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Method for assessing myocardial damage under conditions of perfusion of an isolated heart according to the Langendorff method

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One of the leading mechanisms for the development of a severe cardiovascular pathology is the intensification of free radical processes. With a decrease in the activity of the antioxidant defense, the accumulation of free radicals in the body and, as a consequence, the development of the oxidative stress is natural. The registration of the severity of processes that impair the effectiveness of the antioxidant protection, with the subsequent development of an oxidative stress, can serve as a new reliable method for assessing the degree of a myocardial damage.

The aim of the work was to develop a method for assessing the degree of ischemic and ischemia-reperfusion kinds of damage to the myocardium based on the activity of free radical processes in cardiomyocytes.

Materials and methods. All the experimental work under *in vivo* conditions was performed on 50 white sexually mature mongrel male rats. The physiological and morphological parameters of the hearts, biochemical parameters and the lipid peroxidation level of the perfusate were assessed. The changes in the level of the perfusate lipid peroxidation were assessed in a simple model system simulating the lipid peroxidation. The registration of luminescence was carried out using a chemiluminometer KHLM-003 (Russia). Luminol (5-amino-2,3-dehydro-4-phthalazinedione) was used to detect the reactive oxygen species.

Results. With an increase in the ischemia duration and, as a consequence, the degree of the myocardial damage, an increase in the values of the lipid peroxidation determined by chemiluminescence is observed. When simulating 30 minutes of ischemia, necrosis is formed; it accounts for 8.9% of the total heart volume. With an increase in the ischemia duration to 60 minutes, the necrosis zone increases by 1.4 times (p <0.05), and the light sum of luminescence increases by 9.4% (p <0.05) relative to the 30-minute ischemia. A maximum decrease in pH is recorded at the 5th minute of the reperfusion. Next comes the restoration of pH values, and at the 10th minute, there is no longer any statistical difference between the initial and reperfusion values (7.37 *vs* 7.04 at p >0.05). In turn, the activity indicators of cytolysis enzymes (lactate dehydrogenase [LDH] and creatine phosphokinase-MB [CPK-MB]) show a similar pH trend of the growth in the first minutes of the reperfusion, followed by a decrease in the initial values, which is most likely due to the "washing out" of metabolic products. At the same time, the "freeze–storage (14 days)–defrost" cycle does not affect the indicator of the lipid peroxidation activity.

Conclusion. A new method for assessing a myocardial damage during the perfusion of an isolated heart using the Langendorff method, based on the use of the luminol-dependent iron-induced chemiluminescence of the lipid peroxidation level of the perfusate obtained before and after the perfusion of an isolated heart, can become one of the most effective methods for assessing the damage to the myocardial structure.

Keywords: cardioprotection; oxidative stress; isolated heart; antioxidant protection; reperfusion syndrome

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Abbreviations: AHA – American Heart Association; CVD – coronary vascular diseases; MI – myocardial infarction; HFA – higher fatty acids; LDH – lactate dehydrogenase; CPK-MB – creatine phosphokinase-MB; VCPR – volumetric coronary perfusion rate; SBP – systolic blood pressure; DBP – diastolic blood pressure; PP – pulse pressure; HR – heart rate; EDP – end-diastolic pressure; LV – left ventricular; LP – lipid peroxidation; ROS – reactive oxygen species; HBFP – Haematoxylin-Basic Fuchsin-picric acid.

Способ оценки повреждения миокарда в условиях перфузии изолированного сердца по методу Лангендорфа

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Одним из ведущих механизмов развития тяжёлых сердечно-сосудистых патологий является интенсификация свободнорадикальных процессов. При снижении активности антиоксидантной защиты закономерным является кумуляция свободных радикалов в организме и, как следствие, развитие оксидативного стресса. Регистрация выраженности процессов нарушения эффективности антиоксидантной защиты с последующим развитием оксидативного стресса может послужить новым достоверным методом оценки степени повреждения миокарда.

Цель. Разработать способ оценки степени ишемического и ишемически-реперфузионного повреждения миокарда на основании активности свободнорадикальных процессов в кардиомиоцитах.

Материалы и методы. Вся экспериментальная работа в условиях *in vivo* была выполнена на 50 белых беспородных половозрелых крысах-самцах. Проводилась оценка физиологических и морфологических параметров сердец, биохимических показателей и уровня липидной пероксидации перфузата. Изменение уровня липидной пероксидации перфузата оценивали в простой модельной системе, имитирующей перекисное окисление липидов. Регистрацию свечения проводили на хемилюминомере «ХЛМ-003» (Россия). Для выявления активных форм кислорода использовали люминол (5-амино-2,3-дегидро-4-фталазиндион).

Результаты. При увеличении длительности ишемии и, как следствие, степени повреждения миокарда отмечался рост значений липидной пероксидации, установленных методом хемилюминесценции. При моделировании 30-минутной ишемии формировался некроз, составляющий 8,9% от общего объёма сердца. При увеличении длительности ишемии до 60 мин, зона некроза увеличивалась в 1,4 раза (p <0,05), а светосумма свечения – на 9,4% (p <0,05) относительно 30-минутной ишемии. Максимальное снижение pH регистрировали на 5-й мин реперфузии, а на 10-й мин статистической разницы между исходным и реперфузионным значением уже не наблюдали (7,37 *vs* 7,04 при p >0,05). В свою очередь показатели активности ферментов цитолиза (лактатдегидрогеназа и креатинкиназа – МВ) демонстрировали аналогичную pH тенденцию по росту в первые минуты реперфузии с последующим снижением исходных значений, что, вероятнее всего, связано с «вымыванием» продуктов обмена. При этом цикл «заморозка–хранение (14 сут)–разморозка» не сказывался на показателе активности липидной пероксидации.

Заключение. Новый способ оценки повреждения миокарда в условиях перфузии изолированного сердца по методу Лангендорфа, основанный на применении методики люминол-зависимой железоиндуцированной хемилюминесценции уровня липидной пероксидации перфузата, полученного до и после перфузии изолированного сердца, может стать одним из наиболее эффективных методов оценки нарушения структуры миокарда.

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Ключевые слова: кардиопротекция; оксидативный стресс; изолированное сердце; антиоксидантная защита; реперфузионный синдром

Список сокращений: АНА – Американская кардиологическая ассоциация; ССЗ – сердечно-сосудистые заболевания; ССС – сердечно-сосудистая система; ИМ – инфаркт миокарда; ВЖК – высшие жирные кислоты; ЛДГ – лактатдегидрогеназа; КФК-МВ – креатинкиназа-МВ; ОСКП – объёмная скорость коронарной перфузии; САД – систолическое артериальное давление; ДАД – диастолическое артериальное давление; ПД – пульсовое давление; ЧСС – частота сердечных сокращений; КДД – конечное диастолическое давление; ЛЖ – левый желудочек; ПОЛ – перекисное окисление липидов; АФК – активные формы кислорода; ГОФП – гематоксилин, основный фуксин, пикриновая кислота.

INTRODUCTION

According to the American Heart Association (AHA), in the United States and Canada, the prevalence of cardiovascular diseases (CVD) ranges from 1.5 to 1.9% of the population, and up to 2% in Europe. [1]. In Russia, mortality rates from CVD among men and women of the working age, remain among the highest [2]. Therefore, in Europe, about 4 million people die annually, and the Russian population accounts for about 1 million of the above indicator. [3]. At the same time, one of the leading places in the structure of mortality from CVD is occupied by myocardial infarction (MI).

According to the data of the Department of Medical Statistics of the Central Research Institute of Health Care Organization and Informatization, in 2020, about 170,5 thousand cases of MI among the able-bodied population were registered, 33% of them were fatal outcomes. The presented statistical data cause the interest of many researchers and clinicians to the search for methods of an accurate early diagnostic assessment and prevention of CVD. Among other things, the development of methods to estimate the area of necrotized, stunned and hibernating myocardium in MI is of interest, as it directly correlates with both a high prognosis of a lethal outcome/disability of patients and the processes of myocardial remodelling, which, in turn, determines the preoperative selection of patients and the choice of the most effective cardiac surgical interventions [4].

To date, a number of markers of a cardiac muscle necrosis massiveness have been identified. However, disturbances in the regulation of free radical processes are an important basis for the development of severe cardiovascular pathologies and a number of other diseases. The antioxidant defense system provides an adequate regulation of a free radical formation in the cells, including cardiomyocytes. When its efficiency decreases, free radicals accumulate in the body and, as a consequence, an oxidative stress develops [5, 6]. In addition, ischemia leads to a decrease in the energy supply, which entails disturbances in the transmembrane concentration of Ca^{2+}, Na^{+}, and K^{+} [7–9]. A high concentration of Ca^{2+} inside the cell leads to the activation of a “lipid triad”, which is a source of active detergent substances (higher fatty acids [HFA], lysophospholipids). As a result, free radical oxidation processes increase, a phospholipases and lipases activity increases, free long-chain HFA accumulate [10]. That contributes to the damage of lysosome membranes and release of lysosomal proteases [11–13].

On the other hand, in ischemia, a high intracellular Ca^{2+} concentration is an independent factor of a myofibril damage and an impaired functional activity of mitochondria [14]. Thus, there are many reasons to believe that the registration of the violation processes severity of an antioxidant defense efficiency, with the subsequent development of the oxidative stress, can serve as a new reliable method of determining the degree of a myocardial damage.

THE AIM of the work was to develop a method for assessing a degree of ischemic and ischemia-reperfusion kinds of damage to the myocardium based on the activity of free radical processes in cardiomyocytes.

MATERIALS AND METHODS

Animals

The experimental work was performed on 50 white sexually mature mongrel male rats (225.7±22.4 g), obtained from the nursery of laboratory animals “Pushchino”, on the basis of the Department of Pharmacology of the Bashkir State Medical University (Russia), from January to February 2024. The study has been completed in accordance with the international recommendations of the European Convention for the Protection of Vertebrate Animals in Laboratory Conditions, Rules for Laboratory Preclinical Research in the Russian Federation (3 51000.3-96, 51000.4-96 and GOST 50258-92) and Order of the Ministry of Health and Social Development of Russia No. 708n (dated 23 Aug 2010) “On the approval of good laboratory practice rules (GLP)”.

The study was approved by the Ethical Committee of the Bashkir State Medical University (Protocol No. 1 dated 30 Jan 2024). The animals were kept in accordance with the rules of the European Convention for the Protection

of Vertebrate Animals (Directive 2010/63/EU) and the Guide for Keeping and Care of Laboratory Animals (GOST 33215-2014) in vivarium conditions in accordance with the sanitary and epidemic rules (SP 2.2.1.3218-14). The rats were kept no more than 5 heads in one cage, on an unlimited feed and water consumption, under a fixed light regime of 12 h/12 h. The temperature was maintained within 22–25°C, the relative humidity was 50–70%. The duration of quarantine for all the animals was 14 days, after which the rats were included in the study simultaneously. The animals were divided into 5 experimental groups of 10 rats each: group 1 – Control (without ischemia and reperfusion); group 2 – total ischemia modelling (without reperfusion), group 3 – 30-minute ischemia followed by a 2-hour reperfusion, group 4 – 45-minute ischemia followed by a 2-hour reperfusion, group 5 – 60-minute ischemia followed by a 2-hour reperfusion.

Perfusion of isolated heart according to the Langendorff method

Under the aseptic conditions, the animals were anesthetized using zoletil-xylazine anesthesia according to the following scheme: zoletil 0.3 mg i.m. ("Virbac", France)+xylanite 0.8 mg i.m. (NITA-FARM, Russia) per 100 g of an animal body weight [15, 16]. Further on, a thoracotomy was performed, the main vessels of the heart were cut off (above the capture site). The extracted hearts were placed in the Krebs–Henseleit solution (T_r=+4°C) in order to stop spontaneous contractions. The ascending part of the aorta was cannulated to ensure that the carbogen-saturated Krebs–Henseleit solution (95% O_2 and 5% CO_2) was delivered to the myocardium. To determine the myocardial contractility indices, a catheter with a balloon filled with purified water was inserted into the left ventricular (LV) cavity (volume of the liquid was sufficient to create an end-diastolic pressure; EDP) of 10–15 mmHg). After all the manipulation actions had been completed, a stabilization period of 5 minutes was maintained. Cardiophysiologic parameters were recorded using the PhysExp monitoring system (Cardiprotekt, Russia). The indices of the isolated myocardium contractility were recorded. After all the manipulation actions had been completed, a stabilization period of 5 min was maintained. Cardiophysiologic parameters were recorded using the PhysExp monitoring system (Cardiprotekt, Russia). The indices of the isolated myocardium contractility were recorded: the pressure developed by the LV; heart rate (HR, beats/min); EDP (mmHg).

Under the experimental conditions, the oxygen and plasma replacement solution (Krebs–Henseleit solution) supply to cardiomyocytes, as well as the outflow of the dissimilation products from them was completely stopped (total ischemia model); after the ischemia, the restoration of the cardiac perfusion was performed for 30, 45, and 60 minutes (depending on the tasks); it was followed by the assessment of the perfusate oxidative potential, a pH index, as well as a biochemical analysis and a morphologic evaluation of the myocardium. The perfusate was frozen and stored at -20–22°C, followed by thawing and the parameters re-evaluation to determine the stability of the lipid peroxidation level for 14 days.

Assessment of perfusate oxidative potential

The oxidative potential was assessed by luminol-dependent iron-induced chemiluminescence of the lipid peroxidation (LP) level of perfusate obtained before and after the perfusion of the isolated heart according to the Langendorff method. The changes in the level of the perfusate LP were evaluated in a simple model system simulating the lipid peroxidation. The luminescence was registered on a chemiluminometer HLM-003 (Russia). Luminol (5-amino-2,3-dehydro-4-phthalazindione) was used to registrate the ROS, which oxidizes and forms electronically excited carbonyl chromophores with a high quantum yield, resulting in a sharp increase in the intensity of luminescence associated with the formation of reactive oxygen species (ROS). Chemiluminescence was recorded for 3 min. One conditional unit of chemiluminescence was 5.1×105 quanta/sec. [17]. Luminescence intensity indices were recorded: a light sum and a slow flash amplitude of the model system in the presence of the perfusate obtained before the perfusion, and the luminescence intensity of the model system in the presence of the perfusate obtained after the perfusion. The intensity of the developing luminescence was used to evaluate the LP processes in the model system in the perfusate presence. The greater the values of the chemiluminescence intensity indices of the model system in the presence of the perfusate obtained after the perfusion in comparison with the initial indices are, the more massive the degree of the myocardial damage as a result of ischemic and ischemia-reperfusion factors of the myocardial damage is.

Evaluation of biochemical parameters creatine phosphokinase-MB

Biochemical indices (lactate dehydrogenase [LDH] and creatine phosphokinase-MB [CPhK-MB]) were determined by generally recognized methods on an automatic biochemical analyzer with an open reagent system Dirui CS-T240 (DIRUI Industrial Co., Ltd., China) using original reagent kits and their instructions.

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Morphologic evaluation of hearts

At the end of the reperfusion, half of the hearts in each group (5 hearts from each group) were stained with triphenyltetrazolium chloride followed by a macroscopic evaluation, the other half of the hearts were fixed in a 10% buffered neutral formalin solution. A macroscopic imaging of the hearts was performed using the LSCI laser speckle-imaging system (RWD, China) in the photoregistration mode of the speckle color pattern. The fixed hearts were transversely dissected into 4 parts from the base to the apex of the heart, so that the left, right ventricles and the interventricular septum were included in the slices. Next, a standard histological processing by alcohols of increasing concentrations was performed, after which the preparations were encapsulated in paraffin and 4 μ thick sections were made, which were stained with haematoxylin-eosin, haematoxylin-basic fuchsin-picric acid (HBFP). To improve the accuracy of the results, special staining for ischemia slices was done twice as much as haematoxylin-eosin, with each slice made in a paraffin tape. In this way, the possibility of staging false positive or false negative reactions was reduced. The prepared glass specimens were scanned on a Pannoramic 250 (3DHISTECH Ltd, Hungary) followed by the examination of histological sections under different magnifications using CaseViewer software (3DHISTECH Ltd., Hungary); the measurements were made with straight lines in micrometers (μm), after which the results were uploaded to Statistica 10 (StatSoft Inc., USA). The cardiomyocyte damage severity was assessed by a semi-quantitative method in glass specimens stained according to the HBFP method at a 10-fold magnification of a microscope objective at 10 fields of vision as follows: 0 points – intact myocardium, 1 point – a minimal damage (<26% of myocardium), 2 points – a minimal to moderate level (26–50% of myocardium), 3 points – a moderate level of damage (51–75% of myocardium), 4 points – severe damage (>75% of myocardium).

Statistical analysis

The results of the study were processed using a statistical package Statistica 10 (StatSoft Inc, USA). The test for the normality of distribution of the actual data was performed using the Shapiro–Wilk criterion. A median and an interquartile range were used to describe the groups. The analysis of variance was performed using the Kraskell–Wallis or Mann–Whitney criteria (for independent observations) and the Friedman one (for repeated observations). The critical significance level was taken as 0.05.

RESULTS

The study results of the perfusate collected before ischemia and after the reperfusion, as well as the measurements of cardiophysiological parameters and a histological examination in the experiment at 45 minutes of ischemia and the reperfusion are presented in Table 1.

The data of Table 1 show that the myocardial ischemia-reperfusion changes the indices of physiological constants. For example, systolic blood pressure (SBP) indices are characterised by a maximum 23.7% decrease after the reperfusion (15 min of reperfusion) with a subsequent recovery to 141.3 mmHg (86.6% of initial values) at 45 min of reperfusion. A diastolic blood pressure (DBP) values are characterised by an average increase of 40% (p <0.05) immediately after a blood flow start and during all 45 min of the reperfusion.

It should be noted that perfusate pH after 40 min ischemia is characterised by a 5.0% decrease (p <0.05) relative to the initial values. The maximum decrease in pH is registered at the 5th min of the reperfusion. Further on, there is a recovery of pH values, and at the 10th min, there is no statistical difference between initial and reperfusion values (7.37 *vs* 7.04 at p >0.05). In turn, the indices of the cytolysis enzymes activity (LDH and CPhK-MB) show a similar pH tendency to increase in the first minutes of the reperfusion with a subsequent decrease in the initial values. Evaluation results of the ischemia duration effect on the size of necrotic myocardium, as determined by a macroscopic evaluation by triphenyltetrazolium chloride staining (Fig. 1), and the level of the lipid peroxidation, as determined by chemiluminescence, 10 minutes after the end of the ischemia period, are presented in Table 2.

The data of Table 2 show that with increasing the ischemia duration there was registered an increase in the lipid peroxidation activity determined by the chemiluminescence method. When modelling a 30-minute ischemia, a necrosis was formed, accounting for 8.9% (p <0.05) of the total heart volume (Fig. 1). When the ischemia duration was increased 2-fold (60 min), the area of the necrosis increased 1.4-fold (p <0.05) and the luminescence increased by 9.5% (p <0.05) relative to the 30-minute ischemia.

The evaluation results of a single “freeze–store–thaw” cycle effect on the stability of lipid peroxidation level readings determined by chemiluminescence, are presented in Table 3.

According to the data of Table 3, the cycle “freezing–storage–thaw” did not affect the lipid peroxidation activity index. The storage for more than 14 days in a household freezer did not distort the data obtained.

The microscopic picture after the ischaemic-perfusion of the heart muscle on the isolated heart was characterised primarily by ventricular edema (Fig. 2), and predominantly, at the expense of its epicardial and myocardial layers.

Table 1 – Evaluation results of perfusate properties and morphological picture in 45-minute ischemia experiment and reperfusion, Me [Q1-Q3]

Indicator	Intact values	After stabilisation period (before ischemia)	Reperfusion, min					
			1	5	10	15	30	45
pH	7.37 (7.33–7.42)*	7.07 (6.98–7.12)	6.95 (6.87–7.08)*	6.75 (6.61–6.92)*	7.04 (6.89–7.13)	7.10 (7.01–7.15)	7.18 (7.09–7.21)†	7.31 (7.27–7.36)*, †
SBP, mmHg	–	163.2 (161.5–172.4)	143.5 (140.2–149.7)*	151.2 (147.3–158.4)*	149.4 (142.6–154.2)*	124.5 (120.5–134.9)*, †	144.5 (140.2–151.4)*	141.3 (137.8–145.4)*
DBP, mmHg	–	10.8 (9.1–11.6)	18.5 (17.4–19.3)*	17.4 (17.1–18.2)*, †	16.5 (15.7–17.8)*, †	17.1 (16.8–18.3)*, †	16.5(15.2–17.9)*, †	17.7 (16.7–18.5)*, †
PP, mmHg	–	106.1 (104.2–107.8)	56.1 (54.3–60.2)*	41.8 (38.5–42.7)*, †	42.4 (40.1–45.4)*, †	51.6 (49.3–54.8)*	64.3 (62.7–65.0)*, †	71.3 (70.2–73.5)*, †
HR, bpm	–	241.2 (237.8–245.3)	187.8 (180.2–192.4)*	290.4 (287.3–296.5)*, †	292.7 (284.5–300.2)*, †	252.2 (247.1–263.5)†	264.3 (251.6–274.8)†	231.9 (227.6–233.8)†
VCPR	–	13.8 (12.3–14.7)	19.8 (17.8–21.4)*	15.3 (14.2–17.8)†	14.4 (12.5–15.7)†	10.6 (8.6–12.7)*, †	9.2 (8.7–10.3)*, †	9.4 (8.5–11.2)*, †
CPhK-MB, MU/L	–	10.4 (9.8–11.7)	12.3 (10.7–13.8)	17.4 (15.8–19.2)*, †	21.3 (19.4–22.6)*, †	26.8 (24.3–29.5)*, †	12.8 (11.5–14.6)†	11.9 (10.2–12.5)
LDH, MU/L	–	16.2 (15.4–17.9)	18.3 (17.6–20.4)	28.4 (24.5–30.7)*, †	32.6 (28.4–34.7)*, †	29.3 (28.7–30.1)*, †	17.2 (16.4–18.9)	17.1 (15.7–19.3)
Light sum of luminescence, 5.1×105 quanta/sec	–	30.7 (26.5–33.8)	45.4 (38.1–47.3)*	104.5 (98.5–107.6)*, †	105.2 (96.3–102.4)*, †	104.7 (97.6–107.8)*, †	100.2 (97.3–104.5)*, †	110.3 (106.3–113.7)*, †
Amplitude of slow flash, 5.1×105 quanta/sec	–	16.6 (14.4–18.7)	24.5 (19.4–27.3)*	37.2 (34.4–42.3)*, †	38.7 (35.2–42.6)*, †	36.7 (33.4–40.2)*, †	36.2 (33.3–39.6)*, †	38.4 (36.1–44.3)*, †
Myocardial thickness, µm	11.8 (10.5–12.1)*	13.8 (12.4–14.2)	13.6 (13.1–14.9)	13.9 (12.7–15.2)	14.9 (14.5–17.1)*	16.7 (15.1–17.3)*, †	18.3 (16.8–19.1)*, †	19.1 (17.4–20.1)*, †

Note: VCPR – volumetric coronary perfusion rate; SBP – systolic blood pressure; DBP – diastolic blood pressure; PP – pulse pressure; HR – heart rate; LDH – lactate dehydrogenase; CPhK-MB – creatine phosphokinase-MB; * p <0.05 – values before ischemia *vs* after ischemia, or intact values; † p <0,05 – values immediately after ischemia (1 minute of reperfusion) *vs* the rest of the reperfusion period.

Table 2 – Lipid peroxidation levels determined by chemiluminescence and myocardial damage volume values determined macroscopically by triphenyltetrazolium chloride staining, Me [Q1-Q3]

Indicator	Krebs-Henseleit solution before perfusion	After stabilisation period (before ischemia)	Ischemia, min		
			30	45	60
Light sum of luminescence, 5.1†105 quanta/sec	53.76 (48.3–57.8)*, †	30.7 (26.4–33.6)	100.2 (97.3–102.5)*, †	106.3 (105.1–108.3)*	116.8 (114.7–122.5)*, †
Amplitude of slow flash, 5.1†105 quanta/sec	24.73 (20.1–27.6)*, †	16.6 (14.4–18.7)	35.1 (32.4 –37.2)*, †	38.4 (37.9–39.2)*	40.1 (39.6–44.8)*, †
Infarct area volume to total heart volume, %	–	0.0 (0.0–0.0)	8.9 (6.7–11.9)*, †	21.0 (17.9–23.5)*	39.4 (34.5–40.3)*, †

Note: * p <0,05 – values before ischemia *vs* after ischemia, or intact values, † p <0,05 – values of 45-minute ischemia *vs* intact values, or 30- and 60-minute ischemia.

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Table 3 – Evaluation results of perfusate properties of one “freeze–store–thaw” cycle, Me [Q1-Q3]

Indicator	Solution	Values before freezing	Period, days 1	3	7	14
Light sum of luminescence, 5.1×105 quanta/sec	I	100.2 (97.3–104.5)	101.5(97.5–106.6)†	100.5 (95.6–104.2)†	102.4 (98.4–106.2)†	101.3(96.2–103.4) †
	II	98.5 (96.3–101.4)*, †	99.8 (95.6–100.8)*, †	97.5 (93.1–100.2)*, †	98.6 (97.9–101.4)*, †	98.7 (97.7–101.5)*, †
Slow flash amplitude, 5.1×105 quanta/sec	I	36.2 (33.7 –39.6)	35.2 (32.6 –37.6)†	36.2 (33.5 –37.7)†	38.3 (34.6–41.5)†	36.4 (34.2 –38.6)†
	II	35.4 (32.4–37.3)*, †	33.5 (30.5–36.4)*, †	33.8 (30.2–35.3)*, †	35.2 (35.7–38.2)*, †	36.8 (36.4–39.3)*, †

Note: I – Krebs-Henseleit solution before perfusion; II – perfusate after 45 min of ischemia and 5 min of reperfusion of isolated heart. * p <0,05 – I *vs* II values for the corresponding unfrozen period; † p ≥0,05 – Krebs-Henseleit solution I and II values before freezing *vs* unfrozen samples.

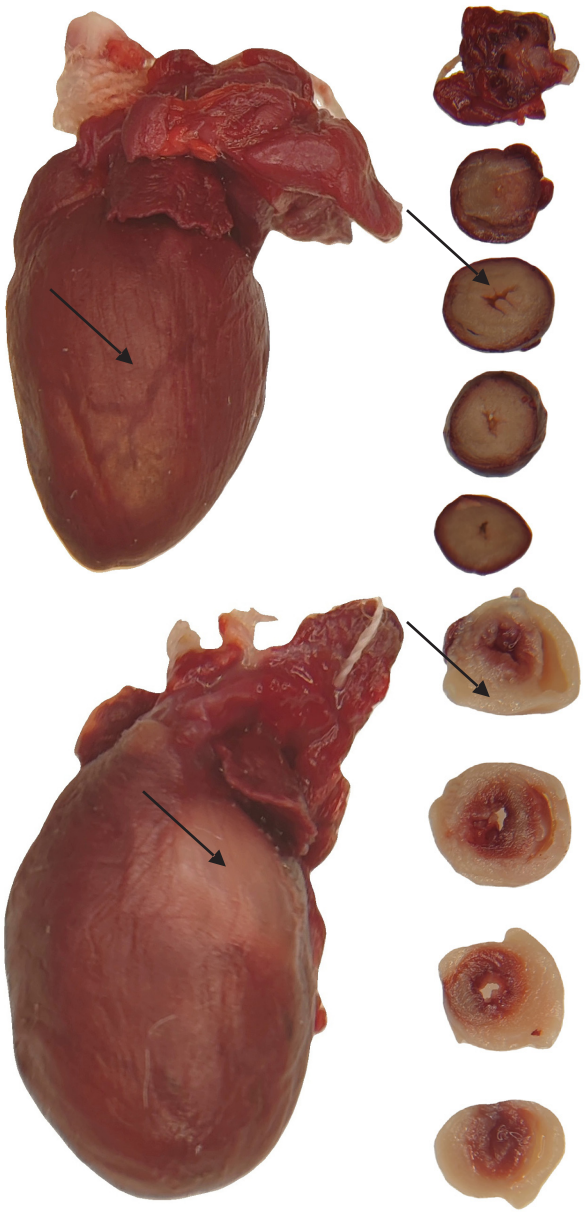

Figure 1 – Pictures of hearts staining with triphenyltetrazolium chloride
Note: areas of viable part of myocardium are coloured red; ischemic white parts are marked with arrows. Macroscopically ischemia was assessed on section with identification of pathology localisation.

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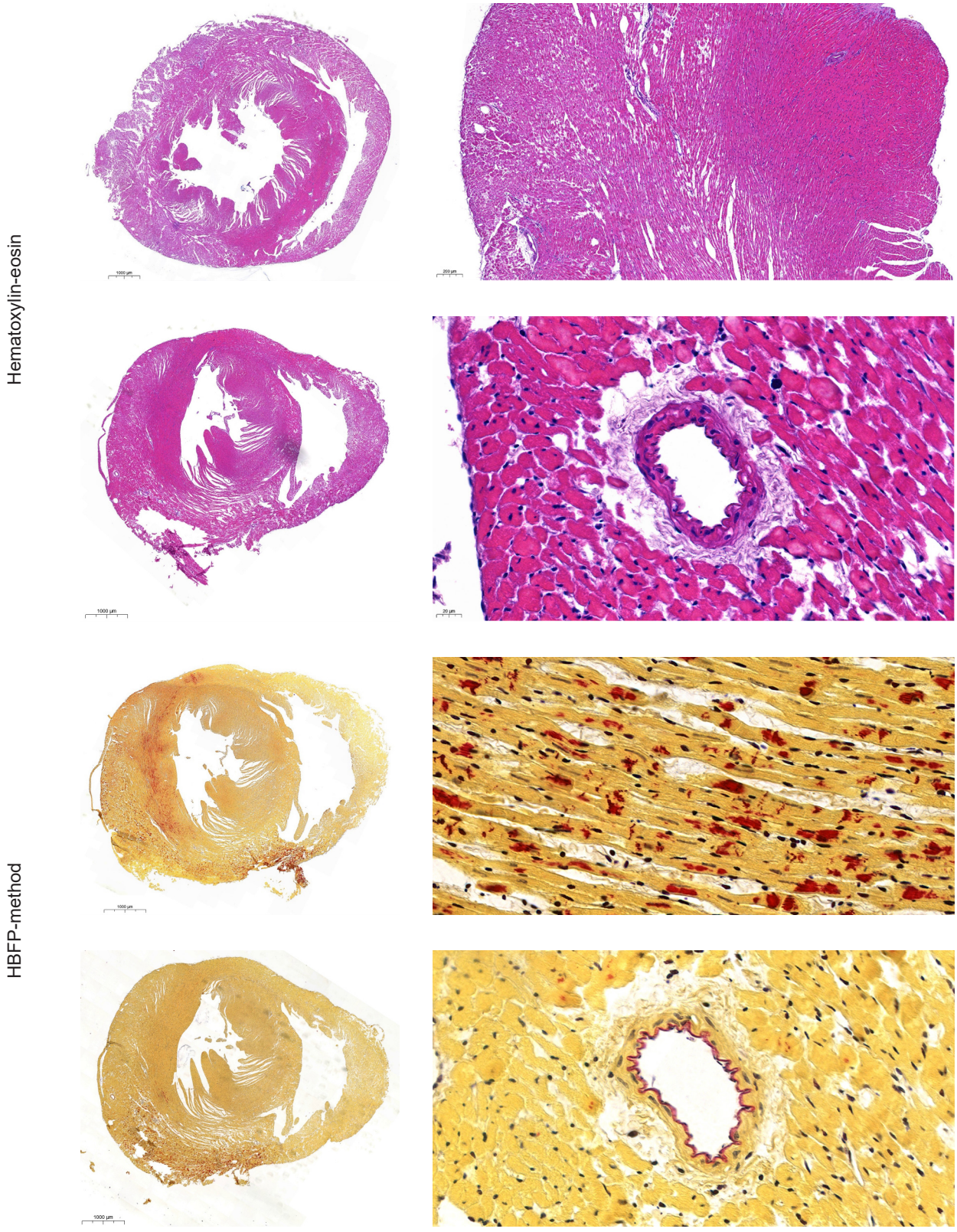

Figure 2 – Micropicture of myocardial cross-section

Note: Ventricular and paravasal edema in the organ is visualised; foci of damaged cardiomyocytes and endothelial cells are noted in specialized staining for ischemia. Hematoxyin-eosin staining, HBFP method; magnification ×15 and ×400.

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It should be noted that the very fact of the heart perfusion under artificial conditions leads to myocardial edema, thus increasing a myocardial thickness by 16.9% (p <0.05) compared to the hearts not subjected to the Langendorff perfusion. A blood flow restoration after 45-minute ischemia resulted in a gradual increase of edema. The median myocardial thickness reached 14.9 µm already at the 10th min of the reperfusion, which was 26.5 (p <0.05) and 7.9% (p <0.05) higher than the values of intact and non-ischaemic hearts, respectively. Edema reached its maximum values at the 45th min of the reperfusion, when the median value of the myocardial thickness reached 19.1 µm (p <0.05), which was 1.3 times (p <0.05) higher than in the hearts without ischemia. At the same time, the arterial wall thickened, the endothelial cells swelled. The application of specialised HBFP staining indicated the areas of myocyte ischemia also in the epicardial and myocardial part of the ventricle, which had previously been visualised macroscopically by the application of triphenyltetrazolium chloride staining. As a part of HBFP staining, basic fuchsin interacted with the breakdown products and picric acid got stained in a different colour. The appearance of a fuchsinophilic substrate was first observed in the nucleus area and then spread throughout the cytoplasm and muscle fibre. Thus, semi-quantitative scoring of a cardiomyocyte damage yielded the following results: control – 0.24±0.43 points; perfusion – 1.78±0.51 points (p <0.05 compared to control) and an ischemia-reperfusion group – 1.54±0.5 points (p <0.05 compared to control).

It should be noted that prolonged ischemia with lysis of cardiomyocytes resulted in either a decreased visualisation of the fuchsinophilic substrate or in the fact that the staining became background due to the dye release into the intercellular space. Thus, a macroscopical comparison was carried between the ischemia areas stained with triphenyltetrazolium chloride, contractural changes in the course of the muscle fibers on haematoxylin-eosin, and positive areas revealed by the HBFP staining. The determination of the lipid peroxidation in the perfusate served as an additional criterion when specialised methods of staining for ischemia become uninformative.

DISCUSSION

To date, there are several ways of modelling a myocardial injury simulating an acute coronary syndrome. There is a known method of studying the degree of a myocardial damage in laboratory rats by a direct ligation of coronary artery branches after thoracotomy with a subsequent wound closure and an elimination of pneumothorax, or a thermal coagulation of coronary arteries using an instrument / electrocoagulator heated with an alcohol burner [18]. However, all methods for *in vivo* studies have a number of drawbacks that limit a widespread use of these techniques. For example, after a coronary artery ligation or thermocoagulation, an assessment of the myocardial necrosis degree by analyzing biochemical markers of cytolysis (troponin, myoglobin, CFK, CFK-MB, ALT, AST, etc.) is insufficiently informative due to the insufficient specificity and the possibility of only indirectly assessing the damage degree. Moreover, a direct method of a myocardial ischemic damage modelling is rather difficult to reproduce due to high requirements to researchers' skills and possible peculiarities of coronary vessels in animals [19, 20].

A more standardized model of myocardial ischemia with a subsequent assessment of the degree of cardiac tissue ischemia is the Langendorff method of an isolated heart perfusion, which consists in a perfusion of an isolated rat heart with an oxygenated Krebs–Henseleit solution [21–24]. The assessment of a myocardial damage in this technique is based on the analysis of the LV contractility by an insertion of a measuring balloon into the LV cavity and the determination of the perfusate biochemical properties. Despite the advantages of this method – a complete control of the ischemia period, an exclusion of the influence of endogenous factors, as well as individual anatomical features of the animals – its significant disadvantage is the analysis of the obtained data. They rely solely on indirect biochemical indicators of the myocardial damage as well as on the contractility of the heart, resulting in the analysis of only a diastolic dysfunction of the heart contractility which is not a reliable and adequate method of assessing the degree of myocardial ischemia either.

It should be noted that the informativity of cardiomyocyte cytolysis markers dynamics in relation to the myocardial necrosis massiveness assessment has been studied for a long time with different approaches to the assessment of these markers. For example, for troponin, the assessment according to the reference values is recommended, while for CPhK-MB, there are no such recommendations, and there are separate proposals to assess CPhK-MB relative to baseline values or the upper limit of normal [25]. The increase in CPhK-MB is not a specific and highly sensitive indicator of a myocardial damage, especially during the cardiac surgery, due to the influence of many factors on this index. It should be emphasized that an important criterion reducing the diagnostic value of this enzyme detection is the possibility of its identification in blood only in the first 9 hours after the first signs of MI. After the above time interval, there is no reliable correlation between the degree of a myocardial damage and quantitative indices of CPhK-MB [26, 27]. The application of the CPhK-MB level estimation in relation to the necrotic changes in myocardium is most reliable from the diagnostic point of view, in case of the detection of large necrosis zones in myocardium with characteristic changes on the ECG [28, 29]. In such a situation, the appearance of an additional parameter allowing to detect a myocardial

damage and its degree in preclinical studies will help to objectify the picture.

Thus, it has been established that the level of lipid peroxidation changes in a regular way in accordance with the results of a biochemical analysis and a morphological study, but persists longer in the perfusate during the reperfusion, which shows the possibility of using the proposed method in order to determine the fact and degree of an ischemic and ischemic-perfusion myocardial damage.

Study limitations

The processes of the interrelation of the antioxidant defense impairment and the severity of necrotic changes in the cardiac muscle in the isolated heart according to the Langendorff method, have not been widely reported in the scientific literature. Within the framework of this study, the sample size is sufficient to confirm the presence of a regularity between the changes in the level of the LP and the degree of the myocardial damage, but within the framework of this paper, it is impossible to fully speak about the presence of strong relationships. This pattern requires a further in-depth study, including the prospect of a further application in the clinical practice as one of the signs of a myocardial ischemic damage in patients with an acute coronary syndrome. Other mechanisms, the use of which can be evaluated as markers of necrotic changes, have not been considered in the present study.

CONCLUSION

The assessment method of a myocardial damage under the conditions of a perfusion of an isolated heart according to the Langendorff method, based on the application of the luminol-dependent iron-induced chemiluminescence technique of the lipid peroxidation level of the perfusate obtained before and after the perfusion of an isolated heart, is one of the most effective methods of the assessment of the myocardial structure disturbance, as the level of the lipid peroxidation was comparable with the results of a biochemical analysis and a morphological study.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHORS' CONTRIBUTION

Wang Yi, Elena A. Smolyarchuk, Dmitry A. Kudlai, Ilkhomjon R. Abdurakhmonov, Mikhail M. Galagudza, Alexander V. Samorodov – development of the concept of the experiment; Vlas S. Shchekin, Ksenia A. Zavadich, Susanna S. Sologova, Lyubov V. Kornopoltseva, Irina D. Krylova – conducting the study; Ilkhomjon R. Abdurakhmonov, Ksenia A. Zavadich, Susanna S. Sologova, Lyubov V. Kornopoltseva – research literature search; Elena A. Smolyarchuk, Alexander V. Samorodov, Mikhail M. Galagudza – writing the manuscript. All the authors participated in the preparation and editing the text of the article. All the authors confirm their authorship compliance with the ICMJE international criteria (all the authors have made a significant contribution to the conceptualisation, research and preparation of the article, read and approved the final version before the publication).

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