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Mobilization of stem cells into the peripheral blood in children with congenital heart disease

Magdalena Borówka[1], Wojciech Stycuła[1], Elżbieta Kamycka[2], Zbigniew Madeja[2], Ewa Zuba-Surma[2], Janusz Skalski[1], Jacek Kołcz[1]

[1]Klinika Kardiochirurgii Dziecięcej, *Collegium Medicum* Uniwersytetu Jagiellońskiego, Kraków
[2]Zakład Biologii Komórki, Wydział Biochemii, Biofizyki i Biotechnologii Uniwersytetu Jagiellońskiego, Kraków

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Abstract

The results of stem cell (SC) research in recent years have revealed real possibilities related to their use in the treatment of heart failure. The mobilization of different types of stem and progenitor cells is stimulated by stress factors. The presence of congenital heart defects and corrective surgery is associated with strong stressors that may stimulate the mobilization of SC. This work presents the preliminary results of research on stem cell mobilization of endothelial progenitor cells (EPCs), hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), and very small embryonic-like (VSEL) stem cells in children undergoing surgical correction of congenital heart defects. Peripheral blood samples were collected from 21 children. The quality and quantity of stem cells were analyzed by classical and imaging (image stream) flow cytometry, while gene expression was evaluated through quantitative real-time RT-PCR. We determined the expression of genes characteristic of SC. Relevant statistical methods were used to assess the influence that some pathophysiological and demographic factors have on stem cell mobilization. In children with congenital heart defects, the mobilization of different types of SC is observed. The degree of mobilization depends on the pathophysiology of the defect and the patient's age. Strong stress factors related to surgical correction of the defect significantly correlate with the degree of stem cell mobilization.

Key words: stem cells, congenital heart disease.

Streszczenie

Badania nad komórkami macierzystymi (ang. *stemcells* – SC) otwarły możliwość wykorzystania ich w leczeniu skrajnej niewydolności serca. Mobilizacja różnych typów komórek macierzystych i progenitorowych stymulowana jest przez bodźce stresowe. Obecność wady wrodzonej serca i jej korekcja wiążą się z działaniem silnych stresorów mogących stymulować mobilizację tych komórek.

W pracy badano mobilizację śródbłonkowych (EPC), krwiotwórczych (HSC), mezenchymalnych (MSC) oraz podobnych do embrionalnych (VSEL) komórek macierzystych u dzieci operowanych z powodu wady serca. Próbki krwi pobierano od 21 dzieci. Obecność badanych populacji komórek oceniano jakościowo i ilościowo (cytometrią klasyczną i obrazową). Oceniano również ekspresję genów charakterystycznych dla komórek macierzystych i progenitorowych. Wyniki analizowano odpowiednimi metodami statystycznymi w odniesieniu do wieku, patofizjologii wady oraz danych okołooperacyjnych.

Poziom mobilizacji komórek VSEL (p = 0,0006), EPC (p = 0,02) i HSC (p = 0,01) dodatnio korelował z młodszym wiekiem pacjenta. Obecność sinicy istotnie zwiększała mobilizację MSC (0,038), HSC (p = 0,014) i VSEL (p = 0,03) po korekcji wady. Geny związane z EPC (VE-kadheryna, vWF) wykazywały największą aktywność po reperfuzji i jej zmniejszenie w kolejnych punktach pomiarowych. Najwyższy poziom aktywacji genów pluripotencji (*Oct-4*, *Nanog*) oraz progenitorów sercowych (*GATA4*, *Nkx2.5*) stwierdzono 24 godz. po operacji. W okresie pooperacyjnym stwierdzono dodatnią korelację mobilizacji EPC z czasem trwania krążenia pozaustrojowego (p = 0,03) i czasem zaklemowania aorty (p = 0,04) oraz ujemną korelację z indeksem katecholamin (p = 0,009).

U dzieci z wadami wrodzonymi serca obserwuje się mobilizację różnych typów SC. Stopień mobilizacji komórek macierzystych i progenitorowych zależy od patofizjologii wady oraz wieku pacjenta. Bodźce stresowe towarzyszące korekcji chirurgicznej wady istotnie korelują ze stopniem mobilizacji komórek macierzystych.

Słowa kluczowe: komórki macierzyste, wrodzone wady serca.

Address for correspondence: Magdalena Borówka, Klinika Kardiochirurgii Dziecięcej, *Collegium Medicum* Uniwersytetu Jagiellońskiego, ul. Pod Strzechą 29, 31-352 Kraków, e-mail: magdaboroowka@gmail.com

Introduction

Congenital heart defects occur in approx. 0.8-1.0% of live-born infants. Owing to the development of prenatal diagnostics, perinatal care, and congenital cardiac surgery, the prognosis for this population has improved significantly within the last few decades. The long-term survival rate of children with complex heart defects, burdened with the highest perioperative risk, is 80-90% [1]. Despite the satisfactory quality of life of the majority of these patients, a significant percentage of the population develops heart failure during the long-term postoperative period. There are only several treatment options available for this group [2]. The limited access to mechanical circulatory support systems and the insufficient number of organ donors stimulate the research concerning the use of various types of stem cells in the treatment of end-stage circulatory failure. There is a small number of reports regarding the use of stem cells in patients with congenital heart defects. Significant efforts have been made to find methods of acquiring and utilizing stem cells in the treatment of cardiac failure, aimed at achieving the best therapeutic effect possible. One of the studied hypotheses states that multipotent stem cells are recruited to blood from different organs (mainly from bone marrow) by tissue-specific signals coming from damaged sites and are homed to the organs sending the signals for regenerative purposes [3].

This work presents the preliminary results of research concerning the mobilization of various types of stem and progenitor cells in children with congenital heart defects. The analysis included the mobilization levels of endothelial progenitor cells (EPCs), hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), and very small embryonic-like (VSEL) stem cells. In order to confirm the results acquired through flow cytometry, the activity level of pluripotency genes (*Oct-4*, *Nanog*) as well as cardiac (*GATA4*, *Nkx2.5*) and endothelial (VE-cadherin, vWF) progenitors in patients undergoing cardiac surgery was assessed. It was demonstrated that, in children with congenital heart defects, various types of stem and progenitor cells are recruited to the blood.

Tab. I. Description of the patient group included in the study

Variable	Mean ±SD	Median (min-max)
age (days)	884 ±1561	165 (5–4876)
body mass (kg)	9.1 ±10.8	5,5 (2.7-51)
ECCT (min)	129.2 ±67.2	110 (75-355)
AoCT (min)	60.2 ±29.7	56 (0-125)
CAI (µg/kg/min)	2.8 ±2.5	3.5 (0-8)
ICU stay duration, days (mean ±SD)	12 ±33.2	10.5 (2-28)
Initial number of stem cells	(Absolute number of cells/ml)	
MSC	17.6 ±21.7	12.2 (4-70)
VSEL cells	41 ±36.9	30 (4-157)
HSC	1563 ±3485	495 (13-16081)
EPC	10.2 ±12.8	5.8 (3-48)

Material and methods

The study included 21 children aged from 5 days to 13 years who underwent surgical treatment of congenital heart defects at the Pediatric Heart Surgery Clinic of the Jagiellonian University Medical College (Tab. I). The research was partially funded by a grant from the Polish National Science Center (No. 2011/01/B/NZ5/04246) and received consent of the Bioethics Committee of the Jagiellonian University (No. KBET/176/B/2010). The group included 11 patients with non-cyanotic defects (atrial septal defect, ventricular septal defect, atrioventricular septal defect) and 10 patients with cyanotic defects (tetralogy of Fallot, hypoplastic left heart syndrome, tricuspid atresia). All patients underwent elective surgical correction of congenital heart defects with the use of extracorporeal circulation. None of the patients experienced any complications during their postoperative stay in the ICU. The necessity of using medications supporting the circulatory system correlated with the degree of heart dysfunction. On the basis of the amount and quality of the drugs used, the catecholamine index (CAI), utilized in postoperative intensive care, was calculated in order to compare the doses (expressed in µg/kg/min) used in individual patients [CAI = dopamine dose + dobutamine dose + 100 × (adrenaline dose + noradrenaline dose)]. In these patients, as part of routine hematological and/or biochemical labeling, blood samples (1-2 ml) were taken immediately before surgery (induction of general anesthesia), during extracorporeal circulation, as well as in the fourth, eighth, twelfth, and 24th postoperative hour.

Cytometric analysis of peripheral blood cells

The samples of peripheral blood collected at each timepoint were centrifuged (10 minutes, 350 × g). The separated cells were then twice subjected to erythrocyte lysis by means of Lysing Buffer 1× (BD PharmLyse, BD Pharmingen 555899), in order to restore the complete white blood cell fraction. The eukaryotic cells of peripheral blood acquired in this manner were rinsed with PBS buffer and suspended in immunofluorescence staining buffer (containing 2% fetal bovine serum; FBS). The leukocyte fractions were stained with cocktails of appropriate monoclonal antibodies coupled with fluorochromes, identifying the presence of stem cell subpopulations: VSEL cells (CD45–/Lin–/CD133+), HSCs (CD45+/Lin–/CD133+), EPCs (CD45–/dim/Lin–/CD133+/KDR+), and MSCs (CD45–/Str–1+/CD105+) (Fig. 1-3). The antibodies used are listed in Table II.

The samples were stained for 30 minutes; the cells were then rinsed and preserved in a 2% solution of paraformaldehyde (15 min). Immediately before cytometric analysis, a nuclear stain, 7-actinomycin D, was added to each sam-

Fig. 1. Representative images of human stem cells acquired with the image stream technique. VSEL cells – very small embryonic-like cells, EPCs – endothelial progenitor cells. Other abbreviations are explained in the text (*Material and methods*)

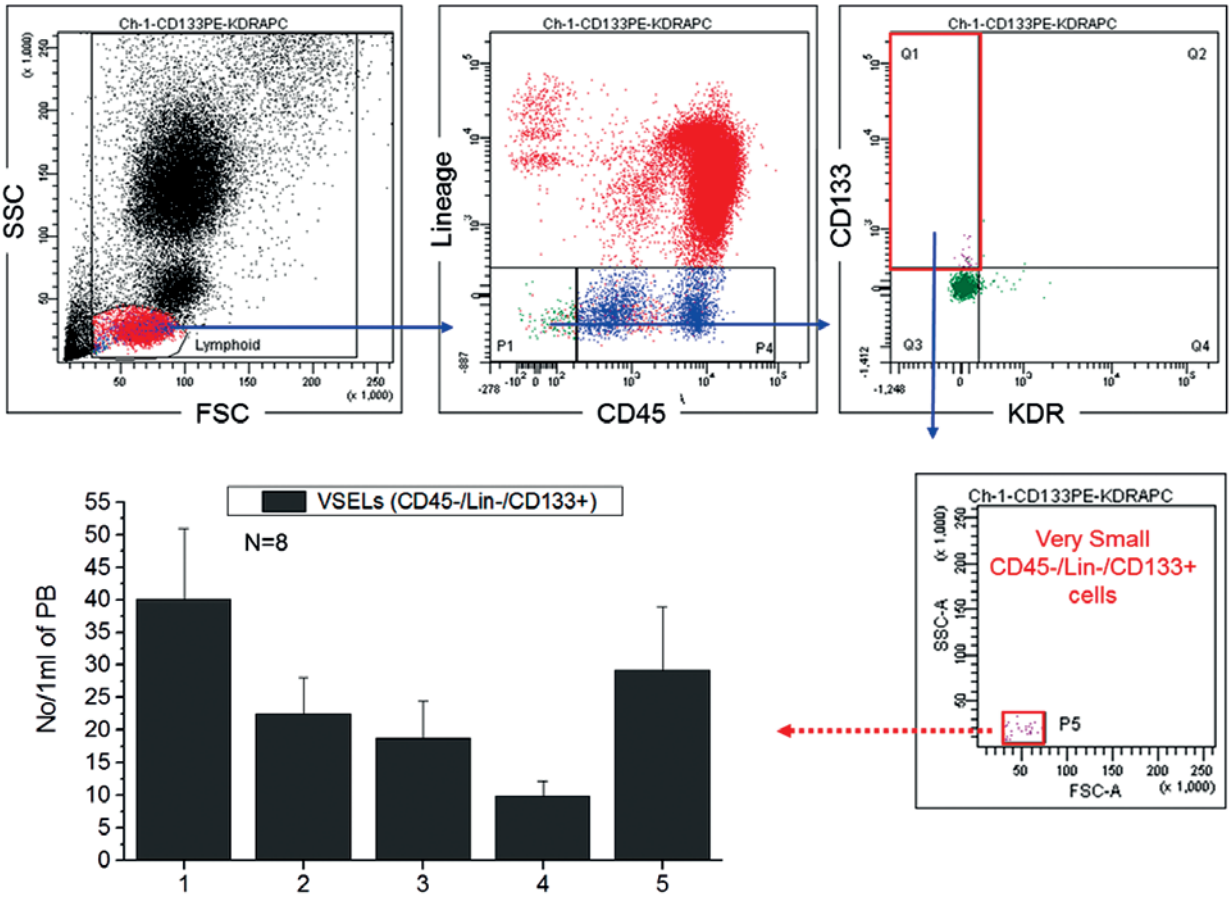

Fig. 2. VSEL cell mobilization level in children undergoing surgical correction of congenital heart defects. VSEL cells – very small embryonic-like cells

ple (7-AAD; BD Bioscience, 559925). The cells prepared in such a way were analyzed by means of an LSR II flow cytometer (BD Bioscience), analyzing the rare stem cell fractions within the fraction exhibiting the presence of nuclei (7-AAD+).

The results were presented as: i) the percentage of cells with a particular phenotype within the leukocytic fraction, and ii) the absolute number of stem cells circulating in a given volume of peripheral blood (calculated on the basis of leukocytosis values for each patient, examined at a given time-point).

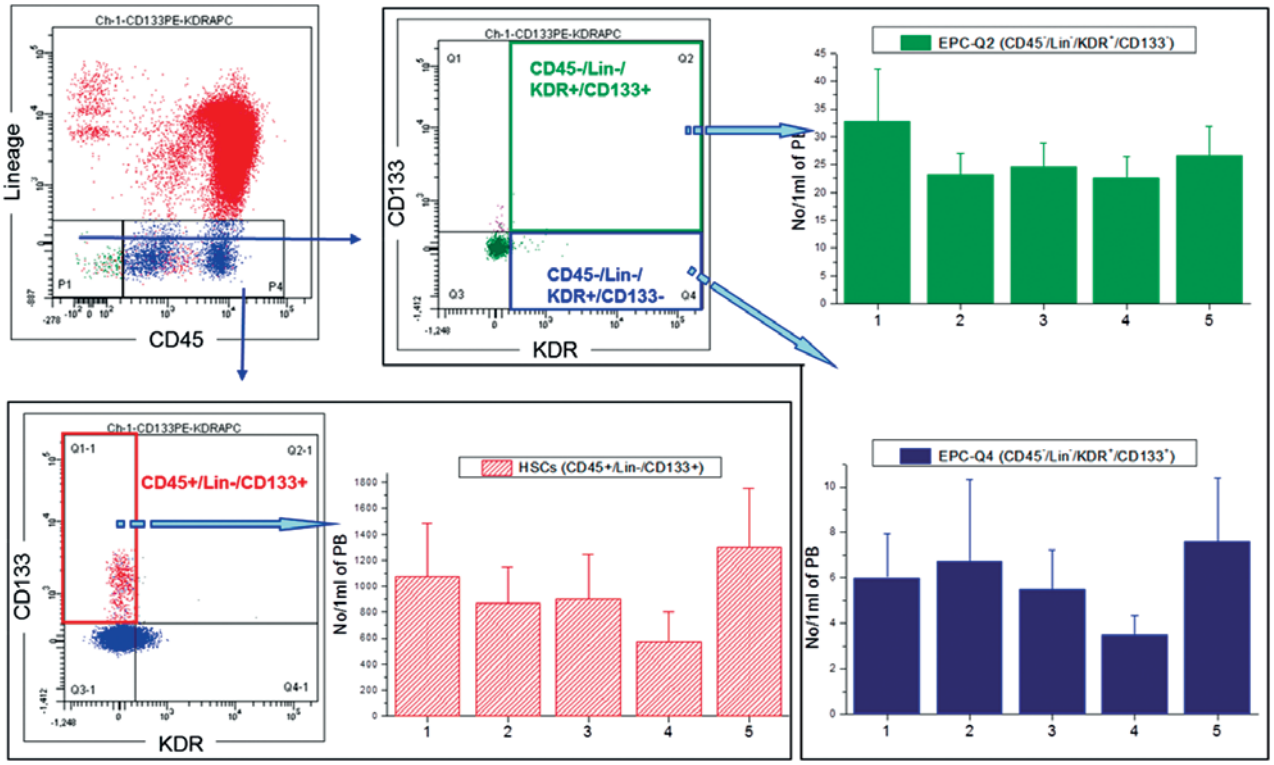

Fig. 3. The level of mobilization of endothelial progenitor cells (EPCs) and hematopoietic stem cells (HSCs) in blood during cardiac surgical treatment

Tab. II. List of monoclonal antibodies used for cytometry staining

Antibody-specific antigen	Fluorochrome
CD45	Pacific Blue
antigens of mature hematopoietic cells (CD2, CD3, CD14, CD16, CD19, CD24, CD56, CD66b, CD235a)	FITC
KDR (VEGF R2)	PE
CD133	APC
CD105	PE
Stro-1	Alx-647

Genetic analysis of the peripheral blood cells of patients

Peripheral blood leukocyte samples were secured for genetic analyses by freezing them (-80°C) in RLT Plus buffer (Qiagen, 1053393) containing 1% of the Bond Breaker reagent (Thermo Scientific, 77720). Subsequently, total cellular RNA was isolated from the secured samples using the GeneMATRIX Universal RNA/miRNA Purification Kit (Eurx, E3599) according to the manufacturer's protocol. cDNA was acquired through reverse RNA transcription, using TaqMan Reverse Transcription reagents (Applied Biosystems, N808-0234). The cDNA acquired in this manner was analyzed in real-time quantitative polymerase chain reaction (qPCR) in order to gain quantitative information concerning the level of mRNA expression in the studied genes. The reaction was conducted using the SYBR® Green PCR Master Mix reagent (Life Technologies, 4309155) and the 7500 Fast Real-Time PCR system instrument (Applied Biosystems). The transcript expression of the following genes was analyzed: Nanog, Oct-4, GATA-4, Nkx2.5, vWF, VE-cadherin, and β2-microglobulin (housekeeping gene). Using the ΔΔCT method, the changes in the level of mRNA expression at individual time-points were calculated, with a control sample harvested from a patient before surgery (Fig. 4).

Descriptive analysis methods were employed for statistical analysis. The data were presented as mean ± standard deviation, median, and value range. The normality of distributions was analyzed with the Shapiro-Wilk test. Differences between the mean values of variables with normal distribution were evaluated using Student's *t*-test. The remaining continuous variables were examined with the Mann-Whitney test with regard to the differences in their distribution. In order to establish the correlation between the studied clinical parameters and the level of stem cell mobilization, simple linear regression was employed. The level of $p < 0.05$ was considered as statistically significant.

Results

In classic or image stream flow cytometry, individual stem and progenitor cell populations were imaged using the previously described methods [4]. Examples of VSEL cell and EPC images created with the image stream technique are presented in Figure 1.

The number of VSEL cells (p = 0.0006), EPCs (p = 0.02), and HSCs (p = 0.01) circulating in peripheral blood significantly correlated with the age of the patient. The highest level of cell mobilization to peripheral blood was found in infants. The number of circulating cells decreased in proportion to the growing age of patients. The children in whom signs of both volume and pressure overload of the heart were found in the preoperative period exhibited a significantly higher number of circulating HSCs (p = 0.04). Similarly, the occurrence of cyanosis before corrective surgery had a significant influence on the mobilization of HSCs (p = 0.014), MSCs (p = 0.038), and VSEL cells (p = 0.03) after defect correction during ICU stay (24 hours after surgery).

Genes associated with EPCs (VE-cadherin, vWF) exhibited peak activity after reperfusion; their activity decreased rapidly at the subsequent measurement points. The highest level of activation of pluripotency genes (*Oct-4*, *Nanog*) and cardiac progenitors (*GATA4*, *Nkx2.5*) was reported 24 hours after surgery. In the postoperative period, a significant positive correlation was found between the level of EPC mobilization and the duration of extracorporeal circulation (p = 0.03) as well as aortic cross-clamping (p = 0.04), while the correlation with the catecholamine index was reported as negative (p = 0.009) (Fig. 4).

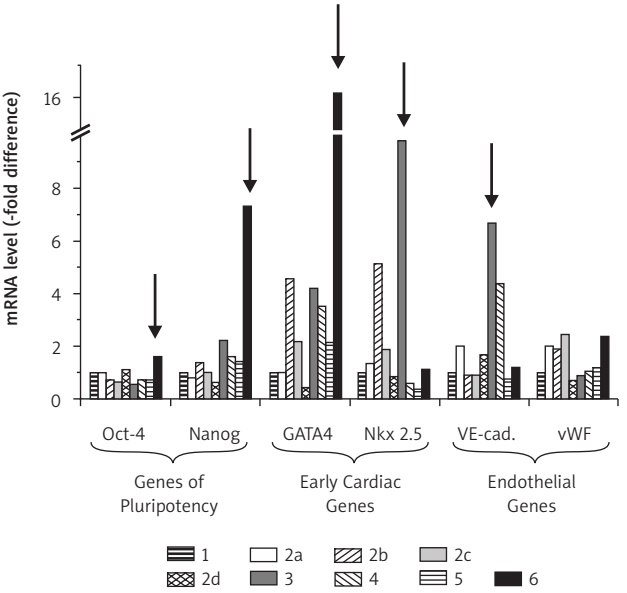

Fig. 4. Activation of pluripotent cell genes, cardiac progenitors, and endothelial progenitors during cardiac surgical treatment. Sample taken 1 – before surgery, 2 – during surgery, 2a – during cooling, 2b – during hypothermia, 2c – during heating, 2d – after stabilizing the patient and turning off the extracorporeal circulation, 3–4 h after surgery, 4–8 h after surgery, 5–12 h after surgery, 6–24 h after surgery

Discussion

Within the last decade, the concept of treating heart failure using stem cells has gained importance and found clinical applications [5]. This strategy was mainly used in adults with ischemic heart disease. Stem cells of different origin were administered to patients through coronary vessels, peripheral vessels, or directly into the cardiac muscle [6]. Even though the mechanisms behind the activity of stem cells are not fully known, there are many indications pointing to their significant clinical effects [7]. The improvement of heart function reported in these cases is attributed to various mechanisms [8]. Apart from the observed differentiation of the cells and their structural integration with tissue (partial substitution of dead cells), these effects are attributed to the combination of signals transmitted by cytokines and growth factors as well as to the process of angiogenesis, the inhibition of apoptosis, fibrosis, and inflammatory response, and the marked activation of local cardiac progenitor cells [9].

The growing problem of treating end-stage heart failure in patients with congenital heart defects stimulates research concerning the potential role of stem cells in this group of patients. The pathological processes leading to myocardial damage are different in the case of congenital defects than in the case of defects described in adult patients. Considerable importance is attributed to immunological, inflammatory, and cytotoxic mechanisms as well as to the processes associated with apoptosis, tissue reconstruction, and fibrosis. The use of stem cells in the pediatric population has been scarce [10, 11]. Both the methods for acquiring cells for therapeutic uses and the most efficient ways of administering them to children are yet to be established. Our material indicated that stem and progenitor cells were present in the peripheral blood of the studied population before the corrective surgery of congenital heart defects, as well as during and after the surgery. The preoperative factors which significantly influence the level of mobilization of stem and progenitor cells include the following: the age of the child (VSEL cells, HSCs, EPCs), concomitant pressure and volume overload (HSCs), and cyanosis, which significantly affects the mobilization level of MSCs, HSCs, and VSEL cells. Moreover, the level of EPC mobilization correlated with significant intraoperative stress factors, such as the duration of extracorporeal circulation and aortic cross-clamping in hypothermia. An evaluation of gene activity demonstrated that the genes of pluripotent cells and the genes of cardiac progenitor cells reached their highest activity around the 24th postoperative hour (Fig. 4). One of the studied mechanisms of providing stem cells to damaged tissue is the recruitment of these cells to blood through tissue-specific mediators and their homing to the damaged tissue [12]. Therefore, enhancing the mobilization of stem cells, e.g. through pharmacological agents, and their movement to damaged tissue may help solve the problem of their acquisition and utilization. It was demonstrated that the use of phosphodiesterase inhibitors (sildenafil) causes a significant increase of stem cell mobilization in patients with pulmonary hypertension [13].

Endothelial progenitor cells (EPCs) are released from bone marrow and may participate in the initiation of angiogenesis, regeneration of vascular endothelium, improvement of myocardial perfusion, and stimulation of

the production of cytokines, which exert a paracrine effect on regenerative mechanisms (stimulation of cardiac progenitor cells) [14, 15]. In our material, the genes associated with EPCs appear first - immediately after the activation of coronary circulation and heart reperfusion. The level of endothelial progenitor mobilization was inversely proportional to the catecholamine index and directly proportional to the duration of extracorporeal circulation and aortic cross-clamping. This phenomenon may reflect the level of endothelial dysfunction occurring during extracorporeal circulation. In the early postoperative period, the catecholamine index, which constitutes the total dose of catecholamine necessary for the maintenance of adequate cardiac output, is closely associated with the level of dysfunction of the heart and vascular endothelium. The value of this factor reflects the level of circulatory failure. In adult patients with significantly dysfunctional systemic ventricles (reduced ejection fraction, higher troponin level) and intensified symptoms of congestive circulatory failure, lower numbers of released stem and progenitor cells were reported [16, 17]. Even though it is not possible to exclude the pharmacological activity of the used catecholamines, the negative correlation between the catecholamine index and the number of cells mobilized to the blood in our material confirms this result in the population of children with congenital defects. Moreover, the level of mobilization of stem and progenitor cells may serve as a marker for myocardial and vascular endothelial damage, as well as a prognostic factor for cardiovascular failure.

Mesenchymal stem cells (MSCs) constitute a developmentally early population of bone marrow cells, providing a stroma for the development of other stem cells. They have the ability to differentiate into muscle cells, osteoblasts, chondrocytes, and adipocytes [18]. *In vitro*, they can differentiate into spontaneously contracting cardiomyocytes [19]. When used in patients with end-stage heart failure caused by previous myocardial infarction [20] they improved left ventricular function, enhanced myocardial reconstruction and the inhibition of myocardial fibrosis, and increased the density of blood vessels [21]. In the studied population, the presence of preoperative cyanosis (chronic hypoxia) constituted a stimulus exerting significant influence on MSC mobilization. As a non-specific stimulus, it also had a significant influence on the mobilization of VSEL cells and HSCs. A similar correlation was found with regard to the age of the patient. The highest percentage of VSEL cells, EPCs, and HSCs was found in infants. The number of these cells was lower in older patients. The presence of various types of stem cells in the peripheral blood of patients undergoing cardiac surgery suggests the possibility of enhancing the mobilization of these cells by various stimuli (paracrine, pharmacological). Taking into consideration the existence of paracrine effects and the stimulation of residual cardiac progenitor cells in inefficient organs, strategies increasing the mobilization of these cells and their penetration into organs could also influence the future directions for research.

Conclusions

In children with congenital heart defects, the mobilization of various types of stem and progenitor cells to peripheral blood can be observed. This mobilization is dependent on the pathophysiology of the defect and the age of the patient. The stressors accompanying surgical defect correction significantly correlate with the level of cell mobilization.

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