The role of muscle tissue in the pathogenesis of chronic heart failure — the potential of exposure (FORMA study)

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Aim. To determine whether the skeletal muscle of patients with chronic heart failure (CHF) retains the ability to regenerate and grow; to compare the effectiveness of long aerobic trainings, calculated by an individualized method, and conventionally calculated trainings (VO_2peak values), in relation to the severity of heart failure, exercise tolerance (ET), and ergoreflex activity (ERGO).

Material and methods. The study included 297 patients with stable III functional class (FC) CHF, receiving optimal therapy. The presence of heart failure was found in all patients at least 6 months before the start of the study (age — 18-65 years, body mass index (BMI) — 19-28 kg/height, m^2. Initially, the study performed a cardiorespiratory test (CRT) with an assessment of gas composition, acid-base balance of the blood and ERGO activity. Patients were randomized into 2 groups: experimental (EG) and control (CG). For EG, based on the determination of the lactate threshold (LT), after 1 and 3 months the CRT was repeated and the training walking mode was dynamically recounted according to the new LT level. For CG, the training walking mode was calculated based on the VO_2peak values. All patients trained for 6 months. At the end of the training, diagnostic CRT was performed, and the activity of EGO was evaluated. Eleven patients with CHF and 3 healthy donors before the start of the training underwent a biopsy of the gastrocnemius muscle.

Results. It was shown that the potential for muscle differentiation of satellite skeletal muscle precursor cells obtained from patients with CHF with a reduced ejection fraction (HFrEF) does not differ in vitro from the potential of satellite cells of healthy donors. After 6 months of training, the severity of CHF decreased to FC II in 75% of EG patients, and among CG patients — in 44%; the main indicators of the stages of compensatory mechanisms activation during physical exertion (VO_2LT and VO_2peak) in EG increased more than in the CG (10,8±0,4, 18,7±0,7 ml/min/kg and 9,5±0,8, 15,3±0,9 ml/min/kg, with $p_1 < 0,01$, $p_2 < 0,05$, $p_3 < 0,01$, respectively).

Conclusion. In vitro, the potential for muscle differentiation, regeneration and growth of satellite skeletal muscle precursor cells obtained from patients with HFrEF does not differ from the potential of satellite cells of healthy donors. Aerobic training in patients with III FC chronic heart failure calculated by definition of LT, relating to safety is not worse than the results calculated by the level of VO_2peak. Aerobic training in patients with III FC chronic heart failure calculated by definition of LT, compared with the usual mode of training walking, significantly reduce the activity of ergoreflex, increase ET, reduce the severity of CHF. In patients with III FC CHF, training walking for more than 1,5 hours/day determined by the level of LT, contributes to the development of physiological reverse myocardial remodeling to a greater extent than aerobic training calculated by the conventional method.

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The prevalence of heart failure (HF) in the Russian Federation has reached the epidemic [1, 2]. By evidence-based medicine, effective methods to combat this pathology were developed, including basic medications: angiotensin-converting enzyme inhibitors (ACE inhibitors), angiotensin II receptor antagonists (ARA II), angiotensin receptor-neprilysin inhibitors (ARNi), beta-blockers (BB), mineralocorticoid receptor antagonists (MCRA). However, to date, it has not been possible to stop the rapid increase in the number of rehospitalizations due to decompensated HF, which significantly burdens the economies of the countries.

Despite the inhibitory effect of BB, ACE inhibitors, ARA II, ARNi, MCRA, neurohumoral activation in HF is increased due to continuous peripheral afferent stimulation (enhanced ergoreflex activity).

One of the possible points of application for HF stabilization is striated muscle tissue. Stimulation of molecular mechanisms for skeletal muscle regeneration, including physical rehabilitation, is a promising strategy to reduce muscle dysfunctions. Therefore, it seems relevant to determine whether the skeletal muscles in HF patients retain their ability to regenerate and grow. Data on such studies was not found.

As any organ or tissue in HF, skeletal muscles suffer from a lack of oxygen and nutrients. There are following differences: muscle tissue is the largest organ by mass in human — 40-45% of body weight; muscles have a special feedback system called "ergoreflex".

Between the skeletal muscles on the one hand and the vasomotor and respiratory centers on the other hand, there are neurogenic connections that are mediated by ergoreceptors. Ergoreceptors are myelinated and non-myelinated afferent nerve fibers in the skeletal muscles, sensitive to all mechanical and metabolic changes in muscle fibers. Ergoreceptors play a major role in feedback control to maintain a balance between muscle load intensity and energy for this. Ergoreflex is a defensive mechanism of the body in response to metabolite accumulation in muscle fiber, aimed at removing metabolites and enhancing aerobic oxidation. In response to the muscle metabolic state, ergoreceptors modulate the intensity of muscle perfusion and the cardiorespiratory response to physical activity in order to meet the metabolic needs of contracting muscles. So, there is an increase in ventilation and a number of circulatory changes due to an enhanced sympathetic nervous system (SNS) activity — increase of heart rate and blood pressure (BP), contraction of the resistance vessels (Fig. 1).

Thus, skeletal muscle is not only the largest organ by mass in the human body, but also an organ that controls the activity of the cardiovascular and pulmonary systems by means of ergoreflex (Fig. 1). However, data on effective influencing methods is currently contradictory. The only and most physiological way to reduce the ergoreflex activity is exercise training (ET).

Physical therapy (PT) in HF patients should be used to improve exercise tolerance and quality of life, reduce the number of hospitalizations for decompensated HF [1, 2]. Currently, individual selection of the type, duration and intensity of physical activity in HF patients is an urgent problem.

There were following aims of the study: 1) to determine whether the skeletal muscle in HF patients retains the ability to regenerate and grow; 2) to compare the effectiveness of individualized and conventional (based on VO_2peak) approaches to selecting exercise mode, in relation to the severity of HF, exercise tolerance, and ergoreflex activity.

Materials and methods

Gastrocnemius muscle biopsy and assessment of muscle-resident cells. Eleven HF patients (mean age 54±12,5 years, body mass index (BMI) — 26,5±6,4 kg/m^2, left ventricular ejection fraction (LVEF) 26,4±1,4%) and 3 healthy donors underwent gastrocnemius muscle biopsy. The preparation of primary muscle-resident cell cultures enriched in satellite cells was performed according to the standard methods [3]. Preparing Geltrex-coated (Invitrogen, USA) culture dishes was performed for 1,5 h in a CO_2 incubator at +37°C in a Dulbecco's Modified Eagle's medium (DMEM) in a ratio of 1:100. The culture

medium was changed every other day. Myogenic differentiation of cells was performed according to the standard methods [3, 4] when cultured in a differentiation medium consisting of a basic culture medium (α-MEM) (PanEco, Russia) with the addition of 1% L-glutamine (Invitrogen, USA), 1% Penicillin-Streptomycin (Invitrogen, USA) and 2% horse serum (Gibco, USA). The primary medium was replaced with a differentiation one when subconfluent state of the culture was observed. During immunocytochemistry, the cells were washed with phosphate buffered saline (PBS) and fixed with 4% paraformaldehyde at +4°C for 10-15 minutes, washed with PBS, incubated with 0,2% TRITONx100 for 5 minutes, washed with PBS, blocked with 15% fetal calf serum for 30 minutes (Gibco, USA) in PBS. Incubation with primary and secondary antibodies were perfomed according to the manufacturer's instructions (MF20 antibodies to the myosin heavy chain (MHC MF20), myogenic factor 5 (Myf5), mitofusin-1 (Mfn1), PAX transcription factors, R&D BioSystems, USA). Immunophenotyping was performed by CytoFLEX flow cytometer (Beckman Coulter). Data was analyzed using CytExpert 2.0 software (Beckman Coulter).

Isolation of ribonucleic acid (RNA), synthesis of complementary deoxynucleic acid (cDNA) and real-time polymerase chain reaction (PCR). Total RNA was isolated using ExtractRNA reagent (Evrogen, cat.no BC032, Russia). cDNA was synthesized from 500 ng total RNA using a reverse transcription kit (Molove, SK021, Russia). Quantitative gene expression was performed using qPCR-HS SYBR + ROX (Evrogen, cat.no. PK156, Russia). Data of qPCR are presented as arbitrary units of mRNA expression normalized to GAPDH expression and expression levels in a reference sample.
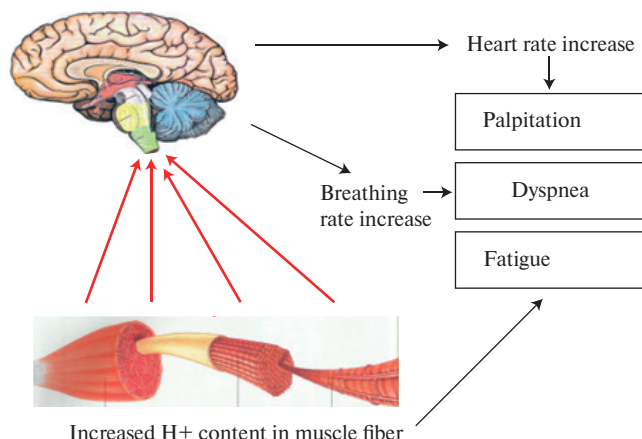
Statistical analysis was performed using GraphPadPrism7 software. All data were analyzed by at least three biological replicates and presented as mean±SEM.

Safety and effectiveness of different exercise methods was assessed as part of the FORMA study. A prospective, randomized study was performed in accordance with Good Clinical Practice guidelines and the principles of Declaration of Helsinki; the study protocol was approved by the ethics committee of the Almazov National Medical Research Center. There were following inclusion criteria: symptoms of class III HF; stable clinical status for at least 2 weeks before inclusion in the study; age — 18-65 years; body mass index (BMI) — 19-28 kg/m^2; completed informed consent; the ability to perform cardiorespiratory test (CRT); LVEF <45%; administration of ACE inhibitors/ARA II/ARNi, BB, MCRA, diuretics; patient education during hospitalization at Almazov National Medical Research Center; follow-up monitoring of HF patients by a cardiologist. Exclusion criteria were moderate and severe chronic obstructive pulmonary disease (COPD), myocardial infarction (MI), pulmonary embolism (PE), surgeries over the past 6 months, severe cognitive disorders, low adherence treatment.

The endpoints of the study were changes in the HF severity, exercise tolerance (VO$_2$peak), ergoreflex, and myocardial contractile function (LVEF, LV end-diastolic dimension (LV EDD), LV end-systolic dimension (LV ESD)).

Clinical characteristics of patients. The study included 297 patients with stable class III HF, which was established at least 6 months before the study. Patients were randomized into two groups: the experimental group (EG) — 237 patients with class III HF (age 18-65 years, BMI 19-28 kg/m^2) and control group (CG) — 60 patients with HF (age 18-65 years, BMI — 19-28 kg/m^2). After 4-6 weeks of exercise, 55 EG patients on their own initiative gradually increased the duration of daily walk to 1,5-2 hours; this subgroup of patients (EGlong) was allocated for additional analysis (Table 1).

Therapy did not differ significantly between groups. Results of clinical and instrumental examinations are presented in Table 1.

The study progress is presented in Table 2. Initially, the subjects underwent a submaximal CRT with a simultaneous assessment of gas composition and acid-base status of the blood (Table 2).

For each EG patient, the exercise mode of walk was estimated according to the CRT results based on the lactate threshold (LT) determination; after 1 and

Fig. 1. The role of muscle tissue in HF pathogenesis.

Table 1

Clinical characteristics of patients

Parameter	Experimental group			Control group	P			
	EG	EGpres	EGlong		EG and CG	EGpres and EGlong	EGpres and CG	EGlong and CG
Demographic characteristics								
Total number of HF patients, n	237	182	55	60				
Age, years, M±m	53,1±4,2	52,3±5,0	57,3±6,5	51,0±6,1	>0,05	<0,05	>0,05	<0,05
Men, n (%)	176 (75)	133 (75)	52 (93)	36 (60)	<0,05	<0,05	>0,05	<0,05
BMI, kg/m^2, M±m	27,5±0,5	27,0±0,9	28,1±1,3	26,2±2,8	>0,05	<0,05	>0,05	<0,05
Etiology of HF								
CAD, n (%)	158 (67)	129 (70)	29 (53)	35 (58)	>0,05	<0,05	<0,05	>0,05
DCMP, n (%)	79 (33)	53 (30)	26 (47)	25 (42)	>0,05	<0,05	<0,05	>0,05
Concomitant pathology								
AF, n (%)	29 (12)	22 (13%)	7 (11%)	6 (10%)	>0,05	>0,05	>0,05	>0,05
Anemia, n (%)	12 (5)	10 (5%)	2 (4%)	5 (8%)	<0,05	>0,05	<0,05	<0,05
COPD, n (%)	85 (36)	67 (35%)	18 (30%)	24 (40%)	<0,05	<0,05	<0,05	<0,05
High tech treatments								
CRT, n (%)	52 (22)	41 (23%)	11 (20%)	9 (15%)	<0,05	>0,05	<0,05	<0,05
CABG, n (%)	73 (30)	61 (34%)	12 (23%)	19 (28%)	>0,05	<0,05	<0,05	<0,05
Left ventricular ejection fraction								
LVEF,%	30±1,3	29±1,5	30±3,5	32±3,3	>0,05	>0,05	>0,05	>0,05
Medication, maximum tolerated doses								
ACE inhibitors/ARA II/ARNi, n (%)	237 (100)	182 (100)	55 (100)	60 (100)	>0,05	>0,05	>0,05	>0,05
Beta-blockers, n (%)	237 (100)	182 (100)	55 (100)	60 (100)	>0,05	>0,05	>0,05	>0,05
MCRA, n (%)	212 (90)	163 (90)	51 (93)	54 (91)	>0,05	>0,05	>0,05	>0,05
Diuretic therapy								
Diuretics, n (%)	237 (100)	182 (100)	55 (100)	60 (100)	>0,05	>0,05	>0,05	>0,05

Abbreviations: BMI — body mass index, AF — atrial fibrillation, COPD — chronic obstructive pulmonary disease, CRT — cardiac resynchronization therapy, CABG — coronary artery bypass grafting, LVEF — left ventricle ejection fraction, CG — control group, EG — experimental group, EGpres — EG subgroup with preserved load during physical rehabilitation, EGlong — EG subgroup with long-lasting exercise.

3 months the CRT was repeated and on the basis of the newly obtained LP values, the mode was re-estimated (walking speed was 95% of the LT speed) [5, 7]. Patients trained for 6 months. At the end of the exercise, a diagnostic CRT was performed. CG patients performed walking at the level of 55% VO$_2$peak 3 times/week. Echocardiography was conducted using Philips iE-33. We used one- and two-dimensional scanning modes, by which the transverse dimension of the left atrium (LA), EDD, ESD, and LVEF were assessed. The CRT was performed using treadmill (GE Medical Systems Information Technologies) and Oxycon Pro system (Jeger, Germany).

Venous blood lactate concentration at rest and during physical exertion. Before the CRT, the catheter was inserted into the ulnar vein. Blood sampling was carried out initially and every minute during the physical exertion. Venous blood lactate concentration was evaluated by i-STAT Portable Clinical Analyzer (Abbott, USA) using CG4 cartridge kits. LT was recorded at the time of the beginning of

Table 2

Progress of FORMA study

Initially	1 m	3 m	6 m
Cardiorespiratory test	+	+	+
Echocardiography	+		+
Assessment of ergoreflex	+		+
Assessment of HF class	+	+	+
Assessment of adverse events	+	+	+

blood lactate concentration increase [5-7]. The assessment of ergoreflex was carried out by post-exercise regional circulatory occlusion (PE-RCO) [8]. During the test, diastolic blood pressure (DBP) was measured; ventilation and gas exchange rates were recorded. The difference between DBP, carbon dioxide ventilatory equivalent (VE/VCO$_2$), minute ventilation (VE) after a three-minute occlusion (+PE-RCO) and the recovery period without occlusion (−PE-RCO) was calculated; percentage ratio of these values was estimated.

Statistical analysis was performed using Statistica 6.0 software. All data were analyzed by at least three biological replicates and presented as mean+SEM. Comparison of mean values was performed using nonparametric statistics (Mann-Whitney U-test). The chi-squared test and the F-test were used to identify confidence in contingency tables. The significance level was p<0,05.

Results

Examination of stem cell population obtained by skeletal muscle biopsy. After isolation of cells and several days of in vitro expansion, we analyzed the expression of surface markers: CD56, CD105, CD166, CD146, CD73, CD140a, CD140b; CD45 was used as a negative control (Fig. 2). We showed that the vast majority of the isolated cells were CD56-positive (marker of satellite cells) and CD45-negative (marker of hematopoietic cells). We also found that a significant fraction of cells expressed stromal markers CD105, CD166 and CD73, and only a small fraction of cells was positive for markers CD146, Cd140a and CD140b. The high level of expression of stromal markers in the population was most likely associated with contamination of the satellite cell fraction with the stromal cell fraction of muscle tissue. Therefore, an immunocytochemical analysis of the obtained samples was carried out, which confirmed the expression of the satellite cell markers Pax7 and

Myf5 (Fig. 3A). The results of a quantitative analysis of immunocytochemical staining and expression of mRNA markers of satellite cells and myoblasts are shown in Fig. 3. The level of mRNA expression of both Myf5 and Pax7 was high and did not differ significantly between samples of healthy donors and patients with HF. The percentage of Myf5+ and Pax7+ cells also did not differ significantly in the samples. The results of the stimulation of differentiation showed that cells obtained from both healthy donors and HF patients have a similar potential for muscle differentiation in vitro. Fig. 4 shows the myotubes obtained after stimulation of muscle differentiation of satellite cell samples in vitro. The fusion coefficient did not differ significantly between the groups and amounted to 19±7% and 23±5% in the samples of healthy donors and HF patients, respectively.

Comparison of safety and effectiveness of conventional and individualized approaches to selecting exercise mode. Of 297 patients, 25 people discontinued participation in trial: 8 EG patients, 17 — CG (p<0,05); there were following reasons: unwillingness to continue exercise (n=10), heart transplantation (n=6), non-HF hospitalization (n=4), 3 — hospitalization due to decompensated HF after URTI. Thus, 229 EG and 43 CG patients completed the study.

After 6 months of exercise, the severity of HF decreased to class II in 75% of patients from EG, and among control patients — in 44%; the main indicators of the stages of the inclusion of compensatory mechanisms in FN (VO$_2$LP and VO$_2$peak) in the EG increased more than in the CG (10,8±0,4, 18,7±0,7 ml/min/kg and 9,5±0,8, 15,3±0,9 ml/min/kg, in p1<0,01, p2<0,05, p3<0,01, respectively).

After exercise, in OG patients there was a more pronounced decrease in the ergoreflex activity compared to CG patients: DBP — by 40%, VE in OG — by 53%, VE/VCO$_2$ — by 38%, and in CG — by 21%, 23% and 15%, respectively (p<0,05) (Table 3).

Table 4 presents echocardiography changes in the studied patients before and after physical rehabilitation. In the EG, LV EDD, LV ESD, LVEF and left atrium dimension were significantly improved. In the CG, there was a significant increase in LVEF; LV EDD, LV ESD, and left atrium dimension were not significantly improved. Against the background of long-lasting aerobic exercise, patients from the EGlong subgroup showed a significant decrease in the end-systolic and end-diastolic volumes of the LV and LA, as well as a more pronounced LVEF increase than in the EG with preserved load (EGpres) and CG (Table 4).
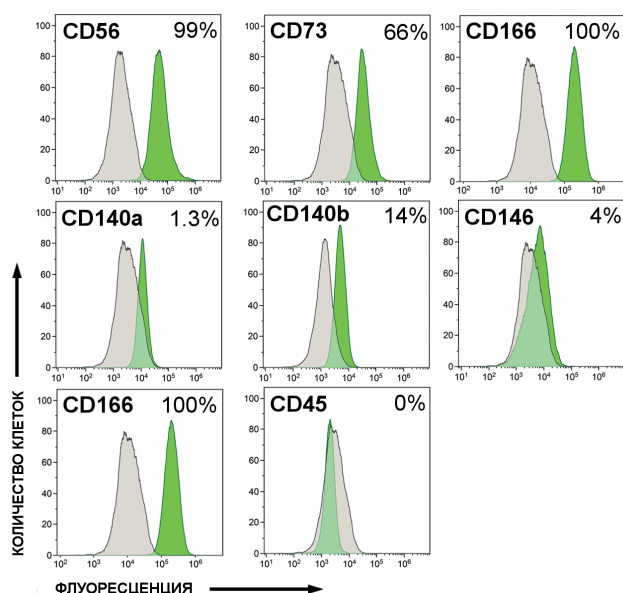
Fig. 2. The results of immunophenotyping of satellite cells isolated from human skeletal muscle and cultured *in vitro*.
Note: histograms represent the immunophenotype of the obtained cells (n=3 in each group).

Fig. 3 (A, B, C). Analysis of expression of the satellite cell markers Pax7 and Myf5.
Note: A. Immunohistochemical staining confirmed the expression of the markers Pax7 and Myf5 in the selected population. Representative photographs are presented; B. Results of a quantitative analysis of immunohistochemical staining; C. Results of analysis of the expression of Pax7 and Myf5 mRNA by real-time PCR.
Abbreviations: HD — healthy donors, HF — patients with heart failure.

Fig. 4. Immunocytochemical staining of differentiated myotubes.
Note: representative photographs are presented. Myotubes are visualized by anti-mitofusin antibodies.
Abbreviations: HD — healthy donors, HF — patients with heart failure.

Discussion

In HF, systemic metabolic changes are accompanied by muscular wasting, which in turn causes deterioration in physical performance and quality of life [1, 2, 5-6].

The aim of the first part of this project was to determine whether the skeletal muscle in HF patients retains the ability to regenerate and grow. The results of the study demonstrated that striated muscle cells of patients with class III HF do not have significant differences with cells obtained from healthy donors. They have similar potential for muscle differentiation in vitro and show a high potential for restoration of muscle precursor cells.

Thus, the skeletal muscle satellite cells under favorable conditions can contribute to the restoration of muscles injured due to HF. The exact molecular mechanisms of skeletal muscle restoration in HF patients have to be investigated. It is obvious that novel therapeutic strategies should be aimed at activating the regeneration potential of satellite cells, which may be partially realized by physical exercise.

The results of applying different exercise modes are reflected in the second part of this study. In 2017, Russian recommendations for the appointment of physical training for patients with chronic heart failure were published [1]. It was proposed to select the regime of physical rehabilitation empirically, based on the six-minute walk test 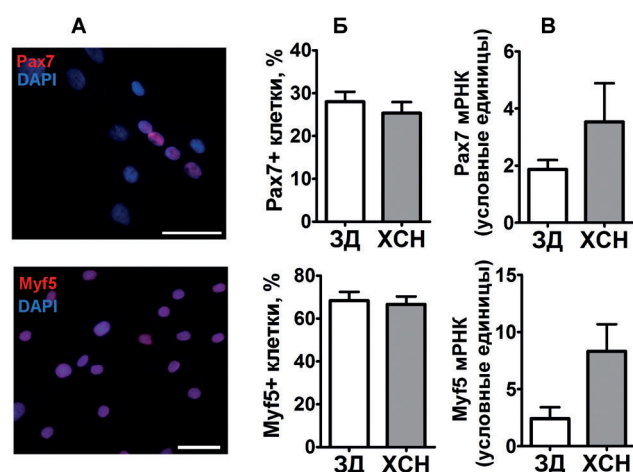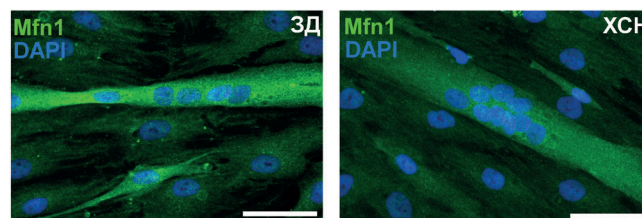(6MWT) or VO$_2$peak. Nevertheless, the 6MWT results largely depend on the motivation of patient and doctor, concomitant pathology and many other factors. Therefore, a physical rehabilitation program estimated by 6MWT can be not accurate [1, 6]. VO$_2$peak is also highly specified by the patient's motivation [1, 6]. Some aspects in determining the exercise regimen for HF patients remain open: there are no uniform principles for controlling the adaptation to physical activity; principles for planning the effective, safe and personalized exercise has not been fully developed [1, 6].

In 2012, we proposed the selection of the walking training mode based on the LT determination [7, 9]. The advantage of this approach is to increase the accuracy of determining the reserves of adaptation to physical activity. This method, in

Table 3

Parameters of ergoreflex activity in patients with class III HF before and after exercise

Parameter	Initially			After 6 months of exercise		
	EG	CG	p	EG	CG	p
DBP changes, mm Hg, %	86,5	89,7	>0,05	56,2	72,1	<0,001
VE changes, l/min, %	93,8	92,7	>0,05	48,5	69,5	<0,001
VE/VCO2 changes, %	33,9	32,2	>0,05	20,7	28,2	<0,001

Note: p — significance of cardiorespiratory test differences in HF patients before and after exercise.
Abbreviations: DBP — diastolic blood pressure, VE — minute ventilation, VE/VCO_2 — carbon dioxide ventilatory equivalent.

Table 4

Echocardiography in patients with class III HF before and after exercise

Parameter	LA, sm		LV EDD, sm		LV ESD, sm		LVEF, %	
	Before PR	After PR	Before PR	After PR	Before PR	After PR	Before PR	After PR
EG	5,52±0,09	5,31±0,05*	6,37±0,08	6,10±0,09**	5,91±0,12	5,68±0,08*	30±1,3	39±1,7**
EGpres	5,51±0,22	5,33±0,05*	6,39±0,15	6,13±0,11**	5,95±0,18	5,75±0,11*	29±1,5	37±2,1*
EGlong	5,54±0,29	5,25±0,05**	6,36±0,19	6,05±0,28**	5,93±0,30	5,55±0,18**	30±3,5	41±2,9**
CG	5,46±0,38	5,41±0,35	6,32±0,37	6,27±0,25	5,91±0,32	5,87±0,29	32±3,3	36±4,1*

Note: p — statistical significance: * -– p<0,05, ** — p<0,001.
Abbreviations: PR — physical rehabilitation, LA — left atrium, LV EDD — left ventricle end-diastolic dimension, LV ESD — left ventricle end-systolic dimension, LVEF — left ventricle ejection fraction, EG — experimental group, EGpres — EG subgroup with preserved load during physical rehabilitation; EGlong — EG subgroup with long-lasting exercise, CG — control group.

comparison with the previous ones, allows developing physical rehabilitation programs for any cardiovascular patients. [5, 7, 9]. This study demonstrated the safety and effectiveness of present approach in class III HF patients. Its using allows to avoid the fatigue and, therefore, to prescribe a longer physical exercise. Described method makes it possible to softly increase the load based on the LT re-determination. As a result, there is a greater decrease in the ergoreflex activity in the EG, followed by decrease in neurohumoral activation [5]. Also longer exercise duration can increase the number of mitochondria and exercise tolerance compared with conventional approaches where the time and load are strictly fixed. This is confirmed by the fact that in patients with LT-dependent exercise load, the tolerance increased more significantly, and in patients with >1,5 hours/day exercise, reverse myocardial remodeling was observed.

Limitations: a relatively small number of patients in the group of long-lasting exercise and multicenter design.

Conclusion
1) *In vitro*, the potential for muscle differentiation, regeneration and growth of satellite skeletal muscle precursor cells obtained from patients with HF with reduced EF does not differ from the potential of satellite cells of healthy donors.

2) Safety of aerobic exercise in patients with class III HF estimated by LT definition is equal with exercise estimated by the level of VO_2peak;

3) Aerobic exercise in patients with class III HF estimated by LT definition, compared with the conventional approach, significantly reduce the activity of ergoreflex, increase exercise tolerance and reduce the HF severity.

4) In patients with class III HF, walking training >1,5 hours/day estimated by the LT level, contributes to the development of physiological reverse myocardial remodeling to a greater extent than aerobic exercise selected by the conventional method.

Conflicts of Interest: nothing to declare.

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