Development and Introduction of Production Standards for Cell Products of Mesenchymal Origin

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The results of the development and introduction of universal production standards for cell products of mesenchymal origin are presented: technology for obtaining and culturing of primary cell cultures from human postnatal organs and tissues, cell product quality and safety control procedures, methods for cell product storage and transportation, and the necessary files.

Key Words: *cell products; certification; transportation; production standardization; standard and legal provision*

Isolation and culturing of mesenchymal cells from human postnatal organs and tissues forms the base for obtaining cell products used in therapy of diseases of various origins. Today there are no standard and legal regulations for the use of cell products or universal standards for their manufacture in the Russian Federation. Amendments to existing legislation and departmental regulations, regulating the use of medical cell technologies, should be made with consideration for the data accumulated by Russian specialists. Studies aimed at development and introduction of production standards for cell products of different origin have been carried out at Laboratory of Medical Cell Technologies of Russian State Medical University during recent 3 years. This paper presents the results for cell products of mesenchymal origin.

Technological scheme for the production of cell products includes the following procedures:

- cell isolation from biopsy material;
- cell passages *in vitro*;
- long-term cryopreservation of cells;
- control of cell culture viability;
- screening for infectious agents;
- certification of cell materials;
- transportation of cell materials to consumers.

Method for isolation of human skin fibroblasts. Adult donor skin fragments are crushed and incubated for 2 h at 37°C in 0.1% collagenase-I (Gibco). The resultant suspension is precipitated by centrifugation, the precipitate is resuspended in growth medium, after which the contents is transferred into Petri dishes (Greiner) and cultured until formation of 80% confluent monolayer (Fig. 1, *a*), after which the cells are transferred into culture flasks (Greiner).

Method for isolation of human umbilical blood mesenchymal cells. Human umbilical tissues after normal delivery at weeks 39-40 of gestation are washed in Hanks' solution (PanEko) and after a short mild mechanical treatment incubated with 0.1% collagenase-I solution (Gibco) for 30 min at 37°C. The resultant cell suspension is centrifuged, the precipitate is resuspended in growth medium, the contents is transferred into Petri dishes (Greiner) and cultured until formation of 80% confluent monolayer (Fig. 1, *b*), after which the cells are transferred into culture flasks (Greiner).

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Method for isolation of mesenchymal cells from human placenta. Human placental amnionic tissues after normal delivery at weeks 39-40 of gestation are washed in Hanks' solution (PanEko) and after a short mild mechanical treatment incubated for 30 min at 37°C with 0.1% collagenase-I solution (Gibco). The cell suspension is then centrifuged and the precipitate is resuspended in growth medium. The contents is transferred into Petri dishes (Greiner) and cultured until the formation of 80% confluent monolayer (Fig. 1, *c*), after which the cells are transferred into culture flasks (Greiner).

Method for isolation of mesenchymal cells from human bone marrow. Cells obtained by separation of adult donor bone marrow aspirate in Ficoll-urografin density gradient are suspended in a concentration of 10^4 cell/ml in growth medium in culture flasks and cultured until formation of 80% confluent monolayer (Fig. 1, *d*), the growth medium being replaced every 3 days.

Cell passages in vitro are carried out by the standard method in culture flasks (Greiner) in a CO_2 incubator (37°C, 5% CO_2, 80% humidity). Growth medium composition: DMEM/F12, 10% fetal calf serum, 100 U/ml penicillin, 100 U/ml streptomycin, 2 mM glutamine, 1 mM sodium pyruvate, 10 ng/ml β-FGF (Gibco). The medium is replaced twice weekly. After formation of 80% confluent monolayer the cells are removed using 0.25% trypsin solution with versen (1:1, PanEco) and suspended in 1:3 proportion. If the cells are intended for medical use, cultures after passage 5 are not recommended, as a detailed cytogenetic analysis of older cultures should be carried out for detecting possible karyotype abnormalities.

Long-term cryopreservation of cells is carried out by the standard method in Dewar's flasks with liquid nitrogen (-196°C). The cells are frozen in cryotubes (Greiner) using MrFroster programmed freezer (Greiner). The composition of cryoprotec-

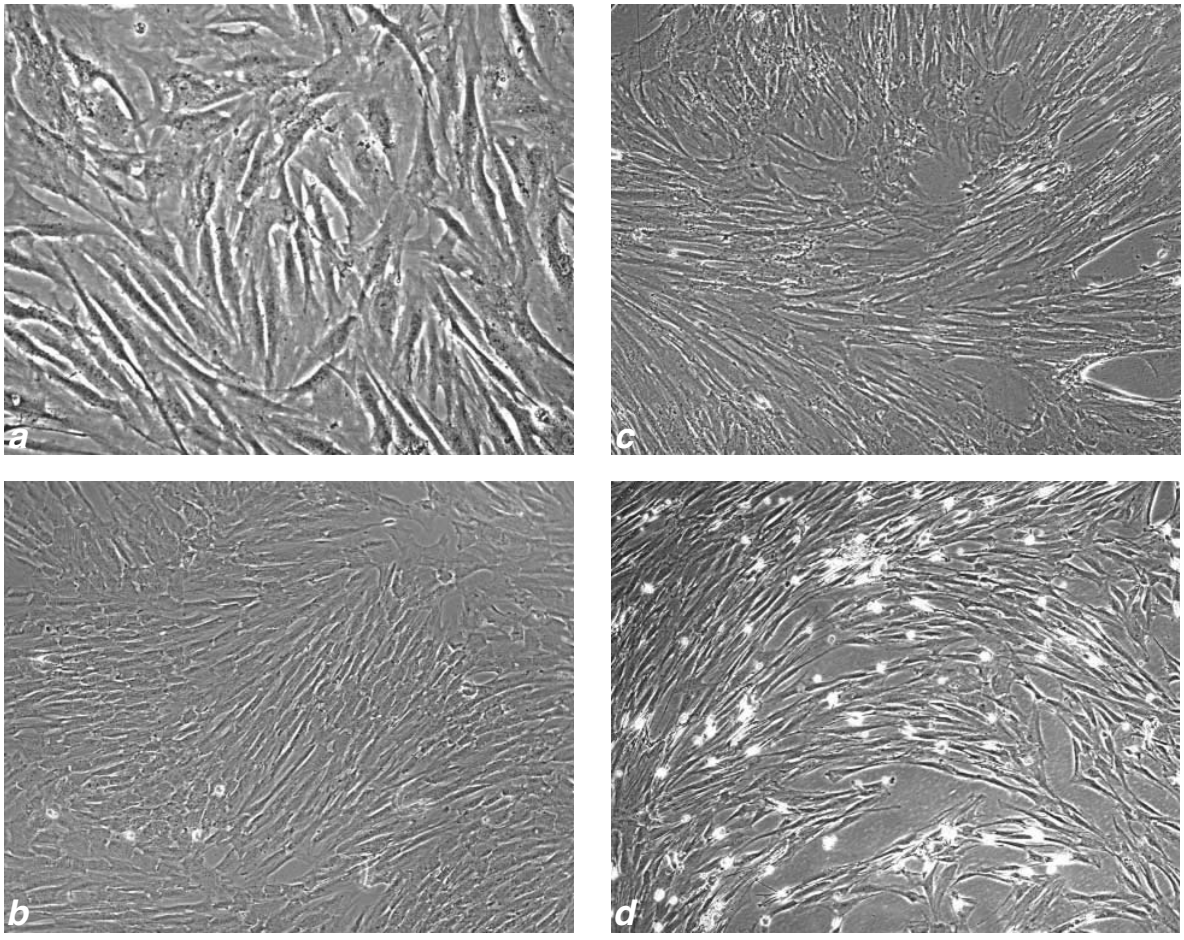

Fig. 1. Mesenchymal cells in culture. *a*) skin fibroblasts (the magnification is greater than for other cells); *b*) umbilical mesenchymal cells; *c*) placental mesenchymal cells; *d*) bone marrow mesenchymal cells.

tive medium is as follows: fetal calf serum (Gibco) and 10% dimethylsulfoxide (PanEco).

Frozen cells are fragile, poorly stand pipetting, shaking, and hence should be handled with particular care during defrosting. Cryotubes with frozen cells removed from liquid nitrogen are placed in water bath (37°C) and left until complete thawing of ice. All manipulations on cell defrosting are carried out as quickly as possible (cells should not be left long in defrosted cryopreserving agent). The contents of the cryotube is transferred into a flask with growth medium, which is placed into a CO_2 incubator. After 24 h, growth medium containing cryopreserving agent is replaced by a fresh portion.

Cell viability control is carried out daily (visually; all cells of the above types should have characteristic fibroblast-like morphology; Fig. 1). Trypan blue staining or MTT test [5-7] are used for evaluation of cell viability, if necessary.

Screening for infectious agents [2,3] starts from testing the donors, as many infectious diseases (HIV-1, HIV-2, hepatitis C) preclude the possibility of biological material donation.

Standard testing of cell material after *in vitro* passages includes testing (by the PCR method) of all types of mesenchymal cells for the absence of the following infectious agents [4]:

- types 1 and 2 immunodeficiency viruses (*Retroviridae*, *Orthoretrovirinae*, *Lentivirus*);
- hepatites B (*Hepadnaviridae*, *Orthohepadnavirus*) and C (*Flaviviridae*, *Hepacivirus*) viruses;
- types 1 and 2 herpes simplex viruses (*Herpesviridae*, *Alphaherpesvirinae*, *Simplexvirus*), cytomegalovirus (*Herpesviridae*, *Betaherpesvirinae*, *Cytomegalovirus*), Epstein—Barr virus (*Herpesviridae*, *Gammaherpesvirinae*, *Lymphocryptovirus*);
- *Treponema pallidum* (*Spirochaetales*, *Spirochaetacea*), *Mycoplasma spp.* (*Mycoplasmatales*, *Mycoplasmataceae*), *Toxoplasma spp.* (*Eucoccidiorida*, *Sarcocystidae*).

Skin fibroblast cultures should be also tested for human papillomaviruses (*Papillomaviridae*), genuses *Betapapillomavirus* (types 5, 9, 49, cand92, cand96) and *Gammapapillomavirus* (types 4, 48, 50, 60, 88).

Mesenchymal cell cultures originating from the umbilical cord and placenta should be additionally tested for urogenital tract infectious agents: human papillomaviruses (*Papillomaviridae*), genus *Alphapapillomavirus* (types 2, 6, 7, 10, 16, 18, 26, 32, 34, 53, 54, 61, 71, cand90), *Ureaplasma spp.* (*Mycoplasmatales*, *Mycoplasmataceae*), *Chlamydia spp.* (*Clamydiales*, *Chlamydiaceae*).

No additional testing of bone marrow mesenchymal cells is carried out, as common requirements to donors of blood and its components are valid for them as well.

Certification is an obligatory procedure, documenting the results of cell material composition, quality, and safety control [1-3]. The passport of cell material includes four obligatory blocks:

1) common information: scientific name of the cells, source of cell material, sample layout, information about cell donor;
2) cell viability, appearance of the sample; cell viability (%), sterility, pH value, sample color and consistency, total cell concentration (cell/ml), volume of sample, time of delivery, useful life, storage conditions;
3) results of sample testing for the absence of infectious agents from the list common for all cell types;
4) results of sample testing for the absence of infectious agents specific of this cell type.

A reference passport for umbilical mesenchymal cells is presented (Fig. 2). It contains all four listed blocks, a conclusion on the possibility of medical use; the passport is certified by the signature of responsible executive and the stamp of the institution.

Cell product transportation. Cell products for medical use can be got only at certified centers [1-3]. Qualified staff and appropriate equipment are obligatory for their functioning. It is impossible to create such centers at any clinical department, and therefore the problem of cell product transportation is inevitable.

Transportation causes unambiguous effects on the cell status, as the cells during transportation are exposed to many factors of different nature. Studies aimed at optimization of cell product transportation resulted in development of media, allowed for medical use, in which cell cultures can be stored and used as intended without additional manipulations. For autologous replacement cell therapy, the cell product is transported in saline with 10% patient's serum [6]. For allogenic replacement cell therapy by cell products from human umbilical cord or placenta, the cultures are transported in transport medium based on NCTF-135 (Filogra). Medium NCTF-135 is a complex of 6 groups of active components: vitamins (A, C, E, D, B, K, *etc.*), nucleic acids, minerals, amino acids, coenzymes, and antioxidants. Initially, NCTF-135 was used in cosmetology for mesotherapy. If cell material is used in cosmetology or trichology, NCTF-135 amplifies the effect of cell therapy.

Analysis of cell viability in transportation vessels of different types and of wishes of practitioners

PASSPORT OF CELL MATERIAL
Human umbilical mesenchymal cells

Parameter	Normal value according to technological standards	Test results
Content of viable cells, %	At least 80%	
Appearance	Cell suspension in transparent pink liquid	
pH	7.0-7.4	
Preparation sterility (bacteria)	No bacteria in preparation	
Preparation sterility (microscopic fungi)	No microscopic fungi in preparation	
Presence of hepatitis B virus DNA in cells	No hepatitis B virus DNA in cells	
Presence of hepatitis C virus RNA in cells	No hepatitis C virus RNA in cells	
Presence of type 1 human immunodeficiency virus provirus DNA in cells	No type 1 human immunodeficiency virus provirus DNA in cells	
Presence of type 2 human immunodeficiency virus provirus DNA in cells	No type 2 human immunodeficiency virus provirus DNA in cells	
Presence of type 6 human immunodeficiency virus provirus DNA in cells	No type 6 human immunodeficiency virus provirus DNA in cells	
Presence of type 1 herpes simplex virus DNA in cells	No type 1 herpes simplex virus DNA in cells	
Presence of type 2 herpes simplex virus DNA in cells	No type 2 herpes simplex virus DNA in cells	
Presence of Epstein—Barr virus DNA in cells	No Epstein-Barr virus DNA in cells	
Presence of papillomavirus DNA in cells	No papillomavirus DNA in cells	
Presence of chlamydial DNA in cells	No chlamydial DNA in cells	
Presence of ureaplasma DNA in cells	No Ureaplasma DNA in cells	
Presence of cytomegalovirus DNA in cells	No cytomegalovirus DNA in cells	
Presence of toxoplasma DNA in cells	No Toxoplasma DNA in cells	
Presence of mycoplasma DNA in cells	No mycoplasma DNA in cells	

Cell material delivered, volume _____

Cell concentration _____

Conclusion

Cell material (human umbilical mesenchymal cells) _____

can be used with medical purpose. Useful life of preparation: 24 h from the moment of delivery at +4-+10°C.

Date and time of delivery: _____

Responsible executive Name

Date

Stamp of Institution

Fig. 2. A passport of cell material from human umbilical cord.

of different profiles resulted in the choice of the optimal package, preserving the initial sterility, viability (the material of the tube was tested for toxicity), and concentration of cell material (no adhesion to the tube walls). We selected the Vacutainer tubes (Becton Dickinson), routinely used in

systems for vacuum collection of blood. The use of Vacutainer with the above-described transportation media allows medical use of the sample without additional manipulations.

Thermal regimen of transportation (2-8°C) and thermoinsulating materials were selected: for transportation no longer than 12 h the tubes with cell material are placed in a Thermos with ice; for longer transportation the Thermos with ice is additionally put into a refrigerator bag with frozen coolants. Adherence to these transportation regulations guarantees 80% viability of cells in a sample.

Introduction of production standards for cell materials [2]. The entire standardization base, from Federal laws to departmental instructions, is in need of revision. For example, the notion "cell technologies" is not mentioned in the Laws of the Russian Federation "On Transplantation of Human Organs and (or) Tissues" (No. 4180-1 of 22.12.1992, version No. 15-FZ of 09.02.2007), "On Donation of Blood and Its Components" (No. 5142-1 of 09.06. 1993, version No. 176-FZ of 24.12.2002), "Fundamentals of the Russian Federation Legislation on the Citizens Health Protection" (No. 5487-1 of 22. 07.1993, version No. 258-FZ of 29.12.2006), "On Drugs" (No. 86-FZ of 22.06.1998, version No. 231-FZ of 18.12.2006), "On Provisional Prohibition of Human Cloning" (No. 54-FZ of 20.05.2002), which creates the possibility of uncontrolled use of products of these technologies. Amendments to these legislative acts should cover all aspects of the use of methods and products of medical cell technologies and bring them into the sphere of legal regulation. There is no regulation and permission system (including the state registration, quality control, and surveillance) in the sphere of cell material production and use; this system should be created as quickly as possible, as the problem is really pressing.

The scientific community, in turn, should adopt universal standards for the production and use of cell preparations in biology and medicine by documenting them in a series of methodological aids, recommendations, and instructions. Obviously, the Russian specialists have ample material for this work. For example, many-year experience gained at Laboratory of Medical Cell Technologies of Russian State Medical University in development of approaches to standardization of technology of cell material production can serve as the base for development of standardization base, regulating safe use of cell cultures in preclinical and clinical studies.

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REFERENCES

1. V. V. Burunova, Yu. G. Suzdaltseva, A. V. Voronov, *et al.*, *Proceedings of Conference of Russian State Medical University*, Moscow, May 30-31, 2007 [in Russian], Moscow (2007), P. 62.
2. V. V. Burunova, Yu. G. Suzdaltseva, I. B. Cheglakov, *et al.*, *Collected Theses of Reports of British-Russian Meeting in Collaboration with European Committee "Stem Cells: Legislation, Studies, and Innovations. International Prospects of Collaboration"*, Moscow, March 16-17, 2007 [in Russian], Moscow (2007), P. 8-9.
3. V. V. Burunova, Yu. G. Suzdaltseva, and K. N. Yarygin, *Proceedings of Conference of Russian State Medical University*, Moscow, May 24-25, 2006 [in Russian], Moscow (2006), P. 64-65.
4. D. K. L'vov, ed., *Medical Virology. Manual* [in Russian], Moscow (2007).
5. M. Yu. Shchelkanov, I. B. Sakhuriya, V. V. Burunova, *et al.*, *Immunologiya*, No. 1, 37-41 (1999).
6. K. N. Yarygin, Yu. G. Suzdaltseva, V. V. Burunova, *et al.*, *Kletochn. Tekhnol. Biol. Med.*, No. 1, 53-59 (2006).
7. T. Mosmann, *J. Immunol. Methods*, **65**, No. 1-2, 55-63 (1983).