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SAFETY OF PLACENTAL, UMBILICAL CORD AND FETAL MEMBRANE EXPLANTS AFTER CRYOPRESERVATION

ABSTRACT

There have been studied morphological safety and functional state of the explants of human placenta, umbilical cord and fetal membranes by vital staining techniques using the MTT and resazurin reduction tests, level of glucose in incubation medium, activity of lactate dehydrogenase and alkaline phosphatase before and after cryopreservation. It has been found that proposed program of cryopreservation allows keeping a high level of viability of the explants of placenta, umbilical cord and fetal membranes, the most informative methods of assessing the safety of these biological objects before and after cryopreservation are method of vital staining, determination of glucose content in incubation medium, MTT-test and resazurin reduction test.

KEYWORDS: placenta; umbilical cord; fetal membrane explants; cryopreservation

Cryopreservation of cells and tissues of placenta, umbilical cord and fetal membranes is becoming increasingly important with the development of modern biology and medicine. Described bio-objects can be used to produce stem cells [14, 20], in an experiment, testing of pharmaceuticals [10], auto-banking [17], for laboratory studies [7, 9]. Fetal membranes are used as scaffolds for constructions in tissue engineering [15, 16], allografts in reconstructive surgery [11, 18] and ophthalmology [13, 6]. Several studies have considered the expediency of isolating cells not from the native, but from the frozen placental material [19], which greatly simplifies the technology of low-temperature banking, eliminating the need to isolate cells in a short time after the birth of the placenta. It has been shown that cells isolated from native or cryopreserved placenta save the phenotype of multipotent mesenchymal stem cells, ability to direct differentiation and migrate in a damaged organ [4, 5]. There have been analyzed the possibility of isolating cells from cryopreserved placenta explants, evaluated the structural safety of explants [10, 21], but there have not been paid any attention to the parameters of their functional state. However, questions of assessment of placental bio-objects viability before and after cryopreservation in the parameters of morphological and functional state of preservation remain relevant for low-temperature biotechnology banking [2, 12]. In addition, methods of cryopreservation and assessment of tissue state after defrosting require optimization and standardization [3, 8].

The **aim** of the study is to evaluate the safety of the placental, umbilical cord and membrane explants after cryopreservation with the determination of the most informative tests for assessing placental structures, stored in a low-temperature bank.

MATERIALS AND METHODS

OBTAINING AND CULTURING OF EXPLANTS

Placental, umbilical cord and fetal membrane explants were obtained after normal delivery from 12 placentas of women aged 18-35 years after their informed consent in compliance with the rules of biomedical ethics in accordance with the recommendations of World Medical Association Declaration of Helsinki, Code Of Ethics Of Ukrainian Doctors, Bioethics Committee of the Institute for Problems of Cryobiology and Cryomedicine NAS of Ukraine. Placentas were delivered to the laboratory within 1-3 hours after birth in a humid chamber. Placental tissue on the maternal side was cut in fragments (up to 0.5 cm) and washed in 50 ml *DMEM* medium (*BioWest*, France) until the blood was washed off. Fetal membranes were separated into fragments (2×2 cm), vessels were removed from the umbilical cord, divided into fragments (up to 0.4 cm), washed twice in *DMEM* medium. For culturing we used 15 mg placental tissue explants, 30 mg membranes explants, or 30 mg umbilical cord explants, which were placed in 24-well cell culture plate (*SPL*, Korea), added 1 ml *DMEM* medium High Glucose w/o L-Glutamine, w/o Sodium Pyruvate (*BioWest*, France) (the growth medium) with 10% Fetal Bowine Serum (*Lonza*, Belgium) (FBS) and 1% solution of Antibiotic-antimycotic (*BioWest*, France). The ratio of an explant weight with a nutrient medium was previously chosen to match the studied parameters to the limits of sensitivity of the used methods. Explants were cultured in a CO₂ incubator (*Thermo Scientific*, USA) at 37 °C and 5% CO₂.

CRYOPRESERVATION OF EXPLANTS

For cryopreservation we used medium with 10% dimethylsulphoxide – DMSO (*Sigma-Aldrich*, USA) and 10% FBS, for which the explant in a medium was added with an equal volume of medium with a double concentration of DMSO and FBS. The exposure of biological objects to cryoprotective medium was 10 minutes at 4 °C, and then the explants were transferred to 1.8 ml cryovials (*Nunc*, USA), cooled by software freezer CP-10 (IPCC NAS Ukraine) at 1 dg/min to -70 °C, and then immersed in liquid nitrogen for storage at -96 °C for 1-7 days. Defrosting was carried out at 37 °C in the water bath (*Micromed*, Ukraine). After defrosting the tissue was washed off cryoprotectants in 15 ml culture medium for 10 minutes. As a positive control we used native explants, as a negative one we used explants in medium without adding DMSO after direct immersion in liquid nitrogen and subsequent defrosting.

THE STUDY OF MORPHOLOGICAL SAFETY OF THE EXPLANTS

Histological examination of the samples was carried by standard methodology [1] by staining with hematoxylin and eosin. Each preparation was studied in 10 fields of view. Vital staining was performed by standard methods using 0.01% solution of neutral red (*PanEco*, Russia) and 0.4% solution of trypan blue (*PanEco*, Russia) for 10 minutes at 37 °C, followed by washing with phosphate-buffered saline (PBS) (*BioWest*, France). While preparing native preparations of fetal membranes we separated amniotic and chorionic membranes to study the state of the amniotic epithelium and used microscope Delta Optical NIB-100 (Poland) and software ToupView V.3.7.1460.

MTT-TEST

In 24-well culture plate we placed 15 mg placental tissue explants, 30 mg fetal membranes explants or 30 mg umbilical cord explants, added 0.5 ml culture medium, 75 µL MTT solution (*PanEco*, Russia) to PSB at a concentration of 5 mg/ml. They were incubated for 4 hours in a CO₂ incubator at 37 °C with 5% CO₂; the medium was removed; formazan was being extracted from the explant for 30 minutes at 37 °C, adding 1 ml of 96% ethyl. Thereafter, there was evaluated the optical density (OD) of the supernatant, dyed with formazan, using a spectrophotometer PV 1251C (*Solar*, Belarus) at a wavelength of 590 nm.

RESAZURIN REDUCTION TEST

In 24-well culture plate we placed 15 mg placental tissue explants, 30 mg fetal membranes explants or 30 mg umbilical cord explants added 1.0 ml culture medium, 200 µL resazurin solution (*Sinbias*, Russia) in PBS at a concentration of 0.15 mg/ml. They were incubated for 24 hours in a CO₂ incubator at 37 °C. OD was measured with a spectrophotometer PV 1251C (*Solar*, Belarus) at a wavelength of 570 nm.

DETERMINATION OF THE ACTIVITY OF LDH, ALKALINE PHOSPHATASE AND GLUCOSE UPTAKE FROM THE ENVIRONMENT

The explants were cultured for 24 hours in 1 ml culture medium according to the above procedure. We used commercial kits “Lactate-L Liquid 100” (*Erba Lachema s.r.o.*, Czech Republic), “Alkaline phosphatase – MEG Liquid 50” (*Erba Lachema s.r.o.*, Czech Republic), “Liquid Glucose 500 C” (*Erba Lachema s.r.o.*, Czech Republic) according to manufacturer’s instructions. OD was measured with a spectrophotometer PV 1251C (*Solar*, Belarus).

Statistical processing of data was performed using Student’s test to assess histological changes in fields of vision and U-Mann-Whitney test for biochemical studies at $p < 0.05$. We used the software Statgraphics V 2.1.

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RESULTS AND DISCUSSION

In studying positive control preparations of placenta explants, stained with hematoxylin and eosin, we observed a typical structure of chorionic villi (**Fig. 1a**). The negative control showed characteristic cryodamages: irreversible structural changes represented by the destruction of terminal villi, destruction of collagen fibers, connective stroma breaks, desquamation of trophoblast and severe pyknosis of the cells’ nuclei (**Fig. 1g**). In defrosted placental samples their general structural form of preparations was similar to the positive control. In most cases there were no pathological changes in the terminal villi of the placenta. Occasionally there was swelling of collagen fibers of connective tissue of villi stroma and breaks of mesenchyme, as well as moderate constriction of hemocapillars. Moreover, this study revealed no damage to vascular endothelium. Trophoblast cells had dense nuclei, there was observed their desquamation. Cellular elements of the connective tissue stroma of the villi remained without visible changes (**Fig. 1d**). Earlier in the literature typical cryodamages were evaluated descriptively [10], we attempted to evaluate them quantitatively to assess the extent of cryodamage, the results are presented in the table.

In the study of the vital-stained preparations of native placental explants there were identified single cells stained with trypan blue and neutral red, and single cases of dye penetration into the stroma in a greater part of the preparation, caused, in our opinion, by a violation of the integrity of the trophoblast, its desquamation (**Fig. 1, b, c**). In the negative control we observed staining with trypan blue and neutral red of both cellular elements trophoblastic and stromal (**Fig. 1, h, i**). In defrosted samples we observed single stained cells, trypan blue penetration into the

 **Table 1.** Histological changes in the placenta explants after cryopreservation (n = 120, at 10 field of view in 12 samples)

	SAMPLE	POSITIVE CONTROL	NEGATIVE CONTROL	PROGRAMMED FREEZING
MORPHOLOGY CHARACTERISTIC				
Villi without pathology, % (M ± m)		93.1±7.1	–	75.5±8.2**
Destructed villi, % of villi (M ± m)		2.3±0.4	89.5±5.7*	5.1±4.5**
Trophoblast desquamation, % of villi (M ± m)		–	74.1±4.1*	11.4±1.7**
Nuclei pyknosis, % (M ± m)		2.9±0.5	85.4±7.4*	17.3±5.8**
Mesenchyme breaks, % of villi (M ± m)		2.6±0.7	87.5±5.7*	24.0±2.8**

Notes:
* – $p < 0.05$ in comparison with the positive control group;
** – $p < 0.05$ in comparison with the negative control group.

stroma, which can be explained by desquamation of trophoblast revealed by histological examination (Fig. 1, e, f).

Analysis of placental explants using the MTT-test showed a significant decrease in OD of negative control. OD indicators of the positive control and defrosted placental explants did not differ (Fig. 2a), indicating the preservation of metabolic activity. The study of resazurin reduction revealed that OD of defrosted placental explants is significantly lower than OD of the positive control, but significantly higher than the OD of the negative control (Fig. 2, b). The study of glucose level in the incubation medium of placental explants showed that the content of glucose in the incubation medium of explants corresponds to the content of the negative control, declared by the manufacturer, i.e. the tissue cannot be considered as viable. In the incubation medium of the positive control sample glucose level was significantly reduced, and in the incubation medium of the defrosted sample it tends to decrease compared with the positive control, which indicates an intensification of oxidative phosphorylation after cryogenic treatment (Fig. 2, f). At the same time, the glucose concentration remains at a rather high level, which, in our opinion, indicates that its concentration in the medium is not a limiting factor for the cultivation of explants for 24 hours. At the same time there was demonstrated a decrease in alkaline phosphatase activity after cryopreservation (Fig. 2, d). Significant changes in lactate dehydrogenase activity were not established (Fig. 2, c), which may be due to the removal of the excluded LDH in the process of explants washing.

Morphological study of the fetal membranes after cryopreservation (Fig. 3, d) showed no damage of the amniotic membrane epithelium. The collagen fibers of the connective tissue of chorionic membranes spongy layer were thickened, with fibrocytes widely spaced between them. Trophoblasts had quite compacted cytoplasm and small nuclei, macrophages were detected between them. There were determined individual trophoblasts in fibrinoid. The changes were more expressed in negative control samples (Fig. 3g). There was a destruction of the amniotic epithelium, thickening of the collagen fibers of fibrous connective tissue of chorionic membrane spongy layer with occasional fibrocytes. Trophoblast layer was thinned, its cells with pycnotic nuclei were more grouped, macrophages were not determined among them. In this case the morphological changes in the membranes were less marked than in the villi, which may be due to a denser connective stroma and fewer cells.

At the vital staining of the positive control samples of amniotic membrane with neutral red and trypan blue $4.1 \pm 2.1\%$ cells were stained (Fig. 3, b, c, $n = 12$), after cryogenic treatment – $23.8 \pm 5.3\%$ cells (Fig. 3, e, f, $n = 12$). Vital staining techniques enabled to count the number of viable cells. In the negative control samples all cells were stained, nuclei in general, that, in our opinion, demonstrates a significant damage to cell membranes and flush of the dye (Fig. 3, h, i). Vital staining of chorionic membranes was not informative because the multilayer structure of the tissue did not allow visualizing the dye interaction with individual cell structures, unlike villi and amniotic membrane, where individual cells were visualized.

Results of the MTT test (Fig. 2, a) and resazurin reduction test (Fig. 2, b) differed significantly for negative control samples, positive control and samples of defrosted fetal membranes. This difference in the metabolic activity of fetal membrane explants and placental explants is due to the greater homogeneity of fetal tissue membranes. Allocation of compatible explants from placental tissue is associated with technical difficulties: different villi with different content of fibrinoid can get in an explant that affects their activity, while the structure of the membranes is homogeneous in case of full separation from the uterus and preservation of their thickness. For this bio-object there were got reliable data of changes in AP activity (Fig. 2, d) and glucose concentration (Fig. 2, e) in incubation medium that suggests preservation of the tissue after applied programs of cryopreservation. LDH activity (Fig. 2, f) did not change significantly.

At cryopreservation of umbilical cord explants there were not expressed morphological changes. It is attributed to a large number of intercellular substance rich in hyaluronic acid. There was noted pyknosis of nuclei and some extension of intercellular spaces. The results of vital staining of umbilical cord explants were not informative because of the difficulty of obtaining single-layer preparations from gel biomaterial – Wharton's jelly. Data of MTT-test (Fig. 2, a), resazurin reduction test (Fig. 2, b) and the concentration of glucose in the incubation medium (Fig. 2, f) differed significantly for defrosted samples, samples of positive and negative controls. The study of enzymatic activity of umbilical cord explants did not provide significant differences, due to the small number of cells in the samples and features of the intercellular substance. Moreover, alkaline phosphatase is more specific enzyme for trophoblast cells that exist in the placental and membrane tissue, but do not in Wharton's jelly of the umbilical cord.

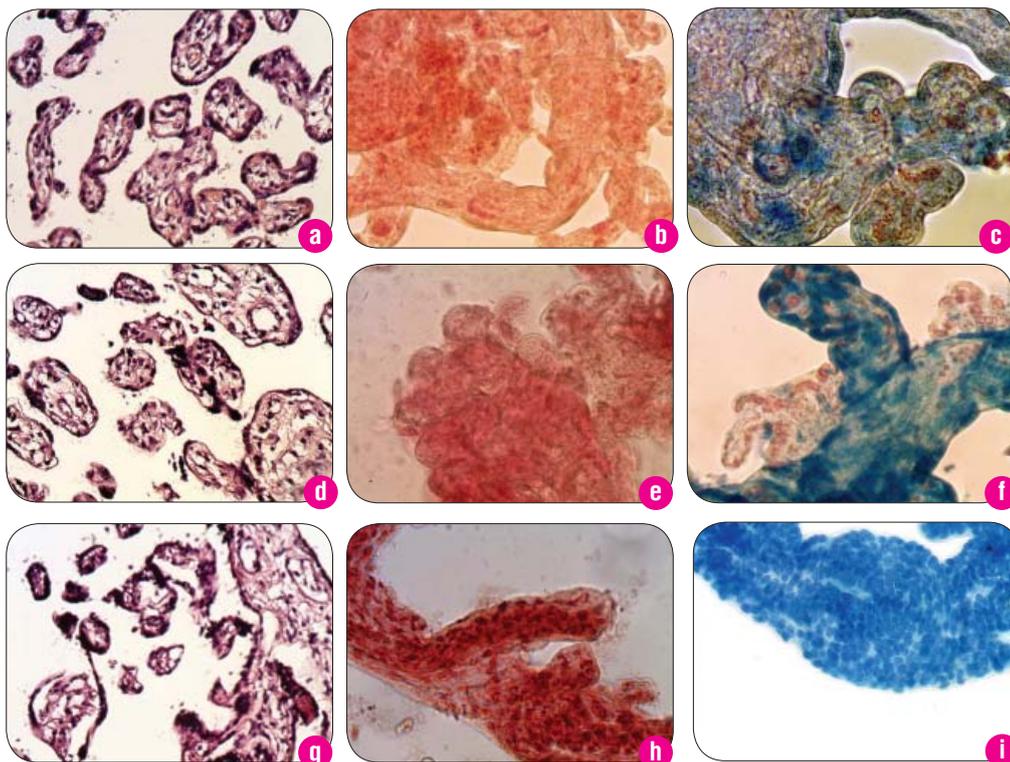
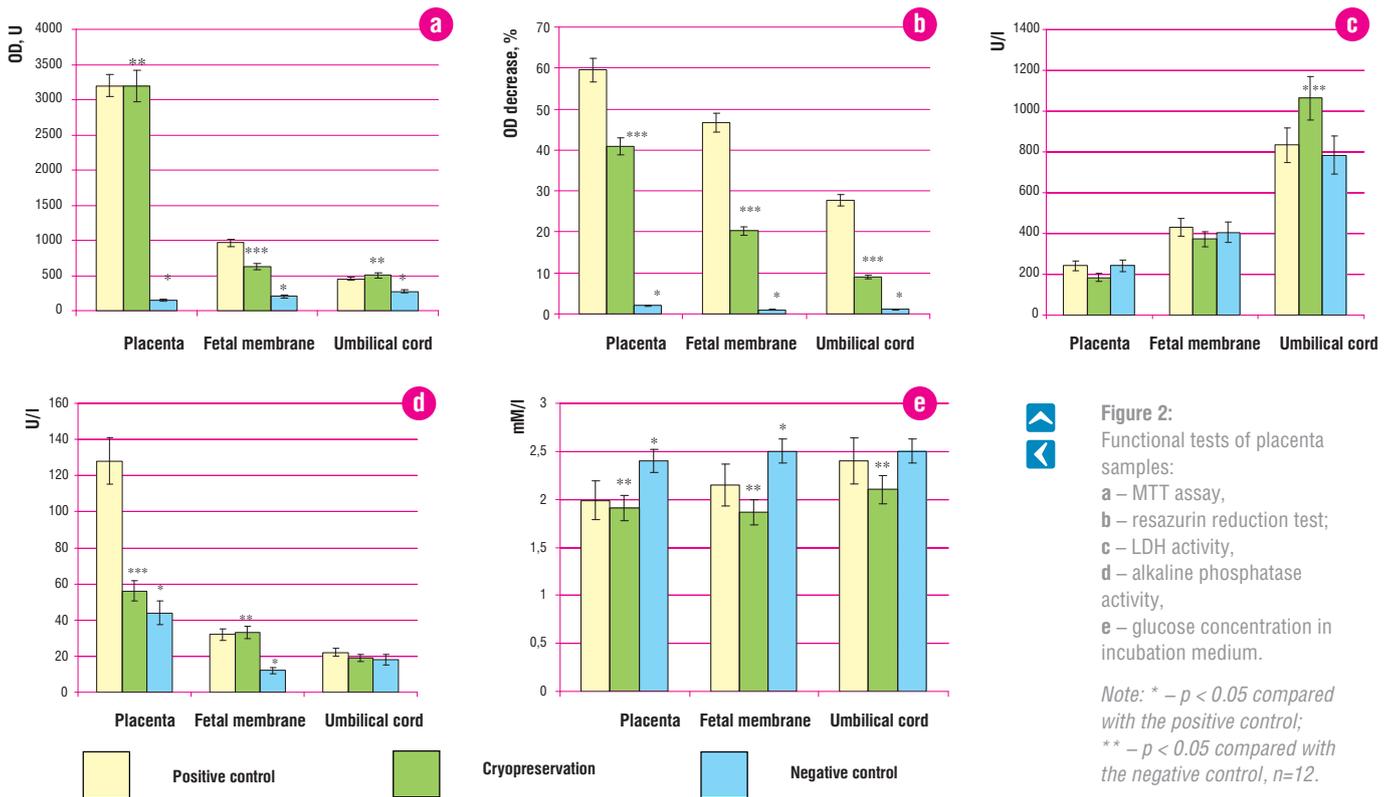


Figure 1: Histology sections of placenta explants: a, b, c – positive controls; d, e, f – after cryopreservation; g, h, i – negative controls. Light microscopy, hematoxylin and eosin staining (a, d, g); neutral red staining (b, e, h); trypan blue staining (c, f, i), $\times 400$.



CONCLUSIONS

Thus, cryopreservation by the developed technique allows saving the structure and metabolic activity of placental tissue, umbilical cord and fetal membrane explants. The most informative methods of assessment of studied explants, as objects of low-temperature bank, are MTT-test, resazurin reduction test and absorption of glucose from the incubation medium. Histological study is more informative for placental villi. Vital staining can be used as an express test for tissues, which individual cells are visualized in native preparations: placental villi and amniotic membrane. Study of LDH and ALP activity does not give reliable results that can be caused by washing of explants.

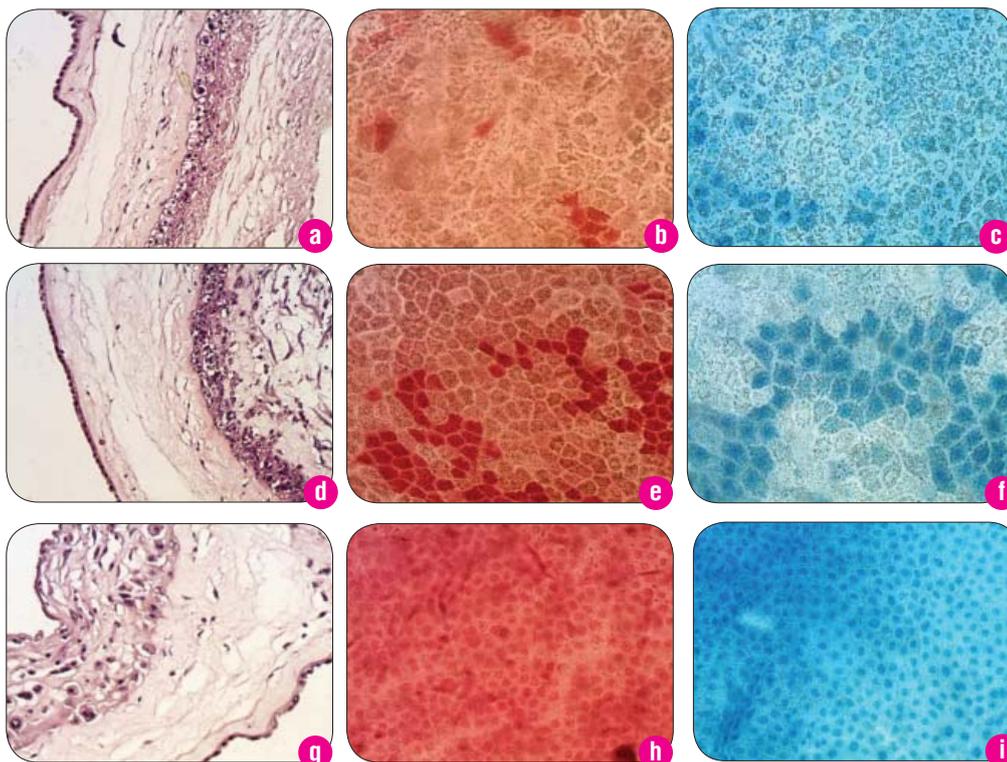


Figure 3: Histology sections of fetal membranes:
a, b, c – positive controls;
d, e, f – after cryopreservation;
g, h, i – negative controls. Light microscopy, hematoxylin and eosin staining (**a, d, g**), $\times 200$; neutral red staining (**b, e, h**), $\times 400$; trypan blue staining (**c, f, i**), $\times 400$.

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