Experimental Validation of Embryonic Cardiomyocyte Implantation in Complex Therapy of Myocardial Failure

Y. L. SHEVCHENKO
Russian Military Medical Academy, St. Petersburg, Russia

Abstract

Embryonic cardiomyocytes, due to their high proliferative potential and low immunogenicity, are of great interest as myogenic material for substitution-cell therapy in the restoration of impaired myocardial function. Cultivated embryonic cardiomyocytes obtained by enzymatic suspension of fetal tissue are able to reorganize cell junctions, not only amongst themselves with the formation of a functional syncytium but also with differentiated cardiomyocytes in tissue culture. A series of experiments was conducted with laboratory animals to confirm results that had been obtained in vivo. The condition of embryonic cardiomyocytes implanted into recipient myocardium was evaluated by comparing initial histological dynamics and electrocardiographic indices with those registered at various intervals over the course of the experiment. Changes in the electrocardiographic indices of animals after injection of a suspension of embryonic cardiomyocytes into both intact- and injured myocardium confirm the possibility not only of building exogenic cardiomyocytes in recipient myocardium structure but also of stimulating reparative processes in differentiated recipient heart cells in cases of ischemia or injury. The regenerative effect of embryonic cell implantation is provided by the substitution of missing cambial elements and by the ability of fetal tissue to synthesize biologically active substances.

Key Words

Embryonic cardiomyocytes, implantation of cultures, ischemia, myocardial infarction

Introduction

It is a generally accepted fact that the myocardium of adult mammals does not have regenerative capacity. Although some papers published in recent years describe the possibility of stimulating partial regeneration of injured myocardial tissue in tissue cultures [14, 16,28], the process of alteration is irreversible for most injured heart muscle and ends with the growth of connective tissue in place of necrotic tissue. Development of compensatory hypertrophy is possible; polyploidy in the cardiomyocytes is also a possibility [3]. But the inevitable result is a decrease in heart function, culminating in heart failure. It is not always possible to correct myocardial function with medication alone. Assuming a long history of disease, a gradual increase in connective-tissue volume with respect to healthy myocardium leads to more frequent heart failure, requiring a continuous increase in cardiotropic-medication dosages and the inclusion of new medications into the therapy. But each of the medications has a certain therapy limit. To date, cardiac surgeons have proposed several methods for correcting cardiac muscle dysfunction, such as heart transplantation, artificial heart implantation, and dynamic cardiomyoplastics. Despite specific advantages and theoretically unbounded possibilities, wide application of each of these methods encounters fundamental difficulties that are an excellent catalyst for improving of present approaches and searching for fundamentally new approaches to correcting disturbances in myocardial function.

In recent years, a method for recovering function in an injured organ by the use of cell and tissue cultures has

found practical application. This became possible due to investigations which revealed that a cell culture is a unique model of the entire organ [24,27]. This method is applicable in surgical endocrinology: namely, allotransplantation of cultures of pancreal island cells is used in the treatment of diabetes [12]. In this case the authors note that cultivation of cells for transplantation leads to a decrease in their antigenicity [19] as well as to the death of leukocytes initiating an immune-system reaction [21]. At present, extracorporeal growth of donor-isolated hepatocytes is used in the treatment of various forms of liver failure [2,4,13]. At the same time, methods of donor-isolated hepatocyte transplantation have been developed in detail, and their effectiveness in the treatment of congenital- and acquired hepathorrhagia has been shown [1,26]. The implantation of cultured epithelium is used in the treatment of thermal skin injury and attains clinical results that could not be achieved before [6,17].

Studies of embryonic cell cultures are extremely widespread due to the cultures' high proliferative potential. Clinical experience is accumulated by using cultured tissue from fetal pancreas in the treatment of diabetes [9,10,21] and the consequences of protein deficiency [20]. Researchers working with nerve tissue have achieved not only restoration of conduction and reconstruction of brain macrostructure [5] but also recovery of some complex functions [15]; additionally, implantation of embryonic myoblasts into somatic muscles has been effective in remedying genetically-caused atrophy, as was hypothesized. Clinical experience with the use of tissue-culture techniques for artificial fertilization is summarized in [23]. The prototype for all these studies was the transfusion of blood and its components, which is now a standard operation [8].

Some researchers regard cell-culture implantation as a method that is to some extent an alternative to organ transplantation. It is not surprising, then, that many scientists attempting to treat different myocardial pathologies pay attention to cell technologies [22,25]. With a great number of variants used in studies on function recovery in injured myocardium, embryonic cardiomyocytes are of great interest as a myogenic material.

Materials and Methods

Obtaining a suspension of fetal myocardial cells.

Myocardial cell cultures were obtained from the fetal ventricle of a rat on the 15 to 19th day of pregnancy. We used the following solutions in the laboratory:

1. Breeding ground [10% embryonic serum + DMEM medium + buffer Hepes 1M (pH = 7.2-7.4; 1 ml / 50 ml of substrate) + hentamycyn (40 µg / ml of substrate)];
2. Hencks solution (without Ca) + hentamycyn;
3. Isotonic phosphate buffer PBS + hentamycyn;
4. Proteolytic enzymes (0.15% solution of tripsin + 0.05% solution of collagenaze + hentamycyn).

Fetuses were removed from pregnant rats under ether anaesthesia after decapitation and laparotomy, cleared from pericarps, decapitated and thoracotomied. Blood was washed out of the removed hearts three times in solution II; the vascular bundle and atria were excised. If necessary, tissues were prepared using binocular MBS-1. Ventricular myocardium was cut up into 1x1 mm pieces, soaked in solution III, flooded with proteolytic enzyme solution IV and set to rocking for 20 minutes with a frequency of 3 a.u. [Wstrasarka universal type WU-4 "Premed" (Poland)] in a thermostat at a temperature of 37ºC ["Terbiol"-320]. Oversedimentary fluid containing a large amount of necrotic cells and cell material was poured after the first step of dissociation. The tissue was again flooded with proteolytic solution IV and placed under the same conditions for 60 minutes. Then supernatant was collected thoroughly into a centrifuge tube. The remaining tissue generally dissociated completely after accurate pipetting with a new solution IV and was mixed with the collected supernatant. To deactivate proteolysis, 2 ml of solution I were added to the cell suspension, and obtained cells were washed three times in solution III by successive centrifuging for 5 minutes at a time with a frequency of 1000 revolutions / min.

Long-term cultivation of cells

For longer cultivation, obtained cells were planted either into Carrel glasses, which are more convenient for observation of native cultures, or onto layer glasses of Petri dish if subsequent fixation and histological investigations were needed. In both cases, the cells were cultivated in the breeding ground (solution I) in CO_2-incubator conditions at 5% CO_2 and 37ºC. The substrate was changed on the third day to remove dead and loose cells, and then after every 3-4 days of cultivation.

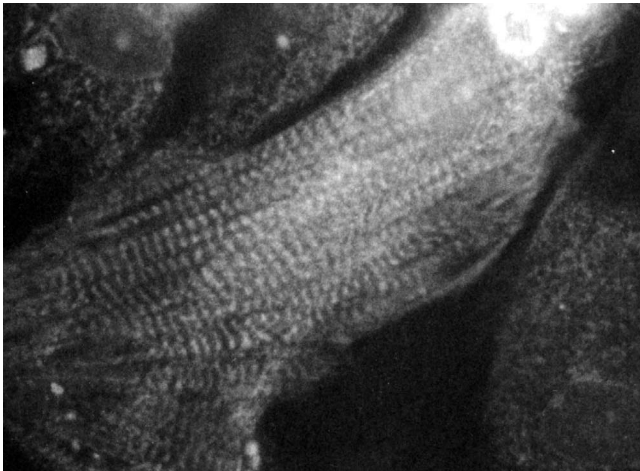

Figure 1. Microphoto: immunofluorescent marking of cell monolayer with a-actinin. Transverse features of reducing cardiomyocite structures is easily visible (x 400).

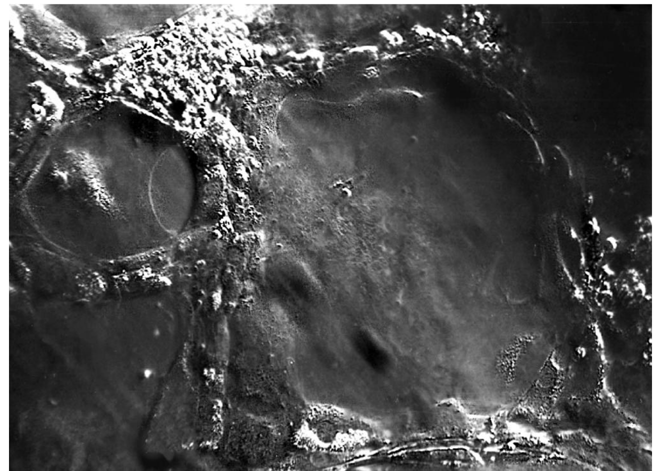

Figure 2. Microphoto: embryonic cardiomyocyte culture on the 7th day. Connection between cell units. Phase contrast (x 160)

Fragmentation of embryonic cardiomyocytes

1 ml of bidistillated water was added to 0.2 ml of concentrated cardiomyocyte suspension. After a 10 minute exposure in the obtained hypotonic medium, the suspension was washed 2-3 times with isotonic solution III and centrifuged at a frequency of 3000 revolutions per minute to complete cell destruction and to recover isotonicity of the solution with cell fragments.

Histological methods of investigation

We used a PGN inverted microscope (GDR) for dynamic observation of native culture in passing light, and a "Biolam" P-1 microscope (USSR) for creation of phase contrast. Muscle cells in the culture were identified by a positive reaction to Schiff reactant (SIC-reaction), imunofluorescent mark to alpha-actinine. For electromicroscopic investigation, tissue patterns were fixed in a 2.5% solution of OsO_4 and put into Epon. Ultrathin sections were colored by uranil-acetate and lead citrate and viewed in a JEM-7A electromicroscope under accelerating voltage 80 kV. The tissue sections were stained with hematoxin-eosine. Preparations were studied with a "Jenaval" microscope (Carl Zeiss, Jena, GDR) at full resolving power. Videorecording with a camera integrated into the microscope was used for dynamic observation of embryonic cardiomyocytic cultures.

Modeling of ischemic injury for cells in the culture

Trays with cardiomyocyte culture were put into an aerostat with rarification of 0.8 atms for 120 min.

Investigations in vivo

Investigations were made with mongrel rats of different genders and ages. Four groups of rats were formed for experimental purposes: Group I, consisting of 10 animals, to implant 0.02 ml of embryonic cardiomyocyte suspension into intact myocardium; Group II, consisting of 6 animals, to inject 0.02 ml of isotonic phosphate buffer into intact myocardium (to control for injection of inactive solution); Group III, consisting of 6 animals, to inject cardiomyocyte fragment suspense into uninjured myocardium (control for dead cell injection); group IV consisting of 10 animals such that branches of left coronary artery were bandaged in order to obtain myocardial necrosis and then 0.02 ml of cardyomyocyte suspension was injected into the necrotic area.

Embryonic cardiomyocyte implantation

Animals were fixed in supine position under ether anaesthesia. Skin was cured, and then a left-side front-side thoracotomy in IV inter-rib was conducted. Injection of 0.02 ml of an embryonic cardiomyocyte suspension was made with insulin syringe that was adapted for experimental purposes. The suspension had a concentration of $4.8 \cdot 10^6$ cells / ml (about $9.6 \cdot 10^4$ cells).

Results and Discussion

With enzymatic suspension of fetal myocardial tissue, up to 95% of viable cells can be obtained. The majority

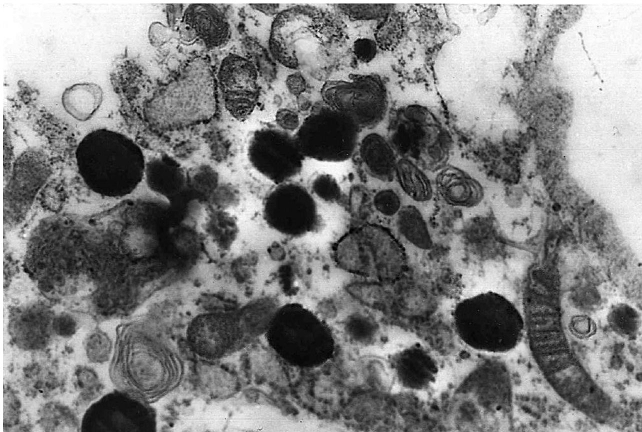

Figure 3. Electrogram: irreversible ischemic injuries of cardiomyocyte: a great number of lysosomes, irreversible injuries of mytochondria, depletion of intracellular glycogen (x 15000).

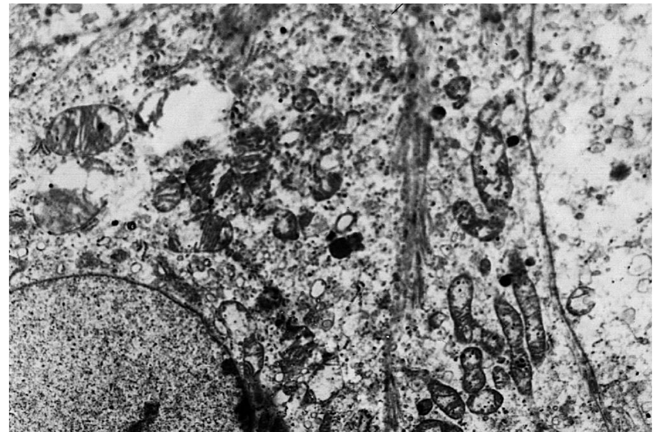

Figure 4. Electrogram: reversible reactive changes of intracellular cardiomyocyte structures: normal nucleus with active chromatin; reactive changes of mytochondria; contractive apparatus (x 5000).

of cells obtained after suspension purification are cardiomyocytes; this was confirmed by the positive reaction of the cell monolayer to glycogen, which is a marker for cardiomyocytes in culture, and by the immunofluorescent marker a-actinin (Figure 1). We observed the step-by-step organization during cell cultivation from individual fixed and dispersed cells on the first and second days of incubation to the creation of a monolayer and its compresses on the 2nd to the 7th days, up to multiple cell units starting on the 7th day of incubation (Figure 2). In addition, only reductions of individual cell groups or asynchronous reductions of several colonies were observable on the 3rd to 7th day of cultivation. However, in glasses with organized cell-units with formed communications that could be seen as transparent pulls, synchronous rhythmic reductions in a great number of cells were observed. At the end of observation, formed structures have a diameter of several mm. This signals a reorganization of functional syncytium already in early period of cultivation.

The main effects of embryonic cell implantation are strong induction of reparative processes in injured areas, as is mentioned in most studies conducted for substitution cell therapy. We put cell cultures into conditions of extended hypoxia for modeling of ischemic injury of cardiomyocytes, then added the suspension of embryonic cardiomyocytes to the cultures under investigation, and added the same quantity of usual breeding ground to control cultures.

During the succeeding days of incubation, the control cell cultures appear obviously injured. Reductions recovered only in individual cells, which corresponds to irreversible ischemic injuries recorded on electrograms (Figure 3). Experimental cell cultures look unchanged and continue to reduce synchronously. Electromicroscopy of these patterns showed reversible reactive changes in intracellular structures.

To confirm the results obtained in vivo, we conducted a series of experiments with animals. We used mongrel laboratory rats of different genders and ages. To evaluate a colony of embryonic cardiomyocytes implanted into the myocardium of an adult rat and to clarify the cells' ability to form a functional syncytium unified with differentiated cardiomyocytes, we analyzed the dynamics of electrocardiogram registered at different stages of the experiment. Due to significant variability of individual animal cardiograms, the initial cardiograms were always recorded for comparison with the later ones.

Electrocardiographic changes for the animals in Group I (implantation of embryonic cardiomyocyte suspension into intact myocardium) show that sinus rhythm is preserved after the injection; intraventricular conduction disturbances and ischemic features were observable, but these phenomena are absent on the cardiogram made on the 21st day of the experiment. This not only signals recovery of heart structure, but also indirectly confirms the hypothesis that the implanted cardiomyocytes are integrated into the architectonics of the adult animal heart, since the existence of any heterogeneous area in the myocardium would lead to conduction disturbances or arrhythmia. The fact that it was

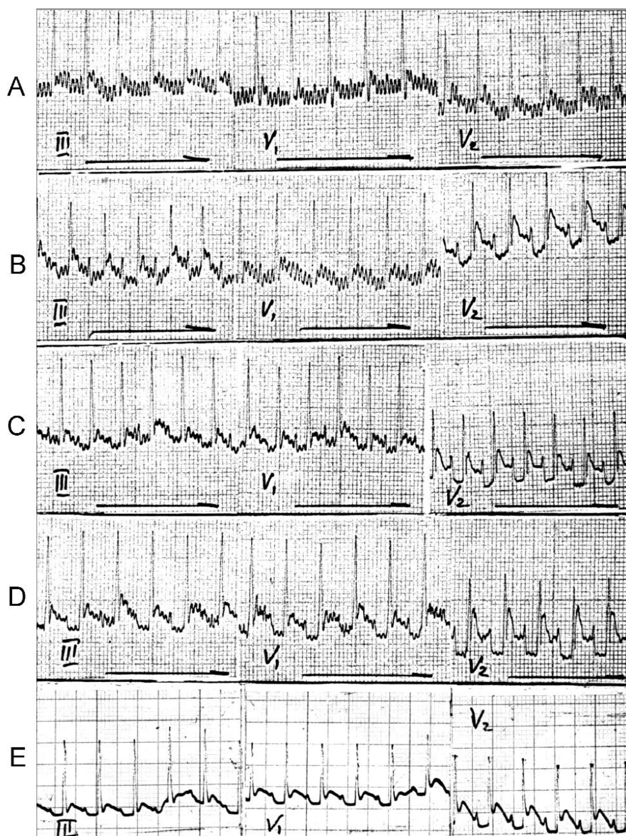

Figure 5. Dynamics of electrocardiographic changes for animals in Group IV: A - initial; B - immediately after experimental myocardium injury; C - 7th day after necrosis; D - immediately after implantation of embryonic cardiomyocytes in necrotic area; E - 21 days after implantation.

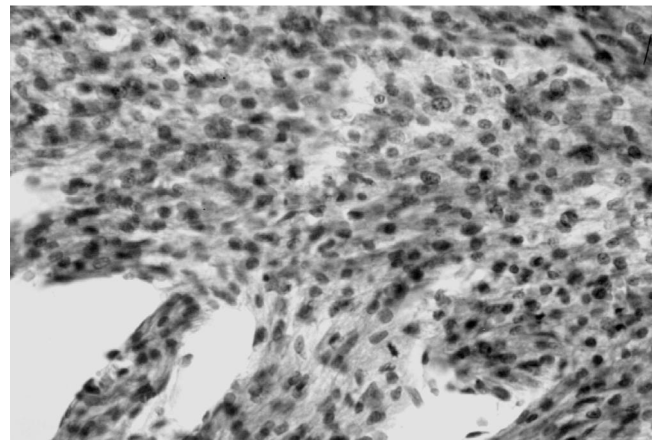

Figure 6. Microphoto: hystosection of fetus myocardium obtained from female-recipient of allogenic embryonic cells. Hematoxylin-eozin (x 100).

the presence of viable cardiomyocytes in injected suspension that provided these electrocardiogram dynamics for animals of Group I was confirmed by control groups II and III. In Group II (Control for the actual injection and injection of the same quantity of isotonic buffer solution) we revealed transitory myocardial ischemia only, which was not diagnosed on the 7th day of experiment. Injection of cell derivates into intact myocardium (Group III) leads to lasting (more than 28 days in our observation) ischemia and electrocardiographic signs of pointed changes in the area of injection.

To simulate myocardial infarction, we banded branches of the left coronary artery. Success of modeling was evaluated by electrocardiographic and histological methods at different intervals. Implantation of embryonic cardiomyocytes into necrotic regions was made 7 days after experimental myocardial infarction (Group IV). As a reaction to cell-suspension injection, some increase of ischemic features in the area of modeled infarction was observed in the electrocardiogram. Nevertheless, the electrocardiogram recorded 21 days after cell implantation (28 days after experimental necrosis) showed a significant decrease of ischemia in the area of injured myocardium as comparison with the situation before the injection (Figure 5).

An important part of cell substitution therapy is the investigation of recipient-organism immune reactions to injected allogenic cells. It is known that there are only two possible reactions of the recipient's immune system to implantation of exogenic cell material: namely, developing one of the types of immunological tolerance with cell-adaptation , or an immune reaction with production of tissue-specific antibodies and rejection of implanted cells. We had not planned any special immunological research. However, one of the rats was found to be pregnant after modeled infarction and consequent cell implantation. Experimental data described in the literature testify to the ability of anticardiac antibodies in immunization of the female to pass through the hemoplacental barrier and accumulate in the myocardium of the fetus and to cause enhanced teratogenic and cytotoxic effects [7,11]. This is an active immune-system reaction in the pregnant female-recipient with production of antibodies against components of implanted cardiomyocytes; with the presence of circulating immune complexes, this would definitely lead to some morphological changes in the developing myocardium of the fetus. The fetus was in the prenatal

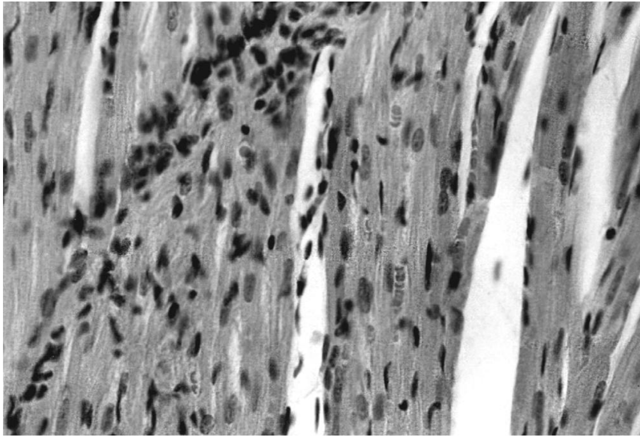

Figure 7. Microphoto: tissue section of adult rat myocardium in the place of embryonic cell implantation. Hematoxylin-eozin (x 100).

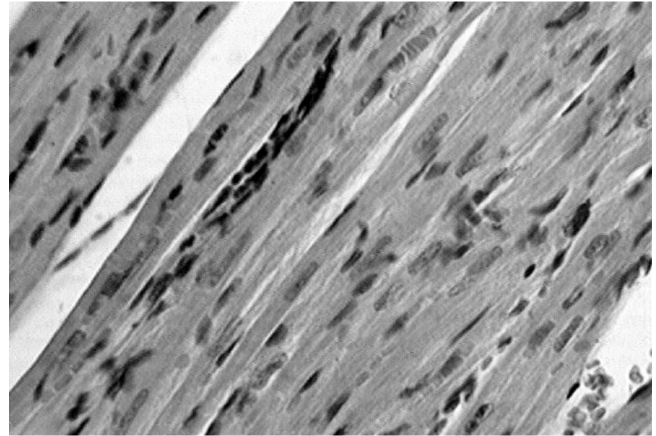

Figure 8. Microphoto: tissue section of site of embryonic cardiomyocyte implantation in myocardial infarction area (21st day). Muscle fiber with underdifferentiated cells. Hematoxylin-eozin (x 200).

period (16-18th day of embryogenesis) when the material was histologically investigated. The structure, form and size of the fetal heart were normal and corresponded to the period of gestation. The heart displayed normal fetal myocardial structure without signs of cell infiltration, with areas of mytos on the tissue section of the fetus' heart (Figure 6). The fetal cardiomyocites had a typical structure, with a relatively large nucleus and a small quantity of cytoplasm. The same cell-type is observed on the 10th day on the tissue section at the implantation site of embryonic cardiomyocytes in the infarction area (Figure 7). One can see vascularization at the implantation site. In a later examination (21st day), one can reveal muscle fibers with underdifferentiated cells (Figure 8).

The described observation indirectly confirms data on immunological inertness of cultivated embryonic cardiomyocytes and the possibility of developing immunological tolerance to them in adult patient.

The data obtained testify not only to the integration of implanted cardiomyocites but also to the possibility of recovering myocardial structure and function in the case of myocardial pathology. These facts give us hope that substitution-cell therapy will have a secure place in complex treatment of heart failure in the future.

Additionally, it is hypothesized that the complex mechanism of substitution-cell therapy can be used to achieve:

- prevention of scar formation in the myocardium in cases of infarction or wounding;
- protection against ischemic injury cardiomyocytes;
- stimulation of angio- and cardiomyogenesis.

At the same time, the ability of this technology to obtain pure cultures of different specialized cells of myocardial tissue indicate other prospective directions for further investigation:

- compensation of incretive function of atrial cardiomyocytes;
- formation of an exogenic pacemaker and areas of the heart conduction system in surgical arrhythmology.

The truth of the great Goethe's words is now evident

"*...Life is the best invention of nature,*
For it, death is just a means to greater life"

Further investigations in this area will inevitably give rise to new questions and problems, but if only one of the prospects described becomes a reality, then cell-substitution therapy will save the lives of many patients in the future.

References

[1] Belkov AV, Onishenko NA, Pisarevskii AA, et al. Experimental premises for use of cryoconserved hepatocytes and spleen fragments in treatment of sepsis patients. Surgery. 1992; 12: 173 (in Russian).

[2] Bocharov AV. Use of cultivated on microcarrier of hepatocytes for liver function artificial support in acute liver failure. Author's summary of Ph.D. thesis in biology. Moscow 1990 (in Russian).

[3] Brodskii VYa. Polyploidy in myocardium: Compensatory reserve of the heart. Bulletin of experimental biology and medicine. 1995; 119(5): 454-459 (in Russian).

[4] Bruslik VG, Loginov AS, Speransky MD, et al. Use of isolated cardiomyocytes in treatment of acute liver failure. Bulletin of RAMS. 1994; 5: 8-14 (in Russian).

[5] Budhram V, Antony F, Persoud S. Transplantation of fetal nervous tissue of rats. Progress in surgery: Thesis of soviet student conference. Moscow 1990: 35-36 (in Russian).

[6] Vasilev AV. Studying of lows of epidermal ceratoncyte cultivation for recovering of skin. Author's summary of PhD thesis in biology. Moscow 1993 (in Russian).

[7] Volkova LS. Immonological interrelation between mother and fetus organisms. Moscow 1970 (in Russian).

[8] Zhuravlev VA, Svedentsov EP, Sukhorukov VP. Transphysiological operations, 2nd edition. Moscow: Medicine. 1985 (in Russian).

[9] Komissarenko VP, Turchin IS, Komissarenko IV, et al. Transplantation of island cell cultures of animal and human fetal pancreas as a method for diabetes treatment. Physician praxis (Vrachebnoe delo). 1983; 4: 52-57 (in Russian).

10] Kulikov VP, Novikov VK, Pisareva TP, et al. Implantation of unconserved fetal pancreas in diabetes treatment. Soviet medicine. 1987; 7: 16-21 (in Russian).

[11] Michailov VM. Anatomical archive. 1965; 6: 9-14 (in Russian).

[12] Pavlovskii MP, Boiko NI. Distant results of allotransplantation of island cell cultures of pancreas. Bulletin of surgery. 1990, 145; 12: 26-29 (in Russian).

[13] Pisarvskii AA. Artificial prototypes for lung and lever. Transplantology and artificial organs. 1994; 1: 55-59 (in Russian).

[14.] Polezhaev LV. A state of problem of heart muscle regeneration. Progress in modern biology (Uspekhi sovremennoi biologii). 1995; 115(2): 198-212 (in Russian).

[15] Polezhaev LV, Alexandrova MA. Transplantation of brain tissue in normal and pathology cases. Moscow, Nauka 1986 (in Russian).

[16] Rumyantsev PP. Cardiomyocytes in processes of reproduction, differentiation and regeneration. St. Petersburg, Nauka 1982 (in Russian).

[17] Sarkisov DS, Alekseev AA, Glushenko EV, et al. Theoretical and practical aspects of use of cultivated fibroblastes in recovery of skin. Bulletin of RAMS. 1994; 6: 6-11 (in Russian).

[18] Sarkisov DS, Alekseev AA, Tumanov VP, et al. Treatment of burns with the use of cultivated human skin cells. Surgery,.1993; 3: 22 (in Russian).

[19] Tribulev GP, Podoplelov II. Analysis of antigen structure of human cells cultivated outside organism. Topical problems of cell immunology and immunogenetics (Aktualnye voprosy kletochnoi immunologii i immunogenetiki). Sofia: Medicina i fizkultura. 1973: 41 (in Russian).

[20] Furugyan P. Transplantation of fetal pancreas for acceleration of rehabilitation period after protein deficiency with rats. Progress in surgery: Thesis of soviet student conference,.Moscow 1990: 34-35 (in Russian).

[21] Shumakov VI, Blumkin VN, Ignatenko SN, et al. Clinical allotransplantation of island cell cultures of human fetal pancreas. Problems of endocrinology. 1985; 31(1): 18-22 (in Russian).

[22.] Delcarpio JB, Claycomb WC. Cardiomyocyte Transfer into the Mammalian Heart: Cell-to-cell interactions in vivo and in vitro. Ann of the New York Academy of Sciences. 1995; 752: 267-285.

[23] Gerrity M, Rinehart JS. Embrio transfer. In vitro fertilization and embrio transfer: A manual of basic techniques. New York, London 1988: 189-206.

[24] Kammermeier H, Rose H. Are isolated cardiomyocytes a suitable experimental model in all lines of investigation in basic cardiology? Basic Res Cardiol. 1988; 83 (4): 343-349.

[25] Kao RL, Chiu RC. Cellular Cardiomyoplasty: Myocardial Repair with Cell Implantation. Austin, Texas: Springer-Verlag 1997: 195.

[26] Weisensee D, Seeger T, Bittner A, et al. Cocultures of fetal and adult cardiomyocytes yield rhythmically beating rod shaped heart cells from adult rats. In Vitro Cell. Dev Biol. 1995; 31(3): 190-195.

[27] Wollenberger A. Isolated heart cells as a model of the myocardium. Basic Res Cardiol. 1985; 80(2): 9-13.