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Evaluation of myocardial regenerative potential in cardiosurgery of middle-aged and elderly patients

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Summary

High mortality from cardiovascular diseases, due to the low regenerative potential of the myocardium, requires the search for new therapeutic approaches for the treatment of cardiac patients. Aim of the present work was to assess the regenerative potential of myocardial cells in cardiac surgery patients of middle and older age.

Materials and methods

The biopsy samples of the myocardial auricles were destroyed by enzyme technique. Confocal microscopy was performed on cells cultivated in the primary culture and on a suspension of fixed cells. In addition, histological and electron microscopic studies of myocardial biopsies were performed.

Results

Regenerative ability of **cardiac cells** was estimated by the presence of cardiac stem cells (CSCs), their progeny, transitory amplifying cells (TACs) inside the colonies, as well as by the presence of "cell-in-cell structures" (CICSs), which were found in each biopsy sample. In this study, *in vitro* experiments demonstrated the release of TACs from the vacuoles of non-encapsulated CICSs of the heart auricles of cardiac patients.

Conclusion

The data obtained enable us to evaluate the level of cardiomyogenesis in each individual patient. Moreover, they open up prospects for the possible use of *in vitro* expanded autologous TACs as a cell product for the treatment of cardiovascular diseases.

Keywords

Cardiosurgery, myocardial biopsies, cardiac stem cells, transitory amplifying cells, cardiomyocytes, "cell-in-cell structures".

Introduction

There is no strict consensus on ability of self-renewal and regeneration of myocardial cells in mammals. For a long time, high mortality rates in heart disorders due to low regenerative potential of myocardial cells were explained by inability of mature cardiomyocytes (CMs) to divide. Meanwhile, further studies in the field revealed cardiac stem cells (CSCs) of three distinct types (c-kit$^+$, Sca-1$^+$- and Isl-1$^+$) [1-4], showing a myogenic potential of resident CSCs, thus suggesting their participation in self-renewal and, moreover, regeneration of myocardium [5, 6]. However, absence of distinct evidence for CSC contribution to cardiomyogenesis in adult mammals, and the results of Porrello et al. [7] on ability of myocardial reconstitution after 20%-dissection of left ventricle in newborn mice led to the hypothesis about regeneration of damaged mammalian myocardium *via* division of mature CMs rather than by proliferation of CSCs which, however,

loose this ability within first week of life. Moreover, some workers presume that the CSCs, in particular, c-kit$^+$-cell population, are absent in adult mammalian myocardium [8]. At the same time, other authors suggest that mature CMs are able to enter the cell cycle after undergoing a de-differentiation step, and to produce new progeny [9, 10].

However, the data obtained by Koudstaal et al. (2013) and Malliaras (2019) confirm participation of c-kit$^+$ CSCs in cardiomyogenesis and consider different approaches to their stimulation aiming for regeneration of damaged myocardium [11, 12].

Participation of CSCs in heart metabolism and cardiomyogenesis in steady state and following myocardial ichemia was also confirmed in Sechenov Institute of Evolutionary Physiology and Biochemistry of Russian Academy of Sciences (IEPhB RAS), Almazov National Medical Research Centre and Pavlov University. Animal studies have shown that the renewal of resident CMs in mammals may proceed from CSCs throughout life in three ways: (1) by means of CSC proliferation within colonies with the formation of transitory amplifying cells (TACs) followed by their differentiation to CMs [13, 14]; (2) by means of intracellular development of CSCs with the formation of encapsulated "cell-in-cell structures" (CICSs) [15, 16], and (3) by intracellular proliferation inside mature CMs with formation of the capsule-free CICSs [17].

Of note, the presence of some cells within other cells resulting into CICSs was revealed as early as 100 years ago for immune cells (cytophagocytosis and emperipolesis) and, later, for malignant cell populations (entosis) and at the sites of inflammation [18]. So far, however, such intracellular patterns were not described in myocardium in terms of CSC development. Unlike Overholtzer and Brugge [19] who suggested entosis to be a form of cancer cell death, the studies by Belostotskaya et al. [17] provide first evidence for intrinsic role of intracellular CSC division in renewal and regeneration of **myocardium**.

Despite some proofs of CSC-mediated cardiomyogenesis obtained in animals of different ages, and in adult female 45 y.o. [16] there are no available data on CSCs proliferation in the older and elderly patients with heart disorders.

The present study was aimed for search and identification of proliferating myocardial cells from heart biopsies of the patients of different age groups who underwent cardiosurgery at the Pavlov University. Experimental results supporting our cardiomyogenesis concept were obtained in IEPhB RAS.

Materials and methods

We have studied cardiac biopsies taken in 24 patients of different age groups (47 to 80 years old) operated over the period of 30 June 2021 to 6 December 2021 with following diagnoses: ischemic heart disease (IHD), 15; aneurism of *a.ascendens*, 3; acquired heart defects, 5; hypertrophic cardiomyopathy, 1. Under artificial blood flow, the IHD patients underwent coronary bypass; in the patients with aortic aneurism the resection of aneurism replaced by a vascular prosthesis with a valve conduit (Bentall operation) and coronary implants. In acquired heart defects, replacement of heart valves, or plastics of mitral valve were performed, and Morrow septal myoectomy was made in hypertrophic cardiomyopathy. When connecting the heart-lung machine, a purse-string suture was placed at the atrial appendage toperform venous cannulation. The biopsy of a fragment from

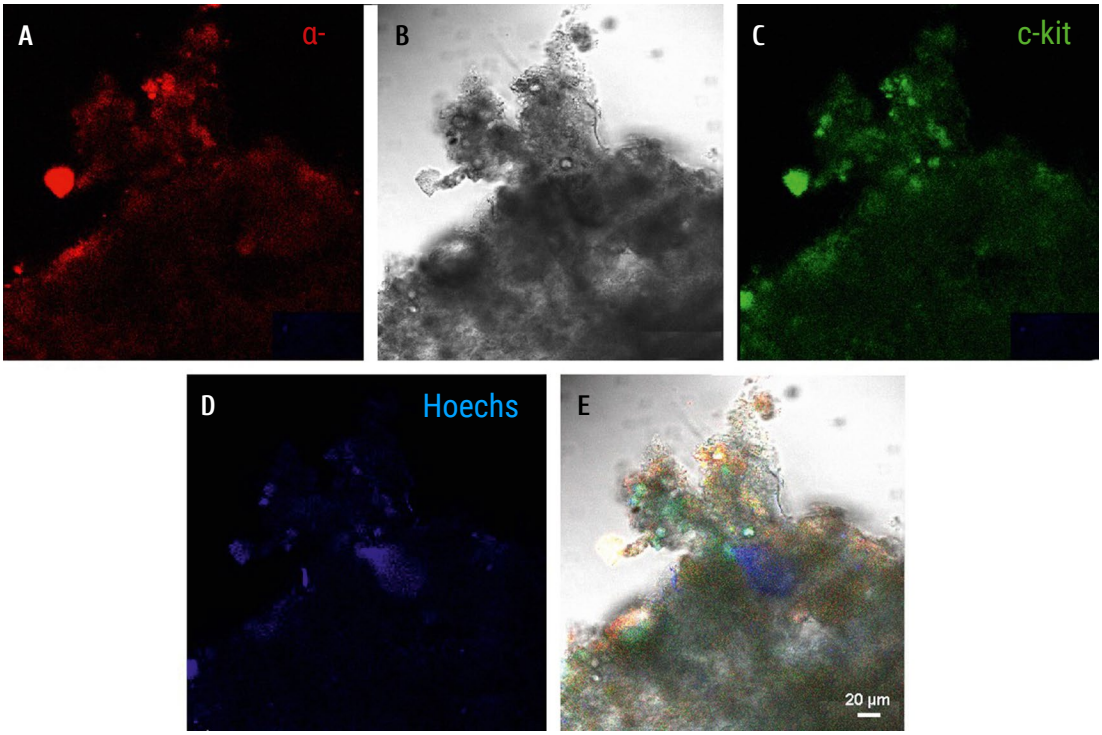

Figure 1. Confocal microscopy of the cells from atrial appendage of a men (74 y.o.) after enzymatic treatment. A, at λ=532 nm (red fluorescence); B, in light transmitted light; C, at λ=496 nm (green fluorescence; D, at λ=405 nm (nuclei, blue light), and E, composite

atrial appendage was made in the center of purse-string suture immediately before the venous cannula was installed. The biopsies of right atrial appendage were performed from the center of purse-string suture immediately before introduction of venous cannula. A portion of biopsy sample was sent to the Laboratory of Morphology (Pavlov University), a the rest of a sample was subject to electron microscopy at the IEPhB of RAS.

The remaining sterile bioptate (a portion of right atrial appendage) was dissected into the fragments 1 to 1.5 mm in size. After its transfer to a tube with sterile saline, the tissues were brought to the IEFB, in order to perform enzymatic disintegration of myocardial fragment in sterile box by a standard protocol according to Lam et al. [18]. The pieces of myocardium were rinsed in Ringer solution (NaCl, 146 mM; KCl, 5 mM; $CaCl_2$, 2 mM; $MgCl_2$, 1 mM; dextrose, 11 mM; HEPES, 10 mM HEPES, pH 7.4), minced and incubated in the same solution supplied with collagenase type IA (Sigma, 1 mg/mL), and trypsin (Biolot, Russia, 0.12%) for 20-30 min. at 37°C. The suspension was then centrifuged at 1500 rpm for 10 min. Subsequent cultivation was performed by transfer of tissue to the warmed DMEM nutrient medium with 10% embryo calf serum (Biolot, Russia) supplied with 50 ME/mL penicillin and streptomycin (50 mcg/mL, Biolot, Russia). The cells were cultured in 35-mm glass Petri dishes (BioVitrum LLC). Incbation was performed in the CO_2 incubator (Binder, Germany) at 5% CO_2, humidity 95%, and 37°C. The medium was changed twice a week without reseeding of cells.

The cultures were observed by means of inverted light microscope (PIM-III, WPI, USA) using a digital camera (Leica DFC300 FX, Germany) with objectives of x4, x10, x25.

To perform confocal microscopy, freshly isolated cells in suspension or cultured cellular monolayer were fixed by means of 2.5-4% paraformadehyde, permeabilized for 10 min. in phosphate buffer with 0,25% Triton X-100. The c-kit$^+$ CSCs were detected by means of FITC-conjugated commercial reagents (Abcam) diluted to 1:100. Cardiac origin of the cells was confirmed with monoclonal antibodies to α-actinin (Sigma-Aldrich) conjugated with Alexa 532, according to Zenon technology (Invitrogen). The cellular nuclei were stained with Hoechst dye 33342 (Molecular Probes, USA, 10 mcg/mL) diluted to 1:1000. The cells were stained at room temperature. Confocal microscopy was performed at the Resource Center of St. Petersburg University using a Leica TCS SP5 microscope with objectives of x10, x25 and x40 (oil), and with a confocal microscope Leica TCS SP5 (x10 and x25 objectives) in IEPhB. According to our concept, we carried out observations of CSCs, their progeny (colonies of TACs), non-encapsulated CICSs, presenting as vacuoles containing TACs within cardiac cells, or as free TACs – containing vacuoles (Fig. 1).

Results

Light microscopy of the heart biopsies stained by H&E has revealed characteristic histological patterns typical to the patients subjected to cardiosurgery. One may see cardiomyocytes with transverse striation, focal sclerosis regions, and fat tissue associated with connective tissue sprawls; intermuscular sclerosis and scarce diffuse lymphocytic infiltration, as well as extended vessel lumens with some full-blooded vessels. The cardiomyocytes are partially hypertrophic, with thickened fibres and enlarged nuclei, looking edematous (Fig. 2).

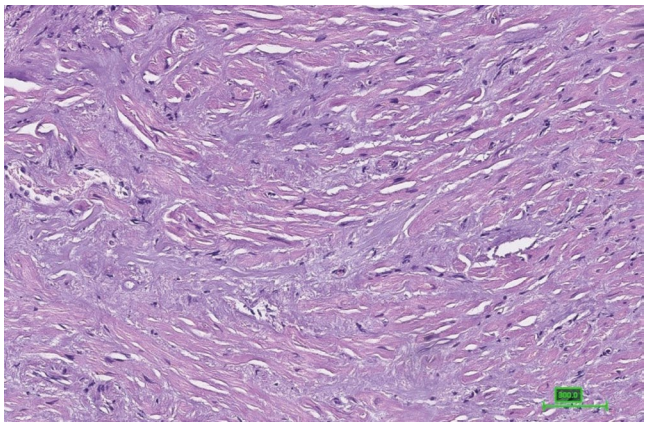

Figure 2. Histological pattern of a sample from right atrial appendage (H&E staining), patient K. (78 y.o.)

Transmision electron microscopy (TEM) of the samples from right atrial appendages made at the IEPhB has shown multiple polymorphic changes in the atrial myocardiocytes. The ultrastructural tissue pattern included multiple polymorphic alterations of contractile structures in the atrial cardiomyocytes, reactive/destructive alterations of cellular organelles, pronounced fibrosis of intercellular connective tissue. Ultrastructural pathology of microcirculatory blood vessels is, generally, characterized by dystrophic changes of endothelium and inhomogeneity of basal membrane (from thinning to notable slerosis). A fragment of contractile myocardiocyte and features of its sarcoplasmic organelles are presented at the Fig. 3.

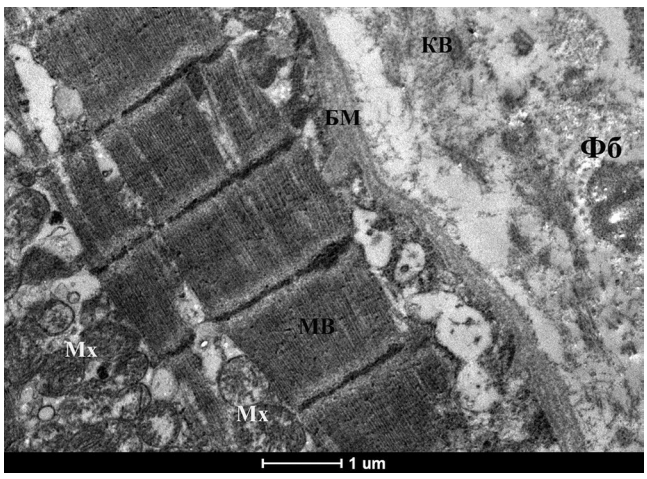

Figure 3. Patient S (75 y.o.). A cardiomyocyte from the right atrial appendage exhibits hypertrophy of the muscle fibres with urregular thickening of Z-membrane and accumulation of small rounded mitochondria with electron-dense matrix. From the outside, the cell is coated with multilayer basal membrane which separates it from the connective tissue with rich collagen bundles and fibroblasts. TEM picture, 11500x.

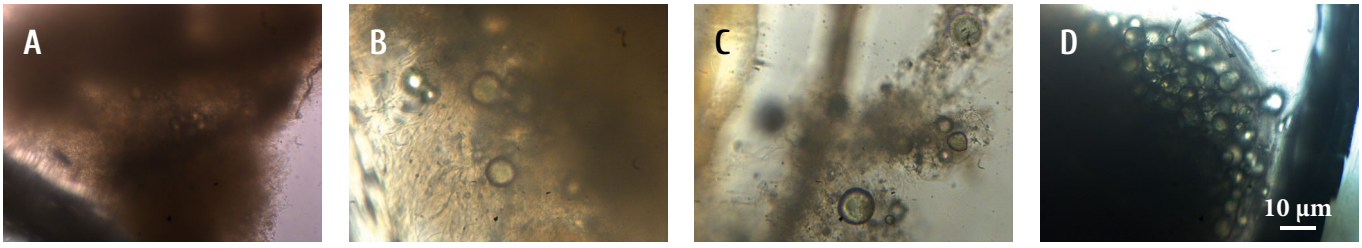

Figure 4. Increased numbers of non-encapsulated vacuoles, released during cultivation of the bioptate from atrial appendage of a female (47 y.o.) at different terms after enzymatic treatment.

A, immediately after 30-min. enzyme treatment; B and C, following 3 days in culture; D, after 30 days in culture.

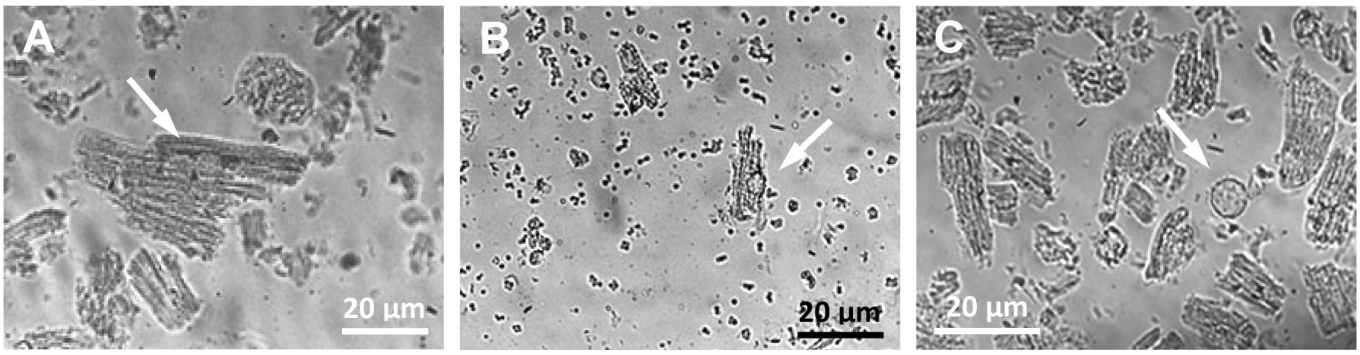

Figure 5. The non-encapsulated cell-in-cell structures (CICS) and freely-moving TAC-vacuoles in freshly isolated cell suspension from the left ventricle myocardium and atrial appendage cells. Patient Z (80 y.o.)

A, two vacuoles within cardiomyocyte (arrow); B, a vacuole within an atrial cardiomyocyte; C, free vacuole with transitional cell (TK) inside (arrow).

Fig. 4. demonstrates an increased number of non-encapsulated CICSs derived from the atrial appendage after enzyme treatment and primary culture for different time periods.

When studying cell suspensions isolated from the atrial appendage and left ventricle (24 clinical cases, the samples of 70-150 mg), we have found spheroid structures in all the preparations. They were described as free vacuoles which have been previously shown to emerge within mature CMs after intracellular development of CSCs [17]. These structures evolve into the non-encapsulated CICSs released as TACs-containing vacuoles from the CMs (Fig. 5, A,B) . Daily light microscopy of the primarily cultured cells derived from the biopsies allowed us to observe the heterogenous vacuoles released from the CICS (Fig. 5, C).

These vacuoles, when released from the atrial appendages by enzyme treatment, may increase in their size due to division of small cells (5-12 μm in diameter) inside them. They are stainable by the stemness marker (c-kit) and cardiomyocyte-specific marker (α-actinin), thus being assigned to the CSC progeny differentiated to cardiac lineage (Fig. 1). Moreover, detection of c-kit$^+$ colonies (Fig. 6) confirms the proliferation of CSCs in myocardium of the patients at 70-80 years old.

The variants of vacuoles from non-encapsulated CICSs are shown in Fig. 7 (A-O). The vacuoles are present in males and females of 47 to 78 years old. Meanwhile, the differences are evident in size and number of vacuoles in the samples presented. Variable size of the cell-derived vacuoles from the patients of different age may result from CSC proliferation

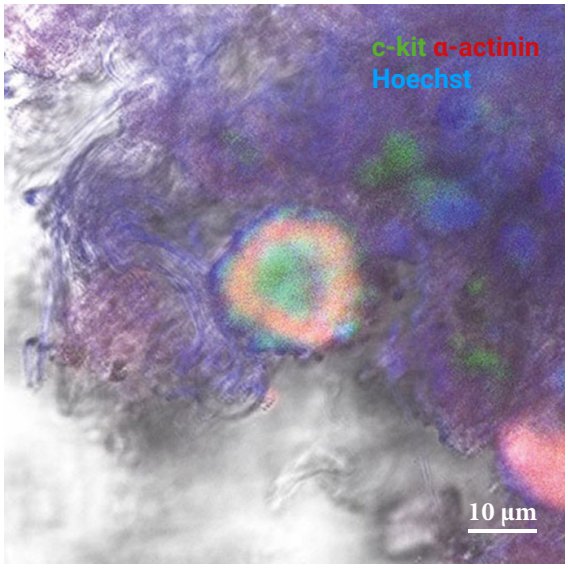

Figure 6. A colony of TACs presumed to be the progeny of c-kit$^+$ CSCs (green) showing a marker of *in vitro* differentiation to myocardial lineage (α-actinin, stained red). Patient K., male, 74 y.o.

within these structures, thus correlating with multiple small cells revealed within large vacuoles (Fig. 7, E, J) in atrial appendages from males (47 and 78 y.o., respectively). Moreover, the data allow us to note that the size of CSC vacuoles is increased upon culturing the biopsy material, either from males (Fig. 7, E, J) or females (Fig. 7, M,O) as well as opening of the CSC vacuoles followed by release of TACs (Fig. 7, D).

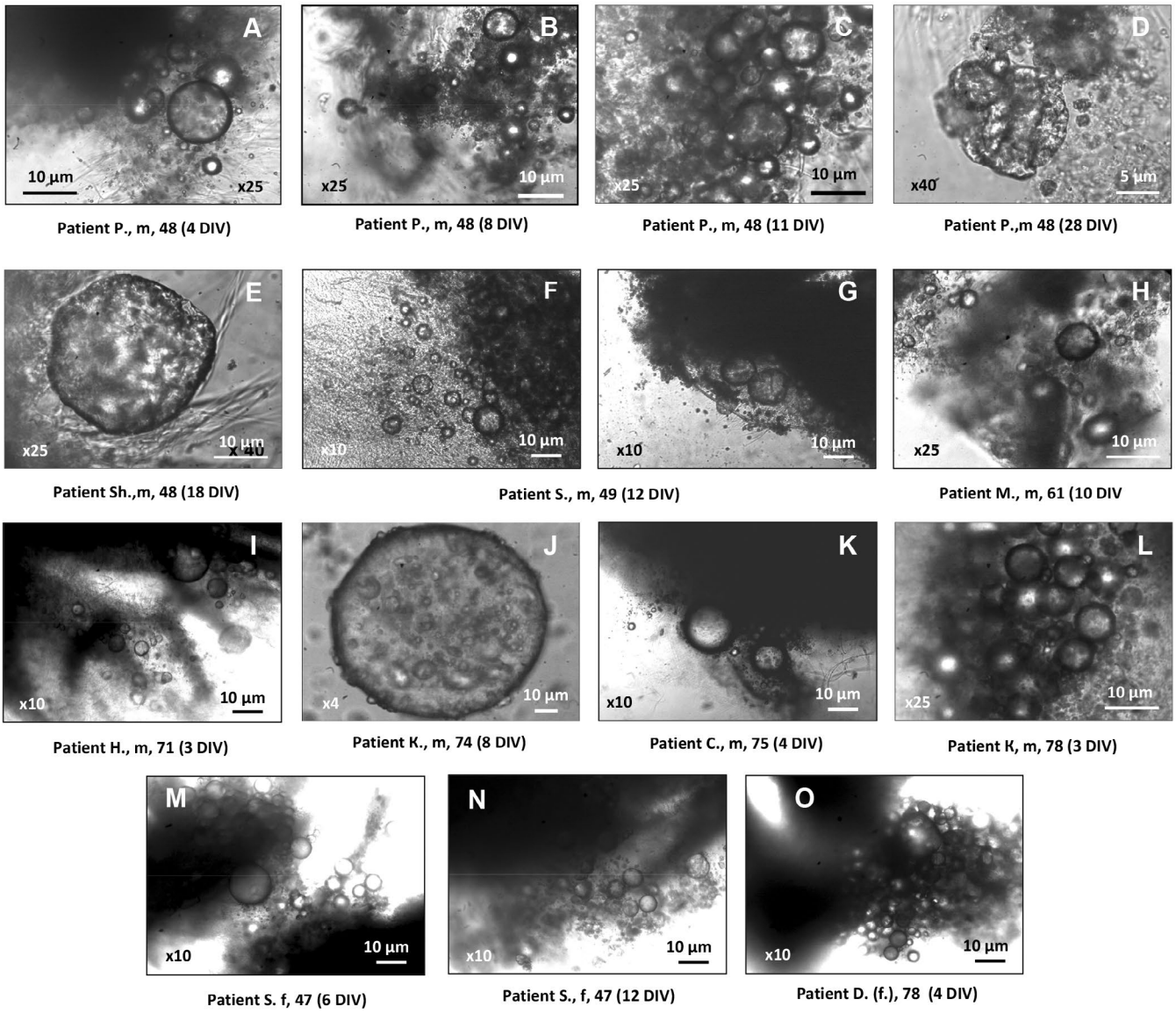

Figure 7. Non-encapsulated cell-in-cell structures (CICS) from the left atrial appendages of operated patients at different age and gender (m, males; f, females) observed in primary cultures at different terms (DIV, days *in vitro*). Light microscopy.

Confocal microscopy of the fragmented atrial appendages from 2 elderly patients detects c-kit-positive CSCs inside vacuoles (Fig. 8).

Meanwhile, a release of TACs was also shown (Fig. 9), both upon long-term cultivation of atrial appendage cells from a young patient (A, B), and in fresh myocardial suspension of an elderly patient (C), thus suggesting the existence of some regenerative potential in human myocardium over the entire lifespan.

However, some sufficient differences are observed when comparing the abundance of CSC-related vacuoles. E.g., a lot of vacuoles was observed in samples C, F, H, L, O, in contrast to their scarcity in the samples A, B, I, K (Fig. 7). Moreover, large amounts of small CSC-related vacuoles in younger patients, males or females (pictures B, F, G, N, Fig. 7) may be caused by low proliferative activity of TACs within the vacuoles thus suggesting independence of these events on age or gender of the patients. However, such effects may be caused by the distinct disorders in the patient, being independent on age or gender factors. Anyway, these issues may be cleared by further experiments with different methods of enzymatic treatment for the atrial appendages taken from surgical patients with different heart disorders.

Discussion

Long-term studies of age-dependent regenerative ability of mammalian myocardium were performed in Wistar rats in IEPhB RAS and Almazov National Medical Research Centre [13, 14, 15]. Similar studies were carried out in both healthy animals and rats following experimental myocardial infarction and ischemia [17]. The present cooperative study was performed at the Pavlov University and IEPhB RAS using myocardial fragments of the patients with different cardiac disorders. For the first time it was concerned to assessment

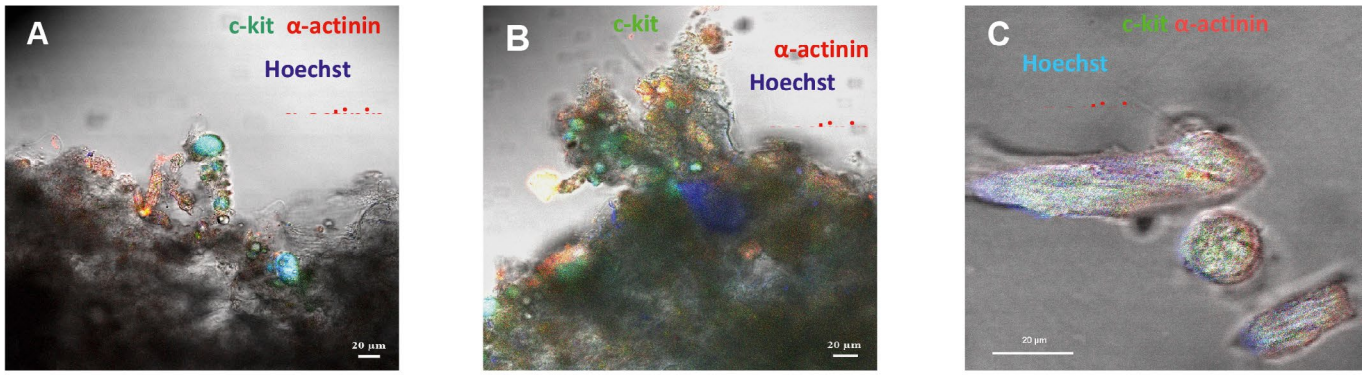

Figure 8. Confocal microsopy of the presumed CSC-containing vacuoles from the atrial appendages: patient K (male), 74 (A, B); patient Z (fem), 80 (C). Scales 20 μm.

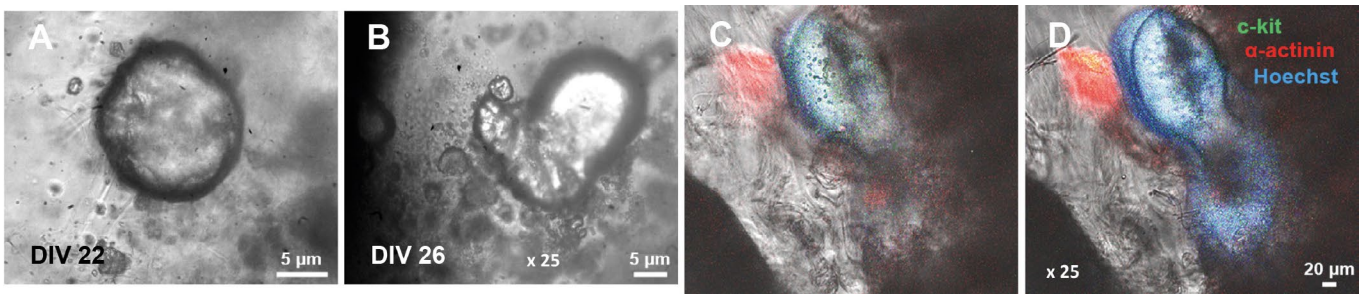

Figure 9. Opening of vacuoles from the non-encapsulated CICSs in the primary myocardial culture of patient Sh., male, 48 (A, DIV 22; B DIV 26); in cell suspension of atrial appendage from patient K., male, 74 (C, D), with triple-stain labeling.

A, B – Light microscopy. C, D – Confocal microscopy.

of regenerative potential in the persons of different gender and age (47 to 80 years old). Extent of cardiomyogenesis in surgical patients was evaluated by detection of stem cells and clusters of TACs, i.e., their progeny in myocardium which may be related to the 1st variant of cell multiplication and differentiation inside CSC colonies, according to our concept (see Introduction). Encapsulated CICS, i.e., 2nd variant of CSC-mediated cardiomyogenesis, were not revealed in the samples from middle-aged and old patients. However, when observing the *in vitro* cultures of cells isolated from atrial appendages, we have revealed intracellular CSC development within atrial cardiomyocytes, resulting into non-encapsulated CICS (3rd variant of CSC multiplication).

However, long-term culturing of atrial cells has shown that development of CSCs within mature atrial CMs with CICS formation represents the main way of TAC reproduction. One may suggest that the encapsulated CICSs may emerge in the cardiosurgical samples from newborns and infants, like as the encapsulated CICSs with intracellular CSCs within immature cells [17]. Prevalence of the non-encapsulated CICSs over TACs production inside the observed colonies may be dependent on poor conditions for CSC proliferation in the patients with severe heart disorders, e.g., ischemic heart disease, acquired heart defects, aortal aneurism, thus making the CSCs to migrate to the mature CMs and multiply within intracellular vacuoles which, upon maturation, could contain a big number of TACs. We guess that the CSC proliferation in mature CMs with development of non-encapsulated CICSs may supply numerous TACs of sufficient maturity for regeneration of myocardium.

Therefore, our data may be interpreted in view of sufficient cardiomyogenesis in the individual patients. In practical aspect, we may suggest injection of the own (autologous) *in vitro* expanded TACs from atrial appendages into the patient's myocardium. Such therapeutic option is confirmed by our results showing increased size of TACs-containing CICSs from young patient of 48 years old (Fig. 7, E) and old patient of 74 years old (Fig. 7, J). Due to individual ability for such proliferation mode, the cell cultures of intrasurgical biopsies would provide selection of those patients eligible for this type of therapy.

Previously, we have already considered potential clinical usage of autologous encapsulated CICSs [21]. However, after detection of non-encapsulated CICSs and their *in vitro* cultivation [17], we suggest that application of CSC-containing vacuoles from non-encapsulated CICSs would provide a more effective cardiomyogenesis, due to larger amounts of more differentiated TACs inside the vacuoles.

The idea of using the autologous myocardial cells for heart regeneration following infarction and ischemia occurred soon after CSC detection [1, 22]. As early as in 2004, Messina et al. [23] proposed to perform intramyocardial injections of *in vitro* produced clusters (cardiospheres) from non-differentiated cells of atrial or ventricular biopsies of murine of human origin. In this respect, special attention was drawn to three clinical trials (SCIPIO, CADUCEUS, Allstar), which studied the opportunity of cardiac cells usage in order to boost myocardial regeneration after heart infarction or ischemia. In the CADUCEUS program (autologous

transplants) and Allstar trial (with allogeneic cells), injections of cells obtained from cardiospheres were associated with myocardial regeneration, decreased size of myocardial scars, and expansion of functional tissues [24]. However, the results of Allstar-trial published 6 months later did not show reduction of scars in left ventricle by the cardiosphere treatment [25]. Meanwhile, other studies in CADUCEUS trial have shown a positive effect of cardiosphere therapy (smaller scare size and improved myocardial function) at 6 and 12 months after the infarction [26, 27]. Moreover, the SCIPIO Trial has shown that application of freshly isolated autologous c-kit$^+$-CSCs in ischemic cardiomyopathy was associated with significant improvement of global and regional left ventricular function, reduced infarction size, and increased area of viable tissue thus suggesting a regenerative effect [28].

Stimulation of TACs proliferation under the ***in vivo*** conditions may be considered an alternative regeneration mode. Meanwhile, the possibility of using growth factors and cytokines for this purpose was earlier suggested [21], followed by assessing the effects of apoptotic bodies from CMs (AbBc) in collaborative studies with A.I. Tyukavin. It was shown that the AbBc stimulates the development of TACs inside the cell colonies in primary cultures [29]. Hence, a hypothesis is proposed that the AbBc contain a RNA complex that stimulates the proliferation of CSCs and the subsequent their differentiation into mature CMs [30].

Conclusion

The present study was performed by the staff of Pavlov University, IEPhB RAS and St. Petersburg State University using the biopsies from cardiosurgical patients with different heart disorders. For the first time, it concerned assessment of potential for myocardial regeneration in the patients of different gender and age groups (47 to 80 y.o.). Cardiac stem cells (CSCs) and their progeny transitory amplifying cells (TACs) were considered as primary substrate for cellular regeneration. These populations have been shown to multiply and differentiate towards cardiomyocytes, either as colony-forming cells, or within "cell-in-cell structures" (CICSs). We have found CSCs with c-kit$^+$ marker in each biopsy of atrial appendages from young and aged patients (up to 80 y.o.). We did not show any distinct dependence between the numbers of revealed structures, stem cells, and patients'age or gender. We were not able to reveal any encapsulated CICSs typical for the 2nd mode of CSC proliferation, probably, due to the age factor. The next tasks include following technical items: to perform exact counting of CSCs, TACs and CICS, to provide their soft isolation from the suspension and *in vitro* expansion. Optimal ways should be found for clinical application of this promising autologous biomaterial in order to enhance myocardial regeneration in the most urgent clinical cases.

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Conflict of interest

None declared.

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Оценка регенеративного потенциала миокарда у кардиохирургических пациентов среднего и старшего возраста

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Резюме

Высокая смертность от сердечнососудистых заболеваний, обусловленная низким регенеративным потенциалом миокарда, требует поиска новых терапевтических подходов для лечения кардиологических больных. Цель работы: изучить наличие регенеративного потенциала сердца у кардиохирургических пациентов среднего и старшего возраста.

Материалы и методы

Биопсийные образцы миокарда ушек предсердий разрушали с помощью ферментов. Конфокальную микроскопию проводили на культивируемых в первичной культуре клетках и на суспензии фиксированных клеток. Кроме того были выполнены гистологические и электронно-микроскопические исследования биоптатов миокарда.

Результаты

Клеточный компонент регенерации в виде кардиальных стволовых клеток (КСК), их потомков – транзиторных клеток (ТК) в составе колоний, а также в виде «структур клетка-внутри-клетки» (СКВК) обнаружены в каждом биопсийном образце. В данном исследовании в экспериментах *in vitro* продемонстрировано высвобождение ТК высокого уровня зрелости из вакуолей бескапсульных СКВК ушек сердца кардиохирургических пациентов.

Заключение

Полученные данные не только позволяют ориентировочно оценивать уровень кардиомиогенеза каждого конкретного больного, но и открывают перспективы возможного использования *in vitro* размноженных аутологичных ТК в качестве клеточного продукта для терапии сердечнососудистых заболеваний.

Ключевые слова

Кардиохирургия, биопсии миокарда, кардиальные стволовые клетки, транзиторные клетки, кардиомиоциты, структура «клетка-внутри-клетки».