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The Antioxidant Effect of Mitochondrially Targeted Antioxidant SkQ1 on the Isolated Rat Heart Model

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Исследование антиоксидантного эффекта митохондриально-направленного антиоксиданта SkQ1 на модели изолированного сердца крысы

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Summary

Mitochondrially targeted antioxidants based on Skulachev ions (SkQ1) are extremely attractive for neutralizing reactive oxygen species directly in the mitochondrial matrix.

The aim was to examine the antioxidant and cardioprotective status of the SkQ1 mitochondrially targeted antioxidant in an isolated rat heart model of ischemia and reperfusion after cold cardioplegia.

Material and methods. The effects of different concentrations of SkQ1 (1200 ng/ml, 120 ng/ml, 12 ng/ml) were explored on isolated hearts of Wistar rats (n=50) during 240-min cold cardioplegia. The levels of oxidative stress, changes in myocardial damage markers (classical and highly specific) and cardiac function (coronary flow velocity, heart rate, systolic pressure) were assessed.

Results. The use of SkQ1 at 12 ng/ml resulted in a significant neutralization of oxidative stress manifestations (P<0.05). The minimum concentration of NO metabolites (nitrates and nitrites) (36.2 [30.8; 39.8] μmol/ml) was maintained at pre-ischemic level throughout the 30-minute reperfusion period, while the malonic dialdehyde concentration (49.5 [41.1; 58.9] μmol/g) was lower compared with SkQ1 use at 120 ng/ml dose. Due to the «mitigation» of oxidative stress, intracellular enzymes and highly specific markers of myocardial damage rose more slowly during reperfusion, while cardiac function recovery occurred at a higher rate and showed stability upon restoration of perfusion.

Conclusion. SkQ1 at 12 ng/ml concentration showed strong antioxidant and cardioprotective properties in an *ex vivo* study.

Keywords: plastomitin; SkQ1; bypass circulation; isolated heart; oxidative stress

Conflict of interest. The authors declare no conflict of interest.

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Introduction

Open heart surgeries under cardiopulmonary bypass remain in high demand. The comorbidity of cardiac surgery patients, cardiopulmonary bypass, prolonged ischemia/anoxia of myocardium during surgery and reperfusion in combination cause accumulation of metabolic products, development of oxidative stress and systemic inflammatory response, which can lead to various postoperative complications including multiple organ failure [1, 2]. Therefore, intensive research is underway to find active agents and develop methods that can minimize postoperative complications in cardiac surgery. Oxidative stress underlying complications is triggered by altered mitochondrial respiration and leads to the production of reactive oxygen species (ROS), initiating a variety of pathological processes [3]. Local neutralization of ROS located in the mitochondrial matrix may become the most effective way of mitigating oxidative stress.

Mitochondrial-directed antioxidants belong to highly promising agents. Plastomitin is a systemic antioxidant drug based on Skulachev ions (SkQ1). The proprietary formula of a plastoquinone-containing antioxidant (SkQ1) was developed and actively studied by a team of scientists led by Professor Vladimir P. Skulachev [4, 5]. The SkQ1 antioxidant as a market product has already found its place in the pharmaceutical and cosmetic industries: Visomitin eye drops are used for the dry eye syndrome and in early cataract, which both involve oxidative stress, whereas Mitovitan face serum has been used to reduce the manifestations of aging, where ROS play an important role [6–8]. Various branches of human and veterinary medicine and agriculture have expressed interest in studies of SkQ1 efficacy [9, 10]. Specifically, our research group is interested in the development of methods to reduce postoperative complications in cardiac surgery, which in most cases involve oxidative stress, and ways to preserve the viability of the heart transplant [11]. This paper is devoted to the study of the effects of SkQ1 on the isolated heart exposed to prolonged ex vivo anoxia and reperfusion. In this case, the lack of systemic regulation allowed us to evaluate the «pure» effects of the drug itself.

The aim of the study was to evaluate the antioxidant and cardioprotective effects of SkQ1, a mitochondrial-directed antioxidant, in an ischemia and reperfusion model of isolated rat heart under cold cardioplegia.

Material and Methods

Plastomitin (with SkQ1 concentration of 1.7 mg/ml) was provided under a scientific cooperation agreement by OOO Mitotech (Russia). Working solutions of SkQ1 were prepared by diluting Plastomitin with perfusion solution in the appropriate proportion.

The experiments were performed on isolated hearts of healthy male Wistar rats (m = 300±50 g), *n*=50. The animals were kept under standard vivarium conditions without food and water restrictions. Experiments and procedures with laboratory animals complied with the rules of the European Convention for the Protection of Vertebrate Animals Used for Experimental or Other Scientific Purposes (Strasbourg, 1986). The study was approved by the local ethical committee (protocol No. 150 of 16.11.2021).

Perfusion of isolated hearts by the Langendorff method. A similar experimental scheme has already been used in studies of the efficacy of other drugs [12]. Initially, rats were anesthetized by intraperitoneal injection of sodium thiopental (25 mg/kg). Then a thoracotomy was performed, the heart with the necessary segment of the aorta was dissected and immediately immersed in Krebs-Henseleit solution (KHS) at t=4°C. Then the aorta was cannulated to allow a retrograde perfusion of the heart using an original system which included gas and temperature circuits, with standard KHS containing NaCl 118.0 mmol/L, $NaHCO_3$ 25.0 mmol/L, glucose 11.0 mmol/L, KCl 4.8 mmol/L, KH_2PO_4 1.2 mmol/L, $MgSO_4$ 1.2 mmol/L, $CaCl_2$ 1.2 mmol/L, enriched with a gas mixture (95% O_2 and 5% CO_2) with pH = 7.3–7.4. During all phases of the experiment on isolated hearts, a stable temperature of the perfusion solution in the acceptable range from 37°C to 38°C was maintained, as well as a constant pressure of the fluid column at 80 cm H_2O.

Perfusion protocol.

Pilot phase of the experiment. Consisted of cardiac stabilization (20 minutes), normothermic hypoperfusion (20 ml/h) with KHS and SkQ1 (20 minutes), and reperfusion (15 minutes) periods. The hearts of the study groups were hypoperfused using the perfusion solution containing various concentrations of SkQ1: 12 ng/ml for the first (SkQ1-12, *n*=5), 120 ng/ml for the second (SkQ1-120, *n*=5), and 1200 ng/ml for the third (SkQ1-1200, *n*=5) ones. The control group (*n*=5) did not receive SkQ1 during hypoperfusion.

The studied parameters. At minute 20 of perfusion (pre-ischemic baseline level, BL) and the minute 15 of reperfusion, coronary flow rate (CFR, ml/min) was recorded. The activity of enzymes such as creatine phosphokinase, myocardial fraction (CPK-MB, units/L) and lactate dehydrogenase (LDH, units/L) was determined in perfusate flowing from the hearts by enzymatic kinetics method on automatic biochemical analyzer «Konelab 30i» (Thermo Fisher, USA).

Main phase of the experiment. Experiment included following stages: stabilization of heart contraction (20 min); connection of the second flow of perfusion solution with SkQ1 (10 min); hypoperfusion (20 ml/h) with cooled (t=4ºC) cardio-

plegic solution (Custodiol, Dr. F. KOHLER CHEMIE GmbH, Germany) (10 min); global cardioplegic ischemia (240 min); reperfusion (30 min). Hearts of the first study group were perfused with double-flow KHS containing 120 ng/ml SkQ1 (SkQ1-120, *n*=10). The hearts of the second study group were perfused similarly to the first group with SkQ1at another concentration, 12 ng/ml (SkQ1-12, *n*=10). In the control group (*n*=10) SkQ1 was not added to the second flow of perfusion solution.

The studied parameters. At a pre-ischemic level, on minutes 1, 10, 20 and 30 of the reperfusion period, CFR, heart rate (HR, bpm), systolic blood pressure (mm Hg) using bedside monitoring device, BSM-2301K (Nihon Kohden, Japan) were recorded.

The methods of measuring levels of such enzymes as CPK-MB, LDH, aspartate aminotransferase (AST, units/L) were similar to those used during the pilot phase of the study. The levels of heart-type fatty acid-binding protein (H-FABP, ng/ml) and cardiac troponin I (pg/L) were detected by enzyme immunoassay (ELISA) using Hycult biotech (USA) and Cusabio (PRC) kits, respectively. Total levels of nitrite and nitrate, mitochondrial superoxide dismutase (SOD-2), mitochondrial DNA peroxidation damage marker (8-OHdg) were studied by ELISA using Biomedica (Austria) and R&D (USA) kits. Malone dialdehyde (MDA) in the myocardial homogenate was measured using a commercial OxiSelect™ TBARS Assay Kit MDA Quantitation (Cell Biolabs, USA).

The data were statistically analysed using «GraphPad Prism 7.0» program. Differences in the measured parameters were determined using non-parametric Mann–Whitney *U*-criterion for unrelated pairs, and Wilcoxon's criterion for dependent groups. Differences between the groups were considered significant at $P<0.05$. Dunn and Tukey corrections for multiple comparisons were applied where needed. Data are presented as *Me* [25%; 75%] and as the values obtained in the studied range or at a specific point.

Results and Discussion

The unique structure of SkQ1 molecule (10-(6'-plastoquinonyldecyl) triphenylphosphonium bromide) allows the molecule to embed into the mitochondrial membrane in conformationally correct manner (Fig. 1).

Pilot phase of the experiment. SkQ1 is known to exhibit high antioxidant activity at nanomolar concentrations [15]. Another mitochondrial-directed antioxidant, MitoQ, is also known to have a strong antioxidant effect at a concentration of 50 nmol/L in a pig kidney ischemia model, resulting in increased total blood flow and urine output [16]. However, given that our study assumed an extremely prolonged stage of anoxia with enevitable severe impairment of cellular respiration and mitochondrial membrane

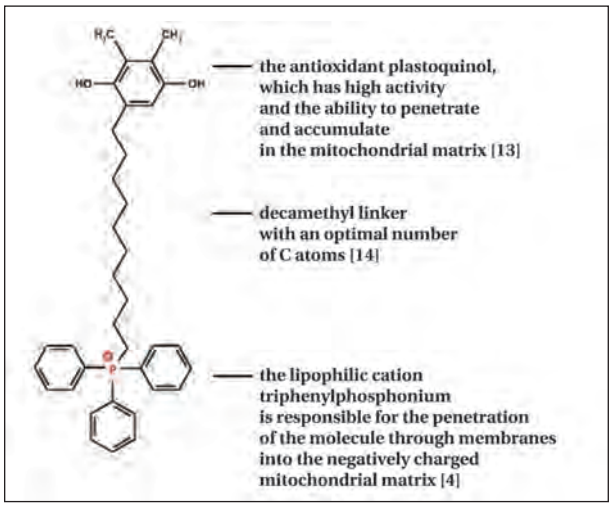

Fig. 1. SkQ1 molecule.

permeability [17], we performed a pilot experiment to select an adequate concentration of SkQ1. Isolated rat hearts were perfused with antioxidant solution of 3 different concentrations, 12 ng/ml, 120 ng/ml and 1200 ng/ml. Initially, the quantity of SkQ1 permeated and deposited in the myocardium was determined.

As expected, the number of ions per 1 g of tissue increased proportionally to the increase in the concentration of ions in the perfusion solution. Thus, the maximum concentration of ions was found in the SkQ1-1200 group at 6234.0 [4569.0; 8867.0] ng/g, in SkQ1-120 at 1059.0 [825.5; 1317.0] ng/g, and in SkQ1-12 at 268.1 [100.2; 293.0] ng/g (Fig. 2, *a*).

Coronary flow rate was restored to baseline in all experimental groups after a period of hypoperfusion and reperfusion. Notably, in SkQ1-120 group, the median AUC (15.1 [14.7; 16.0] mL/min) was significantly higher vs the control group (10.0 [9.0; 14.0] mL/min, $P=0.003$) and 15.8% higher than baseline value of 13.0 [12.1; 15.1] mL/min. However, increasing the SkQ1 concentration in the perfusion solution to 1200 ng resulted in a 3% decrease in CFR relative to baseline (Fig. 2, *b*). No intergroup differences were found in CPK-MB level, but an increase in SkQ1 concentration to 1200 ng/mL was associated with a 38.5% rise in CPK-MB vs the baseline values (Fig. 2, *c*). The level of LDH in the SkQ1-1200 group reached 5.0 [0.0; 8.0] U/L, which was significantly higher than that of the control (1.0 [0.0; 1.0] U/L) and SkQ1-120 (0.5 [0.0; 1.0] U/L, $P=0.003$, Fig. 2, *d*) groups. The increase in both LDH and CPK-MB levels at minute 15 of reperfusion in the SkQ-1200 group may indicate increased intensity of anaerobic respiration, ROC formation and sarcolemma damage of cardiomyocytes in the presence of 1200 ng/ml SkQ1 in perfusion solution, which can be considered as toxic effect of the drug at this concentration.

The pilot phase of the study identified potentially effective and safe concentrations of SkQ1 in

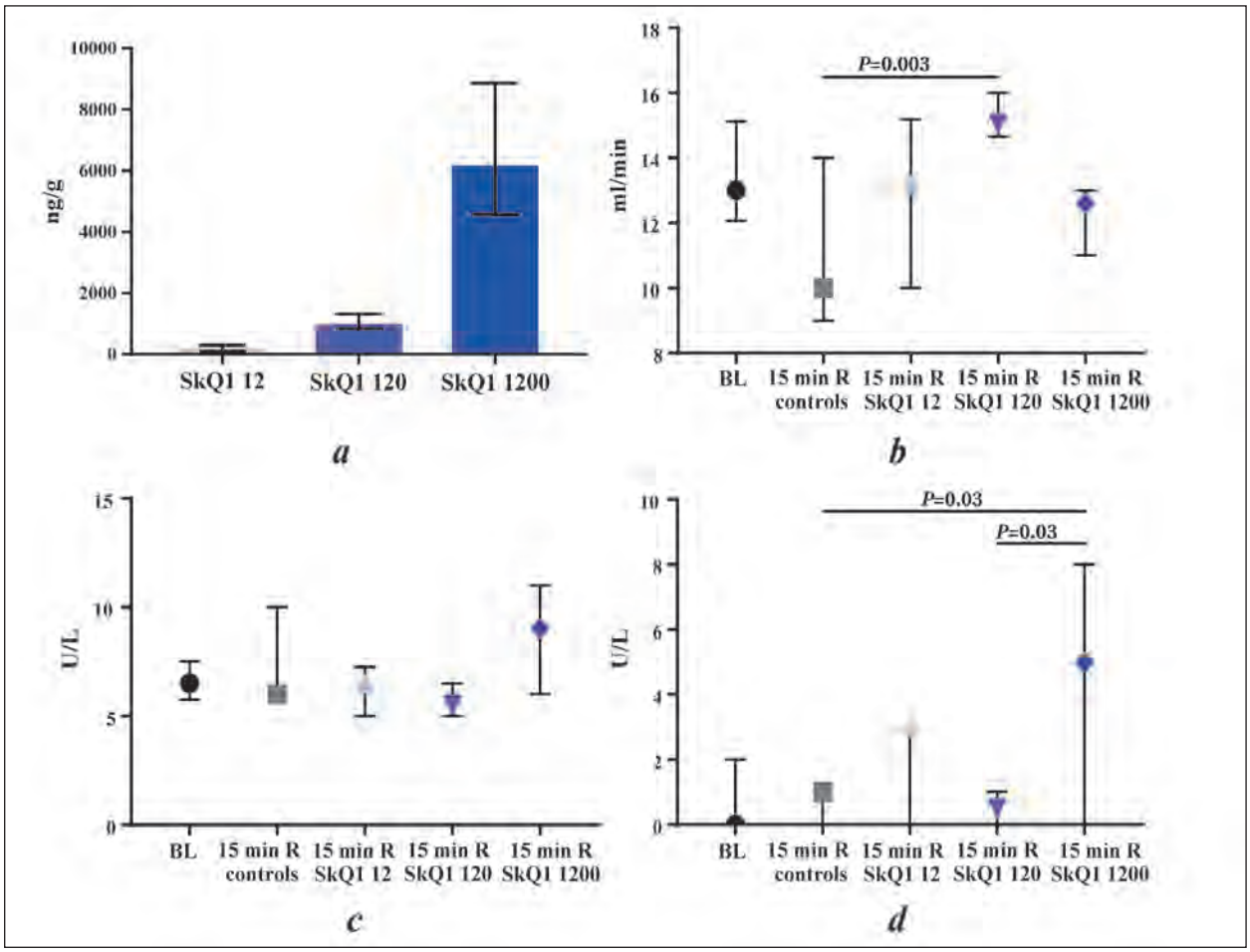

Fig. 2. Results of a pilot study of SkQ1 on an isolated rat heart model: *a* — SkQ1 concentration in myocardial tissue; *b* — coronary flow rate; *c* — creatine phosphokinase, MB fraction; *d* — lactate dehydrogenase level.
Note. For Fig. 2, 4–6: BL — baseline level; R — reperfusion.

an isolated rat heart model without aortic occlusion. Normothermic hypoperfusion with SkQ1 concentration of 12 ng/mL or 120 ng/mL had no effect on the baseline parameters of isolated hearts, in contrast to the 1200 ng/mL concentration, which provoked increased intracellular enzyme release with reduced CFR. Therefore, 12 ng/mL and 120 ng/mL SkQ1 concentrations were included in the main phase of the study.

Main phase of the experiment. The study of a new mitochondrial-directed antioxidant at the laboratory stage involved complication and bringing to clinical conditions of the experiment on the isolated heart, which included 240-minute cardioplegic total ischemia (anoxia) with a target myocardial temperature of +11°C. The range of studied parameters was expanded and grouped into 3 parts including oxidative stress, myocardial damage markers and cardiac parameters.

Oxidative stress. Antioxidant administration at a concentration of 12 ng/mL resulted in a significant decrease in stable NO metabolites compared with controls and the SkQ1-120 group: the total nitrate and nitrite concentration in the studied myocardial tissue homogenate was 36.2 [30.8; 39.8] μmol/mL (P=0.02), whereas in the SkQ1-120 group it was 52.3 [46.6; 55.0] μmol/mL (P=0.0006, Fig. 3, *a*).

NO is known to be a prooxidant and, together with peroxynitrite, directly damages DNA [18, 19]. However, our experiments did not reveal 8-OH-deoxyguanosine, a mitochondrial DNA oxidative stress product, in myocardial homogenate in either study group. The level of MDA, which reflects lipid peroxidation, was lowest in the SkQ1-12 group at 49.5 [41.1; 58.9] μmol/g and was significantly lower vs controls (P=0.02, Fig. 3, *b*) [20]. Normally, ROS are constantly formed in the cell at low concentrations. An antioxidant system that maintains the safe level of antioxidant molecules includes SOD-2 enzyme [21, 22]. However, there were no significant differences in mitochondrial SOD between the study groups; the average level of the enzyme was 14.4 ng/ml (Fig. 3, *c*). Therefore, we can assume that the decrease in oxidative stress in the SkQ1-12 group specifically relates to the antioxidant activity of SkQ1.

Markers of myocardial damage. When measuring the changes in CPK-MB and LDH release simultaneously before and after myocardial ischemia

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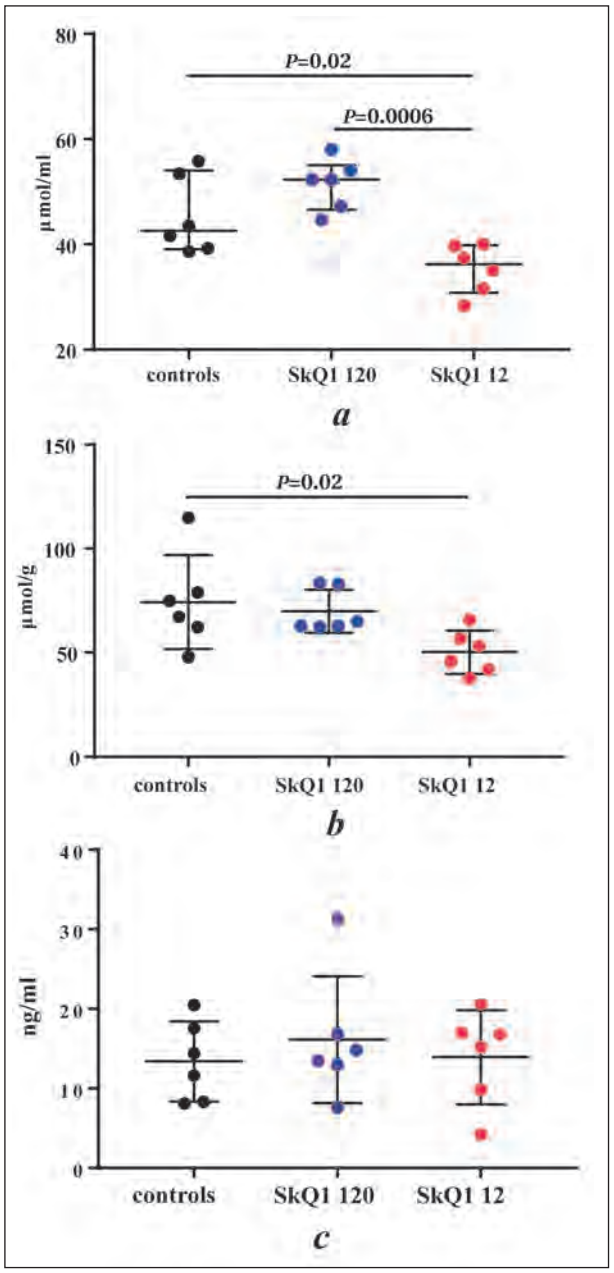

Fig. 3. Products of oxidative stress: *a* — total concentration of nitrites and nitrates; *b* — malondialdehyde; *c* — mitochondrial superoxide dismutase.

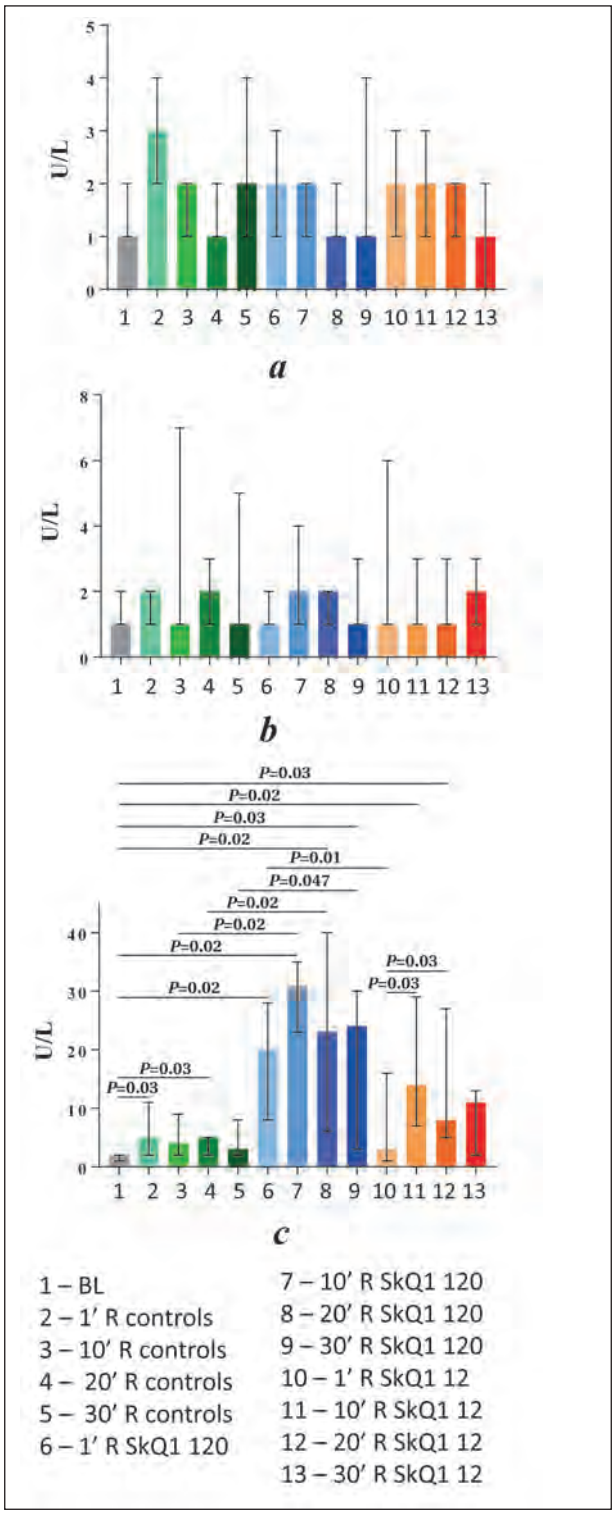

Fig. 4. Changes of the intracellular enzymes levels in the perfusate flowing from the isolated rat heart: *a* — creatine phosphokinase, MB fraction; *b* — lactate dehydrogenase; *c* — aspartate aminotransferase.

and reperfusion, we observed that the change in activity of both enzymes did not exceed 1–2 U/L (Fig. 4, *a*, *b*). However, addition of SkQ1 to the perfusion solution fostered a significant increase in AST release in myocardial outflow relative to baseline values and the control group ($0.01 \leqslant P \leqslant 0.047$, Fig. 4, *c*). The greatest increase in AST release was observed at ion concentration of 120 ng/ml: by the 30th minute, the enzyme activity exceeded the baseline one 12-fold. These changes can be regarded as a sign of cardiotoxicity of SkQ1 at a concentration of 120 ng/ml.

Lower SkQ1 concentration (12 ng/ml) resulted in delay of AST release at minute 1 (3.0 [1.0; 16.0] units/l). By the end of reperfusion, the median level of this enzyme was 5.5 times higher than the baseline one, but these changes were not significant and did not differ from the values at minute 30 of reperfusion in the control group (Fig. 4, *c*).

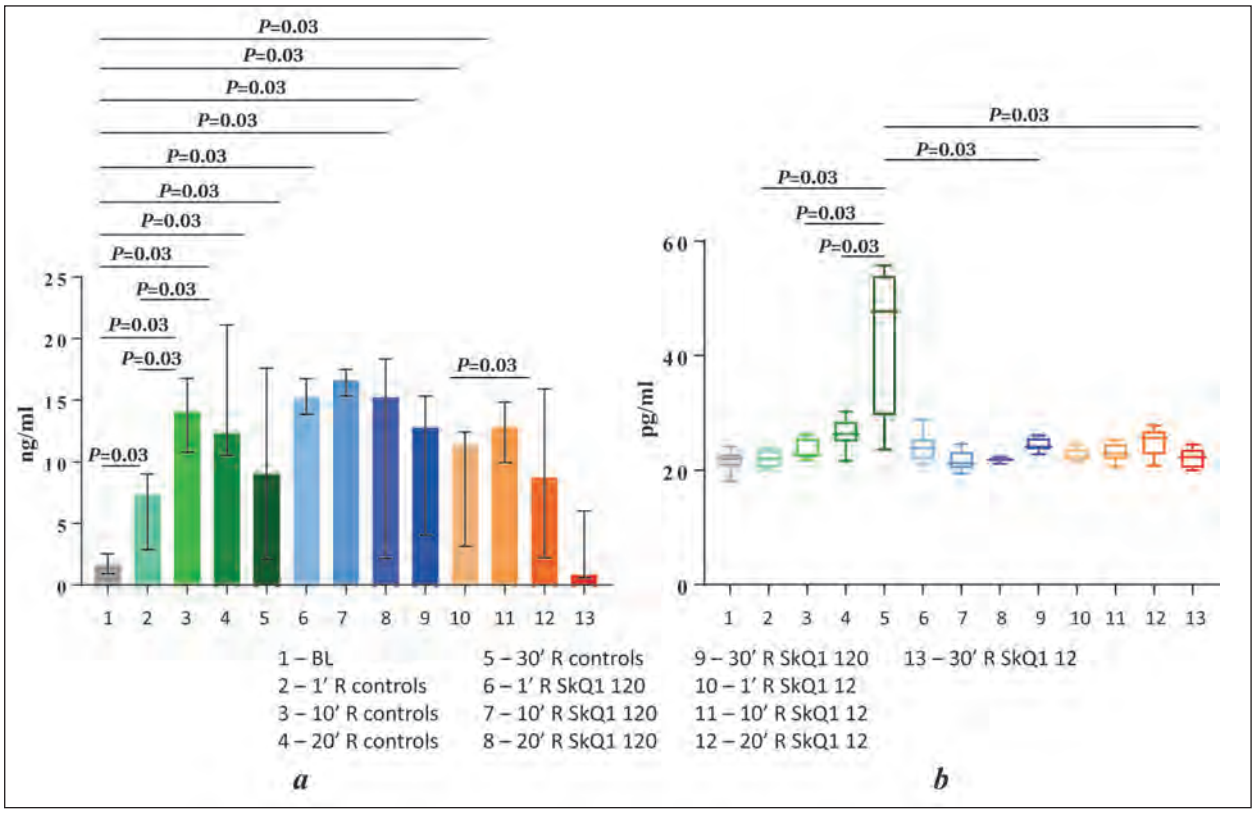

Fig. 5. Highly specific markers of myocardial damage: *a* — heart-type fatty acid binding protein; *b* — cardiac troponin I.

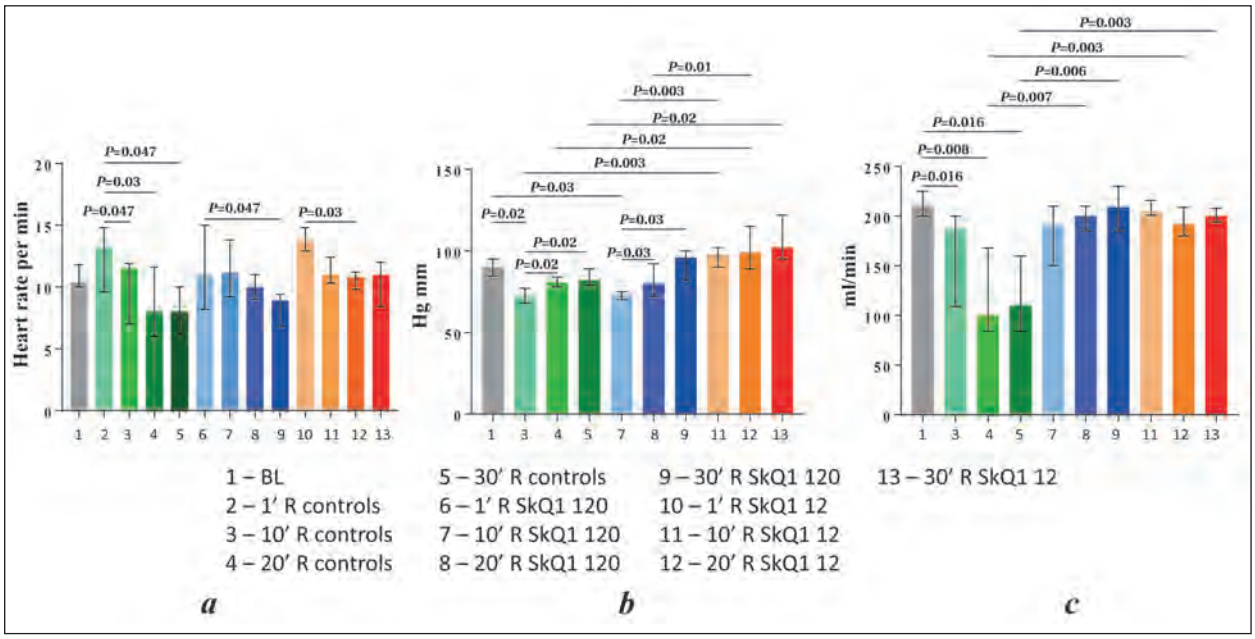

Fig. 6. Physiological parameters of isolated rat heart activity: *a* — heart rate; *b* — systolic blood pressure; *c* — coronary flow rate.

The most active release of H-FABP, a highly specific marker of myocardial damage, was observed in the SkQ1-120 group during the whole period of reperfusion at an average concentration of 14.6 ng/ml. In the SkQ1-12 group, there was an active decrease in H-FABP level in perfusate, which reached 0.8 [0.6; 6.0] ng/ml by minute 30 of reperfusion being significantly lower than that at minute 10 of reperfusion (P=0.03, Fig. 5, *a*)

Adding an antioxidant to the perfusion solution mediated protection of myocardial myofibrils throughout the reperfusion period: at drug concentrations of 12 ng/mL and 120 ng/mL, the intensity of cardiac contractile damage decreased vs the con-

trol group, where the cardiac troponin I level reached 47.4 [29.3; 54.15] pg/mL at 30 minutes after restoration of oxygenated flow of perfusion solution to the ischemic myocardium (Fig. 5, *b*).

Cardiac parameters. Addition of 12 ng/ml SkQ1 into the perfusion solution before the period of cold cardioplegia contributed to positive effects during reperfusion. The HR stabilized and reached the baseline level (211 [200; 225] beats/min) only on exposure to antioxidant, while in the control group there was a significant «fading» of HR by the end of the experiment down to to values that were only 52% of baseline (Fig. 6, *a*). In the SkQ1-12 group, a stable systolic pressure recovery to 98 [90; 102] mm Hg starting from the minute 10 of reperfusion was observed (Fig. 6, *b*).

The CFR recovery was observed on minute 10 of reperfusion only in the SkQ1-12 group and reached 11.0 [8.4; 12.0] ml/min by the end of the experiment. In the SkQ1-120 group, a 14.4% decrease in CFR vs the baseline of 10.4 [10.0; 11.8] mL/min was seen (Fig. 6c). The use of a lower concentration of antioxidant proved to be more effective with regard to cardiac parameters recovery. V. Kapelko et al. in *in vivo* experiments also showed the positive effects of SkQ1 on myocardial contractility [23]. Rhythm disturbances at minutes 1–2 in each study group were observed in 100% of cases. In the SkQ1-120 group, the frequency of short-term arrhythmia periods was 40% from minutes 15 to 30 of reperfusion, whereas in the SkQ1-12 group there was only 1 case of rhythm disturbance. The control group was characterized by maximal arrhythmia frequency, which was 50%, and in 1 case cardiac rhythm disturbances persisted during minutes 16 to 30 of reperfusion. These data correspond to those of V. Kapelko and V. Lakomkin, who succeeded in significant reduction of arrhythmia severity in the isolated heart using minimal doses of SkQ1 [24]. L.Bakeeva et al. also demonstrated that administration of extremely low doses of SkQ1 with food (0.02 nmol/kg/day for 3 weeks) was associated with elimination of cardiac arrhythmia in rats, while its increased dosage (125–250 nmol/kg/day for 3 weeks) resulted in reduced myocardial infarction area [25].

Conclusion

The model of cold cardiac ischemia/anoxia of isolated myocardium helps simulate heart condition under cardiopulmonary bypass. The cardioplegic solution in combination with low myocardial temperature (approximately 11°C) reduces the cardiac metabolic rate during anoxia but cannot completely and safely prevent the accumulation of oxidative stress products. Administration of an antioxidant SkQ1 at a concentration of 12 ng/ml prior to anoxia most effectively neutralized ROS, prevented the increase in the level of molecular markers of myocardial damage and restored its contractility during the reperfusion period. Minimal doses of SkQ1, a mitochondrial-directed antioxidant, possess cardioprotective action.

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