for a more prolonged use of donor organs. It was found that kidney storage under sterile conditions at a temperature near 0 °C does not lead to crystallization of organs. However, the viability of histotypic cells that become sensitive to FMD virus after trypsinization and obtaining a complete monolayer is preserved.

It was determined that that crystallization of the medium and the kidneys during storage at a temperature –2…–4 ° C leads to irreversible changes in trypsinized cells that do not adhere and do not form colonies after being inoculated into the culture vessels.

The experiments showed that when primary trypsinized cell suspensions are stored in a refrigerator at +4 °C for 2days, epithelial cells that are mostly susceptible to FMD virus partially die. However kidney storage at subnormal temperatures (0...+2 °C) for 2 to 6 days allows obtaining of normal populations of primary cells with high sensitivity to the virus after trypsinization.

The proposed method of piglet kidney storage allows to more rationally use donor organs for the preparation of primary cultures used in virology. This organ storage method can be recommended for preserving and transporting organs obtained in the field from domestic and wild animals.

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