

Recovery of Infarcted Myocardium in an In Vivo Experiment

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Summary. Acute myocardial infarction leads to the loss of functional cardiomyocytes and structural integrity. The adult heart cannot repair the damaged tissue due to inability of mature cardiomyocytes to divide and lack of stem cells.

The aim of this study was to evaluate the efficiency of introduced autologous skeletal muscle-derived stem cells to recover the function of acutely infarcted rabbit heart in the early postoperative period.

Material and Methods. As a model for myocardium restoration in vivo, experimental rabbit heart infarct was used. Autologous adult myogenic stem cells were isolated from skeletal muscle and propagated in culture. Before transplantation, the cells were labeled with 4',6-diamidino-2-phenylindole and then, during heart surgery, introduced into the rabbit acutely infarcted myocardium. Postoperative cardiac function was monitored by recording electrocardiograms and echocardiograms. At the end of the experiment, the efficiency of cell integration was evaluated histologically.

Results. Rabbit cardiac function recovered after 1 month after the induction of experimental infarction both in the control and experimental groups. Therefore, the first month after the infarction was the most significant for the assessment of cell transplantation efficacy. Transplanted cell integration into infarcted myocardium was time- and individual-dependent. Evaluation of changes in left ventricular ejection fraction after the induction of myocardial infarction revealed better recovery in the experimental group; however, the difference among animals in the experimental and control groups varied and was not significant.

Conclusions. Autologous myogenic stem cells repopulated infarcted myocardium with different efficiency in each individual. This variability may account for the observed difference in postoperative cardiac recovery in a rabbit model.

Introduction

Cardiovascular diseases present the major and growing public health concern due to the aging of the world's population. According to the World Health Organization, the morbidity due to this reason is estimated to reach a pandemic level by 2020. Myocardial infarction (MI) caused by coronary artery disease is one of the major causes of heart failure (HF). Despite optimal medical therapy and aggressive revascularization strategies, MI with left ventricular dysfunction is a lethal condition for 25% of patients during 3 years after the event. Currently used therapeutic measures are not sufficient to prevent left ventricular remodeling and subsequent development of HF. Patients with New York Heart Association (NYHA) class IV heart failure are treated by pharmacotherapy or sometimes by heart transplantation. Heart transplantation remains the

treatment modality with the best outcome showing the 5-year survival rates of around 65%. However, it is limited by the current shortage of donor organs and the complications of long-term immunosuppression (1). Due to the above limitations, cell transplantation is an area of growing interest in clinical cardiology as a potential method of treating the patients with MI and/or HF. The goal of cell therapy is a replacement of akinetic scar tissue with viable myocardium aiming to improve the cardiac function along with inhibition of the remodeling process. This novel technique of treatment has been intensively researched in the last few decades; however, it has not been widely introduced into clinical practice yet.

The majority of cardiomyocytes are known not to re-enter the cell cycle after their treatment with various agents. Hypertrophy of the existing viable cardiomyocytes may be a reason for myocardium growth. Some recent clinical evidence shows that a small number of cardiomyocytes can re-enter the

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cell cycle; however, it does not always end in cytokinesis. Moreover, the regenerative potential of these cells is very poor (2, 3). Consequently, stem cell transplantation reconstructing damaged myocardium is a novel technique of great promise. There appear more and more clinical studies showing successful experimental transplantation of embryonic, fetal, cardiac and skeletal muscle, bone marrow-derived, or mesenchymal stem cells into experimentally induced myocardial infarction area (4–8).

The successful transplantation of skeletal myoblasts into the damaged myocardium made by Kao et al. (1989) has generated a great interest all over the world (9). Skeletal muscle satellite cells have clinically relevant and attractive characteristics, such as an autologous availability, a high degree of proliferation in culture in vitro, a myogenic lineage direction, and a high resistance to ischemia (10). Successful outcomes using myoblasts for cellular cardiomyoplasty were confirmed by experimental studies with animals and in some clinical studies (6, 11). Menasche et al. were the first who performed clinical cellular cardiomyoplasty with myoblasts (12). Since then, a number of small-scale uncontrolled clinical trials have been reported by different groups. Their presented outcomes were very promising; however, later large-scale, placebo-controlled, double-blind, randomized trials were not so optimistic (13). Poor therapeutic effectiveness has been explained by a high rate of transplanted cell death (up to 90%), poor electromechanical integration, sometimes causing arrhythmias, and insufficient perfusion in the transplantation area (14, 15). In order to enhance the therapeutic efficacy of cellular cardiomyoplasty, multiple measures improving stem cell survival, inducing intercellular contact formation with existing cardiomyocytes, have been studied. The role of various molecular signaling pathways – Cx-43 gene, encoding gap junctions between cardiomyocytes, angiogenesis-inducing factors, such as VEGF and TGF- β , etc. – is still being investigated (16, 17). Despite numerous experimental studies on cellular cardiomyoplasty, many questions still wait to be clarified, e.g., an ideal type of stem cells, an optimal number of cells for transplantation, cell transplantation timing, administration mode, therapy long-term efficiency, etc.

Our purpose was to investigate the postoperative recovery of cardiac function after autologous myogenic stem cell transplantation using a model of experimental laboratory rabbit heart infarct. Although being not similar to pathologic processes in human body, this model allows evaluating the survival of transplanted stem cells under pathologic conditions when they are affected by various inflammatory

factors as well as functional myocardium recovery under modeled circumstances. The laboratory rabbit is not a rare experimental model in cellular cardiomyoplasty research; however, the strengths and weaknesses of this experimental model have not been fully disclosed yet. The aim of this study was to evaluate the efficiency of introduced autologous skeletal muscle-derived stem cells to recover the function of acutely infarcted rabbit heart in the early postoperative period.

Material and Methods

Animals. For this experimental study, California rabbits weighing 3.0–3.5 kg were used (Vivarium of the Institute of Biochemistry, Vilnius University). All animal surgical procedures were performed under general anesthesia. The animals received humane care according to the “Law on the Care, Welfare and Use of Animals” of the Republic of Lithuania. License for the use of laboratory animals in stem cell research (No. 0171, 31-10-2007) was obtained from the Lithuanian Food and Veterinary Office. In this study, 13 animals were used: 6 in the control group and 7 in the experimental group.

Preparation of Autologous Skeletal Muscle Stem Cells. Under general anesthesia of the animal (Bioketan 35 mg/kg, xylazine 5 mg/kg), a longitudinal incision was made intramuscularly over the projection of the *biceps femoris* muscle, and a 0.5-cm³ piece of muscle tissue was removed and placed into the transportation medium (Dulbecco’s modified Eagle’s medium, DMEM, Sigma-Aldrich) with penicillin (100 U/mL) and streptomycin (100 mg/mL; both from Biological Industries, Israel). Subsequently, the tissue was minced and treated with a mixture of enzymes – 0.125% trypsin-EDTA (Sigma) and 1 mg/mL collagenase type V (Sigma) – prepared in phosphate buffered saline (PBS) as described by Širmenis et al. (18). The resulting cell suspension was centrifuged; cell mass was washed twice with growth medium (Iscove’s modified Dulbecco’s medium, IDMEM, Sigma-Aldrich) supplemented with 10% fetal calf serum (Biological Industries, Israel) and antibiotics. Afterward the cells were harvested in the culture medium and counted; their viability was evaluated by the trypan blue exclusion test. The isolated cells were maintained in cell growth medium with supplements. Approximately in 2 weeks, skeletal muscle-derived stem cells formed a monolayer. Then the cells were propagated in growth medium with supplements, passaging them twice a week. After the quantity of cells reached the required amount, they were examined.

Cell Viability Test. Viability of the cells prepared for transplantation was examined using a mixture

of acridine orange (AO, 100 mg/mL, Molecular Probes) and ethidium bromide (EB, 100 mg/mL, Sigma-Aldrich) dissolved in PBS. The stain (4 μ L) was mixed with 100 μ L of growth medium containing cells. Staining with AO/EB distinguishes living cells from dead cells based on membrane integrity: AO intercalates into DNA of viable cells and stains them green; EB binds to DNA of nonviable cells and stains them orange (19).

Labeling of the Cells Prepared for Transplantation. Cell labeling is exclusively an experimental procedure oriented at tracing transplanted cells in heart muscle in order to assess their survival in the recipient's tissue. In this study, the cells prepared for transplantation were stained with DNA-intercalating vital dye, 4',6-diamidino-2-phenylindole (DAPI). The cells in an exponential growth phase were treated in vitro with DAPI (10 μ g/mL at final concentration). In 24 hours, the dye was washed with PBS; the cell monolayer was dispersed by trypsin-EDTA mixture and washed again. Three million cells were suspended for further procedures in 300 μ L of medium without serum.

Histological Analysis of Host Tissue. Histological preparations of heart muscle were prepared according to the standard procedure: the tissue was dehydrated in ethanol, embedded in paraffin, and sectioned in 5- μ m slices. Preparations were stained with hematoxylin and eosin (H&E); the images were analyzed by light microscopy ($\times 150$ to $\times 300$) (20).

Induction of Rabbit Experimental Myocardial Infarction. The induction of anesthesia for laboratory rabbits was performed using the intramuscular injections of Bioketan (35 mg/kg) and xylazine (5 mg/kg). Glucose solution (5%) was infused through a cannula introduced into the ear vein. To observe functioning of the heart and overall status of the animal, ECG was continuously recorded during the experiment. The first ECG was recorded just before the operation. The intubation was performed using a 2.5-mm endotracheal tube, which was later immobilized. Lung ventilation was performed by a Harvard Inspira (Massachusetts, USA) mechanical ventilation machine sustaining 3-L respiratory minute volume and respiratory rate of 40 breaths per minute. Further anesthesia was maintained by the intravenous injections of Bioketan (0.3 mL) and xylazine (0.3 mL) every 15 minutes. A slow infusion of 5% glucose solution was administered continuously during the operation. The rabbit was put on the right side, and the operating field was prepared on the left side of the chest. The fur on the heart projection was clipped; the operation field was later disinfected. Thoracotomy was made in the third or fourth intercostal

spaces. Lidocaine (0.2 mL, 20 mg/L) was administered intravenously to avoid arrhythmias. The left lung was pushed off, pericardium was cut, and the left anterior descending artery was ligated using a 5-0 Prolene suture approximately in the middle of its length. ECG was recorded after coronary artery ligation in every 5 minutes, and characteristic acute myocardial changes of ECG, such as ST-segment elevation of 2 mm or more and QS complex formation, were evaluated. Acute myocardial infarction was visually defined by darkening in the myocardium zone or local contractility impairments in the infarction area below the ligature.

Stem Cell Grafting Into Infarcted Myocardium. Characterized, counted, and stained cells were injected directly into the zone of infarcted myocardium below the ligation of the coronary artery. Transplantation was performed with an insulin syringe by 5–6 injections. The suspension of IMD medium (300 μ L) with approximately 3×10^6 stained cells was administered to each experimental rabbit. The suspension of IMD medium (300 μ L) without cells was injected into the experimental animals in the control group using the same scheme. The site of transplantation was marked with a purse-string 5-0 Prolene suture, and it was assumed that this string held injected cells in the myocardium. After transplantation, the chest incision was closed in layers with running sutures of 3-0 Vicryl. The postoperative ECG was recorded. Finally, animals were removed from ventilation and recovered under a warming lamp. The injections of Reflinim (50 mg/kg) were administered to each animal for 3 days after the operation. During 3 days after the operation, Diclofenac natrii (25 mg/mL) as a painkiller and Dexamethasonum (4 mg/mL) to avoid laryngeal edema after intubation, which sometimes makes great problems, were administered.

Assessment of Cardiac Function. Echocardiography was performed before the experimental operation and on days 7, 14, 21, and 28 after it. The rabbits were anesthetized by the intramuscular injections of Bioketan (35 mg/kg) and xylazine (5 mg/kg). Cardiac ultrasonography was performed using a commercially available echocardiographic system (MyLab30CV, ESAOTE S.p.a., Italy) equipped with a 7.5-MHz broadband sector transducer. The heart was imaged in a two-dimensional mode in long axis four-chamber and two-chamber views to evaluate the contractility of myocardial infarction zone, and the left ventricular ejection fraction was calculated by the Simpson's rule. ECGs were recorded at every critical moment of the experiment: in the normal state before the beginning of the experiment, after induction of myocardial infarction, cell transplanta-

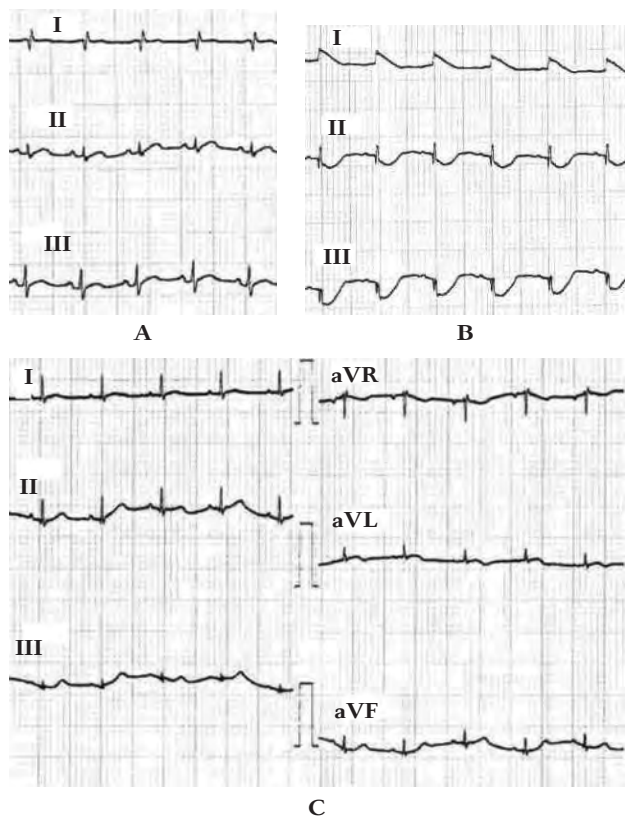


Fig. 1. Electrocardiograms (ECGs) at different time points
 A, ECG after induction of anesthesia: normal heart sinus rhythm, each P wave is followed by a QRS. B, ECG in 30 minutes after ligation of left descending coronary artery and transplantation of stem cells: normal heart sinus rhythm, each P wave is followed by a QRS. Typical ECG changes during acute anterior myocardial infarction: ST-segment elevation in I lead, ST-segment depression and negative T in II and III leads. C, ECG in 30 days after stem cells transplantation: normal heart sinus rhythm, each P wave is followed by a QRS. Changes of conduction are seen in III and aVR leads. Right His bundle branch block is seen in III and aVR leads.

tion as well as before euthanasia.

Statistical Analysis. Data are expressed as mean (SD). Comparisons were performed using the Student *t* test. Differences were considered statistically significant at $P < 0.05$.

Results

Our previous long-term research when rabbits were investigated during a 3-month period showed that the rabbit cardiac function in the control group generally recovered within a month after experimental myocardial infarction. Consequently, in this study, to evaluate the effect of grafted cells on the rabbit heart function, special attention was given to the first month after cell transplantation. During this period, the results of histological examination and echocardiographic cardiac function assessment were analyzed and compared in order to understand the early processes undergoing in the infarction area.

Functional myocardial infarction and postoperative period was observed and evaluated during ECG (Fig. 1) and echocardiographic (Fig. 2) examinations.

The most common echocardiographic findings (Fig. 2) typical of myocardial infarction were as follows: a new hypokinetic zone in the left ventricle wall, the apex of the heart or interventricular septum, changed echogenicity in the infarction zone, reduced left ventricular ejection fraction as well as systolic volume. All these disorders revealed cardiac function deterioration. During the first postoperative month, the obtained data showed comparable dynamics of echocardiographic changes in both the groups:

- The most significant deterioration of left ven-



A



B

Fig. 2. Two-chamber short axis (A) and four-chamber long axis (B) views during echocardiography of an experimental rabbit

tricular ejection fraction was observed 7 days after myocardial infarction; the myocardial infarction zone was hypoechogenic, with undefined boundaries; reduced myocardium contractility was observed in the apex and/or in the lower part of the septum;

- Left ventricular ejection fraction improved after 14 days; however, contractility of infarction zone was reduced, and hypoechogenic zone was more noticeable in the apex of the heart;
- During 21 days after the operation, left ventricular ejection fraction continued to improve; contractility of the heart apex remained reduced or just started to improve; the aneurysmatic apex was observed quite often, and blood flow regurgitation through the mitral valve caused by the left ventricle dilation was registered several times;
- Left ventricular ejection fraction was mostly restored in the period of 28 days after myocardial infarction induction; the infarction zone contractility improved, although with a few exceptions, it remained hypoechogenic.

Most attention was given on the processes in the infarcted area. Despite the fact that the changes in left ventricular ejection fraction after the induction of myocardial infarction varied both in the experimental and control groups, the data revealed better recovery in some test animals with grafted stem cells. Although the difference among animals in the experimental and control groups varied and was not statistically significant, a trend toward a slight improvement was observed in the experimental group. In particular, it was most clear at the end of the month, when left ventricular ejection fraction reached the baseline level more quickly (Fig. 3).

For the transplantation, to obtain a sufficient number of stem cells, they were propagated in cul-

ture by repeated passages. Before the grafting, cell viability was checked using AO&EB dye test; the results demonstrated a high viability of cells prepared for the transplantation (Fig. 4A).

To identify donor cells *in vivo*, they were labeled with vital fluorescent dye DAPI and injected into cardiac tissue during heart surgery immediately after the experimental infarction. Our previous *in vitro* studies have demonstrated that DAPI-labeled cells (Fig. 4B) remained labeled, and the intensity of labeling did not decline significantly during one month (data not shown). In our experimental model, during the first postoperative month, no detrimental effects of cell transplantation on the rabbits were detected. Throughout this time, the ECG demonstrated normal heart sinus rhythm; each P wave was followed by a QRS (Fig. 1C). Therefore, our data did not demonstrate arrhythmias in the infarcted heart after autologous skeletal muscle-derived cell transplantation.

The histological appearance of grafted cells was analyzed at different time points after cell injection. In order to identify DAPI-labeled cells, heart tissue sections were examined using fluorescence microscopy; light microscopy was used to evaluate the integration of grafted cells in the slices stained with hematoxylin-eosin as well. The last-mentioned examination method also allowed us to detect the occurrence of infarct. Integration of transplanted cells was confirmed in serial heart sections (Fig. 5–7). Histological evaluation enabled to detect stained cells and demonstrated that the injected cells proved to be able to survive in the damaged cardiac tissue. Nevertheless, during the first postoperative month, rather great variability in donor cell engraftment in the damaged rabbit myocardium was observed. The example of the most early image (1 h after injection) of cell distribution in the myocardium was obtained in one case when the experimental infarct and cell transplantation was followed by a sudden animal death, and the histological examination was conducted immediately after the section. In this case, histological images showed the discreet loci of unspread transplanted cells (Figs. 5A and 5B). Later, following 2 weeks after cell transplantation, in some cases, similar images of unspread cell loci were also observed (Fig. 6A). As well at this time, transplanted cells spread over a large myocardium area were found in the slides of other test animal hearts (Fig. 6B). When DAPI- and hematoxylin-eosin-stained heart sections were prepared 4 weeks after cell injection, large loci of donor cells fully integrated into myocardial tissue prevailed in the heart slides of all animals in the experimental group (Figs. 7A

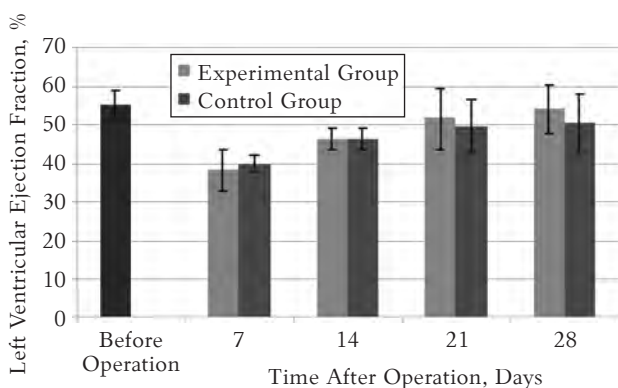


Fig. 3. Changes of rabbit left ventricular ejection fraction during 4 weeks after operation (n=13)

There was no statistical significance between the experimental and control groups ($P>0.05$).

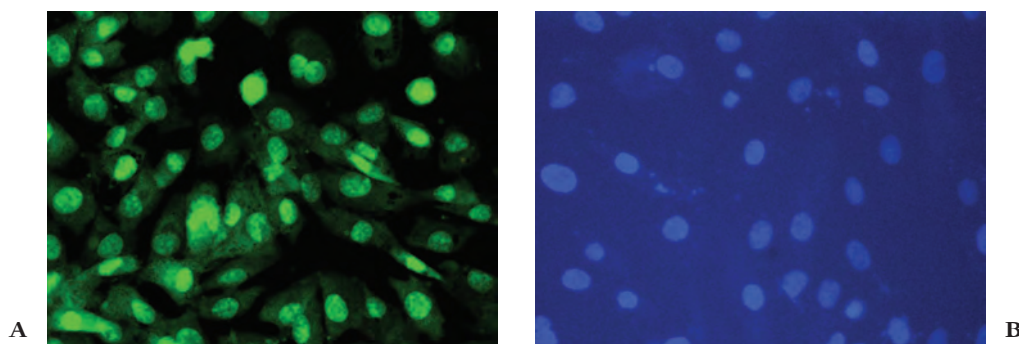


Fig. 4. Rabbit muscle stem cell imaging in vitro after propagation in culture before transplantation into acutely infarcted myocardium. A, cell viability confirmation by staining with a mixture of AO and EB dyes. Viable cells are stained in green. B, corresponding cultured DAPI-labeled cells before transplantation (blue nuclei). Cells were analyzed using fluorescence microscope.

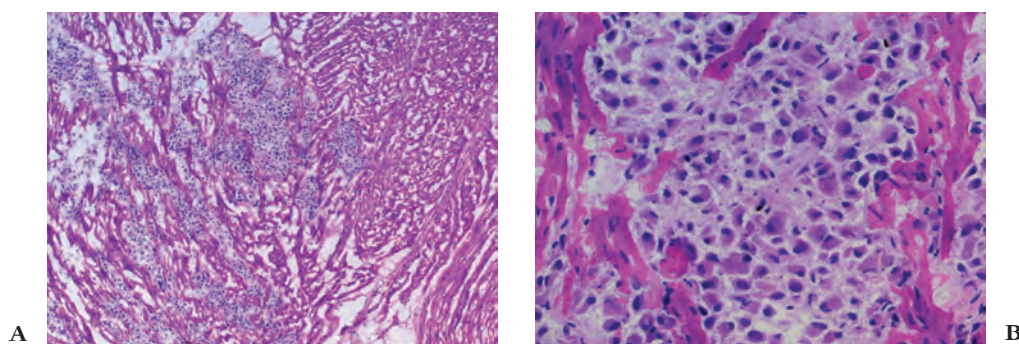


Fig. 5. Histological examination of transplanted cells immediately (1 h) after inoculation them into infarcted myocardium. Hematoxylin and eosin stained rabbit heart sections with injected cell areas show a discreet loci of unspread transplanted cells clusters. Dark blue stained nuclei of donor cells are larger than nuclei of host cells in the area. A, $\times 40$; B, $\times 100$.

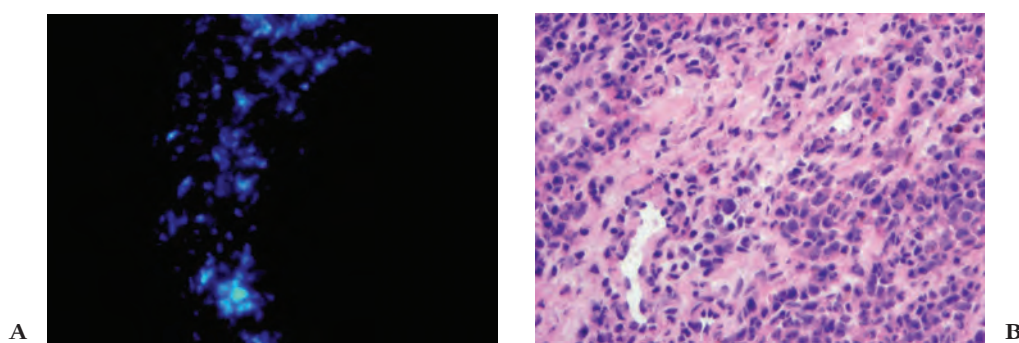


Fig. 6. Infarcted area of rabbit myocardium with transplanted skeletal muscle donor cells 2 weeks after transplantation ($\times 100$). A, a section of the scar with the transplanted cells examined using fluorescence microscopy shows engrafted DAPI-labeled cells (blue fluorescent nuclei). B, a light microscopy micrograph of hematoxylin-eosin-stained heart tissue section showing transplanted cells expansion over a large myocardial infarction area (dark blue cells).

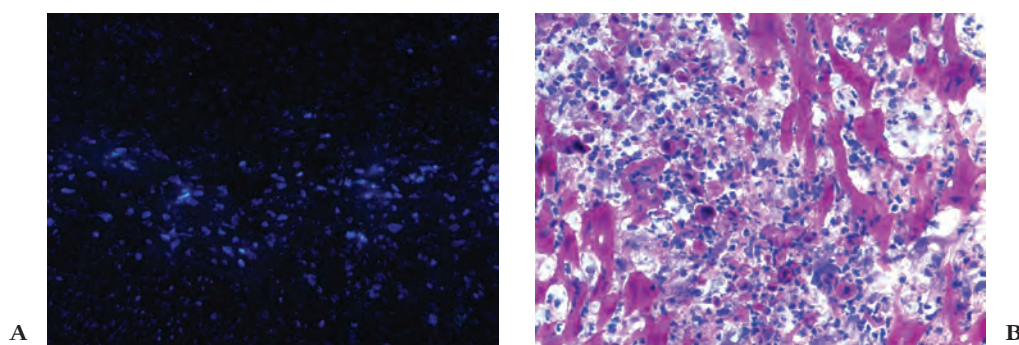


Fig. 7. Areas of cell transplanted rabbit myocardium 1 month after cell transplantation. A, a fluorescent micrograph of heart tissue section showing the nuclei of DAPI-labeled engrafted skeletal muscle donor cells (blue fluorescence) ($\times 100$). B, a light microscope micrograph of hematoxylin-eosin-stained heart section with transplanted cells (blue stained cells) ($\times 200$).

and 7B). Therefore, the diverse integration of transplanted cells was found in individual animals at different times. Moreover, transplanted cell grafts varied in size and the degree of integration into the surrounding tissue. Such variability may be influenced by a variety of factors. Consequently, obtained differences in the improvement of heart function by transplanted cells may be attributed to different levels of cell repopulation of the damaged myocardium.

Discussion

Up to now, numerous clinical or animal studies conducted to evaluate a particular class of cells for its regenerative potential in the infarcted heart have demonstrated an improvement in cardiac function of various degree. Various cell types including cardiomyocytes, skeletal myoblasts, mesenchymal stem cells, bone marrow-derived mononuclear cells, etc. were used in preclinical or clinical trials and showed their own advantages and limitations in cardiomyoplasty. Distinct mechanisms have been proposed for different cell types in improving the cardiac function.

Rabbit skeletal myogenic cells used in our experiments to restore damaged rabbit tissue after induction of myocardial infarction were described in our previous papers (20, 21). The present study was focused on the grafted cell integration into myocardium and subsequent postoperative functional cardiac recovery within the first month. Analysis of the results obtained from animals in the control group revealed that damaged rabbit myocardium maintains the ability of self-repair within the period of one month after injury. Therefore, the data obtained on our experimental rabbit model indicate that a period of up to one month is critical for the assessment of stem cell therapy effectiveness in the improvement of heart function. Evaluation of the role of transplanted cells in the restoration of cardiac functions during later periods is problematic.

However, during the first postoperative month, the results of the assessment of dynamics in cardiac function and cell therapy effectiveness were also ambiguous and varied among animals both in the experimental and control groups. It may be a result of several reasons, e.g., anatomical variants of coronary artery networking among rabbits, causing a different size of infarction zone, complications during intubation, different recovery in postoperative period, etc. Definitely, the dynamics in cardiac function depends on operation extent and progression as well as on the individual animal status before and after surgery. The major factor determining different animal recovery after the experimental operation could be an

unequal integration of stem cells into myocardium. Our investigation of cell engrafting revealed the differences in the integration of injected cells in rabbit myocardium at various time points during the period of one month after induction of experimental infarct and cell transplantation. Unlike in other experimental studies (22), no specific side effects related to muscle stem cell transplantation, including events of arrhythmia or other adverse events, were detected in our study. However, there was variability in the levels of autologous cell engraftment, e.g., in the extent of donor cell integration into surrounding tissues, which was time-dependent and varied among the animals. According to our data, the effectiveness of cell therapy for heart repair may depend on the size of donor cell grafts and degree of their integration into the surrounding damaged heart tissues.

Numerous previous studies have proposed that functional improvement of injured heart may depend on various factors including the number of injected cells, mode and locus of cell delivery, the number of injections, cell type and/or combinations of different cell types (4–6, 11, 12, 22, 23). Better restoration of damaged myocardium usually is related to the higher quantities of donor cells. On the other hand, excessively condensed cell suspensions may result in poor results due to a decrease in cell survival after transplantation. In our study, the efficacy of myocardium regeneration was not related to the number of injected cells. Moreover, there are data that even a very small graft generated by implanted cells may account for functional improvement of the heart (24). Local signals in the site of cell injection, which depends on the extent of experimental infarct and individual properties of a host, may be responsible for the initial retention of injected cells and their subsequent survival. Pathophysiological factors in the infarcted myocardium, such as inflammatory cytokines, hypoxia, reactive oxygen species, or unbalanced supply of nutrition, may influence the outcome of cell therapy (25). The qualitative properties of transplantable cells may be another factor responsible for the restoration of heart function. Muscle-derived progenitors, which possess a high capacity to regenerate both skeletal and cardiac muscle, are among the most exploited cells used for improving the efficiency of injured heart function (23). These cells were shown to be able differentiate into cardiomyocytes *in vitro* (26) and *in vivo* (27). It is supposed that the main limitation of skeletal myoblasts as a therapeutic agent for the treatment of chronic and acute heart damage is their inability to express gap junction proteins (28). Regardless the data that cultured skeletal myoblasts were found to express N-cadherin and connexin-43,

the major proteins responsible for mechanical and electrical coupling, their expression was downregulated following cell intramyocardial implantation (29). It was reported that skeletal myoblasts improved cardiac function by preventing ventricular dilation and preserving matrix architecture in the remote region, likely mediated by paracrine effects (30).

Therefore, our studies of skeletal myogenic stem cell engraftment show that obtained differences in the improvement of heart function by transplanted cells may be attributed to different levels of cell repopulation of the damaged myocardium. Moreover, the rabbit cardiac functional parameters may vary depending on the individual.

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Conclusions

The transplanted autologous skeletal muscle-derived myogenic cells were proved to be able to survive in the acute infarction site; however, the level of cell integration varied. These differences in integration may account for the differences in cardiac function recovery. Establishment of strategies applying physical, biochemical, and genetic methods aimed at an improvement in stem cell integration and survival in damaged tissues is currently underway.

Statement of Conflict of Interest

The authors state no conflict of interest.

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