

Morphologic characteristic of rat myocardium in comorbid pathology

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of the article

Diabetic cardiomyopathy is a serious complication of diabetes mellitus (DM).

Aim. Therefore, we aimed to study myocardial changes in adult rats with streptozotocin (STZ)-induced DM exposed to chronic immobilization stress (CIS).

Materials and methods. A total of 26 adult albino male rats weighing 180–200 g were examined. All the animals were divided into three groups: Group I included 10 rats with STZ-induced DM exposed to CIS; Group II comprised 10 rats with STZ-induced DM; Group III included 6 intact animals. The samples were collected on the 14th and 56th days of the experiment. Histological, histochemical, electron microscopy, and biochemical methods were used.

Results. On the 14th day of the experiment, in Group I and Group II, increased blood flow was observed in the capillaries, venules, and veins, while an arteriolar spasm in the microcirculation was found. In addition, cardiomyocyte surface area in different myocardial regions reduced due to low glycogen content as confirmed by histochemical and ultrastructural studies.

On the 56th day of the experiment, in Group I and Group II, hyperemia occurred due to red blood cell aggregation and microthrombi. The surface area of all microcirculatory vessels increased as compared to that of intact animals, as evidenced by an increase in their wall surface area leading to an increase in their wall-to-lumen ratio. Such morphometric changes in the microcirculatory vessels were indicative of decreased vascular permeability and impaired myocardial blood flow. At the histological level, in Group I and Group II, focal cardiomyocyte lysis, moderate to diffuse stromal edema, lymphohistiocytic infiltration were seen. Such changes pointed to sterile inflammation, probably due to myocardial infarction secondary to diabetic microangiopathy. In cardiomyocytes, karyolysis, vacuolar degeneration, apical ballooning, subsarcolemmic edema, fibrosis and lysis of myofibrils, colliquative necrosis were observed.

Conclusions. STZ-induced DM and stress resulted in pronounced destructive changes in the myocardium of rats, including interstitial edema, focal cardioclerosis, myolysis. Such changes occurred on the background developing diabetic microangiopathy. The most pronounced myocardial changes were recorded in animals with a comorbidity.

Key words:

diabetic cardiomyopathies, heart, myocardium, microcirculation, diabetes mellitus, experimental diabetes mellitus, heart failure.

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Морфологічна характеристика міокарда щурів за умов коморбідної патології

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Вагоме місце серед ускладнень цукрового діабету (ЦД) посідає діабетична кардіоміопатія.

Мета роботи – дослідити зміни міокарда статевозрілих щурів при стрептозотоциновому ЦД (СЦД) за умов хронічного іммобілізаційного стресу (ХІС).

Матеріали та методи. Для дослідження використано 26 статевозрілих білих щурів-самців (масою 180–200 г), яких поділили на 3 групи: перша – тварини з експериментальною коморбідною патологією (10 щурів) – СЦД і ХІС; друга – з СЦД (10 щурів); третя – інтактна (6 щурів, яким не робили жодних маніпуляцій). Матеріал забирали на 14 і 56 доби експерименту. Використали гістологічний, гістохімічний, електронномікроскопічний і біохімічні методи дослідження.

Результати. На 14 добу експерименту в 1 і 2 групах виявили повнокров'я капілярів, венул і вен, а в приносній ланці гемомікроциркуляторного русла визначили спазм артеріол. Крім того, встановили зменшення площі кардіоміоцитів різних ділянок міокарда внаслідок зменшення в них вмісту глікогену, що підтверджується гістохімічними й ультраструктурними дослідженнями.

На 56 добу експерименту в 1 і 2 групах виявили повнокров'я в усіх мікрогемосудинах внаслідок переповнення їх еритроцитарними складками, мікротромбами. Площа усіх судин гемомікроциркуляторного русла збільшилася порівняно з інтактними показниками внаслідок збільшення площі їхньої стінки, що спричиняє підвищення індексу Вогенворта. Така морфометрична перебудова мікрогемосудин свідчить про зменшення їхньої пропускної здатності та порушення кровопостачання міокарда. В 1 і 2 групах тварин на гістологічному рівні визначили вогнищевий лізис кардіоміоцитів, набряк строми від помірного до дифузного з явищами гістолейкоцитарної інфільтрації. Такі зміни вказують на асептичне запалення, яке може бути наслідком інфаркту міокарда, зумовленого розвитком діабетичної мікроангіопатії. У кардіоміоцитах визначили каріолізис, явища вакуольної та балонної дистрофії, підсарколемальний набряк, розволокнення та лізис міофібрил, колікваційний некроз.

Висновки. Стрептозотоциновий цукровий діабет і стрес призводять до виражених деструктивних змін у міокарді щурів: інтерстиційного набряку, поодиноких дрібних вогнищ кардіосклерозу, міолізу. Такі зміни відбуваються на тлі розвитку діабетичної мікроангіопатії. Найбільш виражені зміни в міокарді зафіксували у тварин із коморбідною патологією.

Ключові слова:

діабетична кардіоміопатія, серце, міокард, мікроциркуляторне русло, цукровий діабет, експериментальний цукровий діабет, серцева недостатність.

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On February 24, 2022, Russia launched a full-scale invasion of Ukraine. Constant shelling of the entire territory of our country and the occupation of parts of the regions have led to the majority of the population living under prolonged stress. No person can live with long-term stress as it subtly undermines their health and contributes to the development of chronic pathological conditions, including diabetes mellitus (DM) which has a considerable socioeconomic impact due to its complications. Diabetic cardiomyopathy is a serious DM complication which is often asymptomatic [1,2,3].

The risk of myocardial infarction and death is twice as high in DM patients. DM increases the absolute risk of coronary death by 2.5 times in men and 4.7 in women [4,5]. The high rate of atypical and painless presentation of chronic coronary artery disease and myocardial infarction poses a high risk of “sudden death”. Approximately one-third of patients hospitalized due to heart failure have DM [6].

Aim

Therefore, we aimed to study myocardial changes in adult rats with streptozotocin (STZ)-induced DM exposed to chronic immobilization stress (CIS).

Materials and methods

A total of 26 adult albino male rats weighing 180–200 g were examined. All the animals were divided into three groups: Group I included 10 rats with STZ-induced DM exposed to CIS; Group II comprised 10 rats with STZ-induced DM; Group III included 6 intact animals. STZ-induced DM was modeled by a single intraperitoneal injection of STZ (Sigma, USA) dissolved in 0.1 M citrate buffer, pH 4.5 (6 mg per 100 g body weight). Animals were exposed to CIS by placing them in a closed plastic container for five hours a day (Ukrainian Patent for the Invention No. 125623). In Group I, STZ-induced DM was modeled and on the 14th day of the experiment, animals were exposed to CIS. The samples were collected on the 14th and 56th days of the experiment.

To exclude the influence of circadian rhythms and biological activity on rat metabolism, the samples were collected prior to morning feeding. To measure fasting blood glucose levels in standard vivarium conditions, a droplet of blood was daily collected from the incised tail vein of rats using a portable blood glucose meter Accu-Chek® Active (Roche Diagnostics GmbH, Germany) with a standard set of test strips. Animal testing was performed in the Educational and Scientific Laboratory of Morphological Analysis, the Ivano-Frankivsk National Medical University (IFNMU) Bioelementology Centre, DIAMEB laboratory.

All experiments were conducted in compliance with ethical norms and requirements of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986), EU Directive 86/609 (Council of the European Communities, 1986), the Law of Ukraine “On Protection of Animals from Cruel Treatment” as of December 15, 2009, Orders of the Ministry of Health of

Ukraine No. 690 dated September 23, 2009, No. 616 dated August 03, 2012 (Advisory Opinion of the Ethics Committee of IFNMU Protocol No. 111/19 dated November 19, 2019).

Immediately after animals were euthanized and blood samples were collected into tubes at the IFNMU Bioelementology Centre, glucose concentration was measured by the glucose oxidase method with a standard “Glucose-FKD” (Ukraine) reagent kit. Blood glycated hemoglobin (HbA1c) and cortisol levels were determined at the certified laboratory DIAMEB.

Histological (hematoxylin and eosin staining), histochemical (glycogen determination by the Shabadash method), electron microscopy, biochemical, and statistical methods were used. Cutting speed and feed rate were followed when collecting the samples of the ventricular myocardium for generally accepted electron microscopy [7].

For morphometric studies, the histological sections were observed under a Leica DM750 light microscope, then images of histological sections were recorded using a digital CCD camera with a resolution of 1200x1600 and saved as TIF files. Morphometric analysis was performed using ImageJ version 1.47t. The results obtained were statistically processed using the statistical package Statistica (StatSoft, Inc. (2011) STATISTICA (Data Analysis Software System), Version 10). Table variables are presented as M – sample mean, SD – standard deviation, n – sample size (the number of individuals within a group), p – the statistically significant level.

Results

On the 14th day of the experiment, blood glucose and HbA1c levels were the highest in Group I as compared to intact animals: Group I – 15.21 ± 1.33 mmol/l ($p < 0.001$) and 7.78 ± 0.58 % ($p < 0.01$), respectively; Group II – 13.72 ± 1.53 mmol/l ($p < 0.001$) and 6.08 ± 0.45 % ($p < 0.01$), respectively; Group III – 4.85 ± 0.63 mmol/l and 1.78 ± 0.18 %, respectively. Such biochemical changes in Group I and Group II were indicative of developing decompensated STZ-induced DM. Blood cortisol levels were significantly higher in the experimental groups as compared to intact animals: Group I – 32.17 ± 2.14 ng/ml; Group II – 18.29 ± 2.27 ng/ml (in all cases $p < 0.05$); Group III – 10.06 ± 0.98 ng/ml.

Histological specimens obtained from intact animals clearly showed three layers of the ventricular myocardium (Fig. 1a). Most myocardial cardiomyocytes appeared oval and contained moderate amounts of glycogen granules (Fig. 1b). The myocardium was well vascularized, with the sinusoids facilitating efficient venous drainage (Fig. 1a).

On the 14th day of the experiment, in Group I and Group II, increased blood flow was observed in the capillaries, venules, and veins (Fig. 1c), while most afferent microcirculatory vessels appeared with slit-like lumen due to their spasm (Fig. 1d). In Group I and Group II, the caliber of arterioles reduced significantly to 234.12 ± 20.31 μm^2 and 241.48 ± 189.12 μm^2 , respectively (intact animals – 269.35 ± 18.34 μm^2 , $p < 0.05$) due to narrowing of their lumen to 46.58 ± 7.26 μm^2 and 56.35 ± 7.89 μm^2 ,

respectively (intact animals – $83.34 \pm 7.35 \mu\text{m}^2$, $p < 0.05$). Such morphometric changes in arterioles caused a drastic reduction in their permeability, as evidenced by a significant increase in their wall-to-lumen ratio (WLR) to $402.62 \pm 39.15 \%$ and $328.53 \pm 27.64 \%$ in Group I and Group II respectively ($p < 0.05$), as compared to intact animals – $223.19 \pm 21.04 \%$.

Morphometric analysis revealed a significant reduction in the surface area of ventricular cardiomyocytes in the early stages of modeling STZ-induced DM and CIS (Table 1). The surface area of cardiomyocyte nuclei remained unchanged and their nuclear-cytoplasmic ratio (N:C ratio) increased (Table 1).

At the ultrastructural level, in the lumen of the microcirculatory vessels, RBC aggregation was detected. Cardiomyocytes underwent cell shrinkage and nuclear fragmentation. The highest degree of variability was observed in the mitochondria: from disorganization and destruction of mitochondrial cristae to mitochondrial swelling and inner mitochondrial membrane damage (Fig. 2a, b). Focal destruction of the intercalated discs was observed (Fig. 2b). Segmental contractions were occasionally found in the myofibrils of ventricular cardiomyocytes. The above-described changes in cardiomyocytes were equally detected in both Group I and Group II. In cardiomyocytes of Group I, however, distinct clusters of large, electron-dense mitochondria with disorganized and partially destructed cristae were seen underneath the sarcolemma and close to the nucleus.

On the 56th day of the experiment, blood glucose and HbA1c levels were the highest in Group I as compared to intact animals: Group I – $20.61 \pm 3.23 \text{ mmol/l}$ ($p < 0.001$) and $9.25 \pm 0.72 \%$ ($p < 0.01$), respectively; Group II – $18.55 \pm 2.13 \text{ mmol/l}$ ($p < 0.001$) and $8.34 \pm 0.48 \%$ ($p < 0.01$), respectively; Group III – $4.58 \pm 0.56 \text{ mmol/l}$ and $2.89 \pm 0.17 \%$, respectively. Such biochemical changes in Group I and Group II were indicative of developing decompensated STZ-induced DM. Blood cortisol levels were significantly higher in the experimental groups as compared to intact animals: Group I – $14.17 \pm 2.14 \text{ ng/ml}$; Group II – $17.58 \pm 2.15 \text{ ng/ml}$; Group III – $10.06 \pm 0.98 \text{ ng/ml}$; however, in both experimental groups, blood cortisol concentration decreased significantly as compared to the previous observation period (in all cases $p < 0.05$).

At the histological level, in Group I and Group II, cardiomyocyte lysis, moderate to diffuse stromal edema, lymphohistiocytic infiltration were occasionally seen (Fig. 1e–f), being more pronounced in animals of Group I (Fig. 1e). Such changes pointed to sterile inflammation due to myocardial infarction secondary to diabetic microangiopathy. Pronounced hyperemia was found; cardio-sclerosis, however, was rarely seen (Fig. 1g). PAS staining showed low quantities of glycogen granules or their absence in the cytoplasm of cardiomyocytes (Fig. 1h). Cardiomyocytes were found to show vacuolar degeneration and apical ballooning (Fig. 1h). The surface area of cardiomyocytes and their nuclei reduced significantly as compared to the values of intact animals and those from the previous observation period, thereby indicating cell atrophy. On the 56th day of the experiment, in Group I and Group II, hyperemia occurred due to RBC aggregation

Table 1. Morphometric analysis of cardiomyocytes in rats with streptozotocin-induced diabetes mellitus exposed to chronic immobilization stress ($M \pm m$)

Group	Myo-cardial region	Morphometric indicators of cardiomyocytes		
		Cardiomyocyte surface area, μm^2	Nucleus surface area, μm^2	N:C ratio, %
14th day				
Group I	LV	$309.73 \pm 19.46^*$	19.92 ± 1.19	$6.43 \pm 0.15^*$
	RV	$231.54 \pm 14.73^*$	17.78 ± 0.92	$7.68 \pm 0.23^*$
Group II	LV	$312.49 \pm 19.81^*$	19.92 ± 1.19	$6.37 \pm 0.14^*$
	RV	$242.93 \pm 18.70^*$	17.78 ± 0.92	$7.32 \pm 0.19^*$
Group III	LV	349.26 ± 21.85	19.61 ± 1.72	5.61 ± 0.14
	RV	271.69 ± 19.48	17.47 ± 0.53	6.43 ± 0.13
56th day				
Group I	LV	$259.73 \pm 20.46^{*\#}$	$16.53 \pm 1.24^{*\#}$	6.36 ± 0.15
	RV	$201.54 \pm 16.73^{*\#}$	$15.63 \pm 1.25^{*\#}$	7.75 ± 0.12
Group II	LV	$278.31 \pm 19.13^{*\#}$	$17.98 \pm 1.86^{*\#}$	6.46 ± 0.14
	RV	$219.61 \pm 20.34^{*\#}$	$16.93 \pm 0.53^{*\#}$	$7.89 \pm 0.17^{\beta}$
Group III	LV	354.28 ± 19.26	19.65 ± 1.04	5.56 ± 0.18
	RV	278.69 ± 18.63	17.34 ± 1.59	6.22 ± 0.19

LV: left ventricle; RV: right ventricle; *: $p < 0.05$ – the probability of differences as compared to the intact group; #: $p < 0.05$ – the probability of differences as compared to the previous study period within the same group; β : $p < 0.05$ – the probability of differences between Group I and Group II indicators during the same study period.

and microthrombi (Fig. 1g). In animals of Group I and Group II, the surface area of all microcirculatory vessels was higher than that in intact animals, as evidenced by an increase in the WLR of arterioles to $331.32 \pm 29.56 \%$ and $312.56 \pm 24.83 \%$, respectively (intact animals – $223.19 \pm 15.63 \%$, $p < 0.05$); an increase in the WLR of capillaries to $190.05 \pm 16.56 \%$ and $161.46 \pm 14.72 \%$, respectively (intact animals – $72.00 \pm 7.84 \%$, $p < 0.05$); an increase in the WLR of venules to $165.02 \pm 24.33 \%$ and $137.07 \pm 17.32 \%$ (intact animals – $62.29 \pm 5.73 \%$, $p < 0.05$). Such morphometric changes in the microcirculatory vessels were indicative of decreased vascular permeability and impaired myocardial blood flow.

At the ultrastructural level, on the background of hyperglycemia, pronounced microangiopathy was observed, which was manifested by RBC aggregation, microthrombi, platelet and RBC vascular adhesion, vacuolar dystrophy of endothelial cells and myocytes (Fig. 3a). Most capillary basement membranes showed thickening and proliferation in the form of separate layers, thereby indicating DM (Fig. 3b). Severe microangiopathy led to pronounced hypoxic changes in the myocardium. In cardiomyocytes, karyolysis, vacuolar degeneration, apical ballooning, subsarcolemmic edema, fibrosis and lysis of myofibrils were observed (Fig. 3c, d). Such changes were most pronounced in Group I. At the same time, in the myocardium of animals belonging to Group I, colliquative or partial necrosis of cardiomyocytes was occasionally seen (Fig. 3d). The death of individual cardiomyocytes was observed, leading to the subsequent development of cardiosclerosis (Fig. 3e). In Group II, cardiomyocytes exhibited vacuolar degeneration and apical ballooning, colliquative cardiomyocyte necrosis was rarely seen (Fig. 3f). Along with cardiomyocytes with pronounced destructive changes, preserved cells were visualized as well; however, all cardiomyocytes underwent more or less pronounced changes.

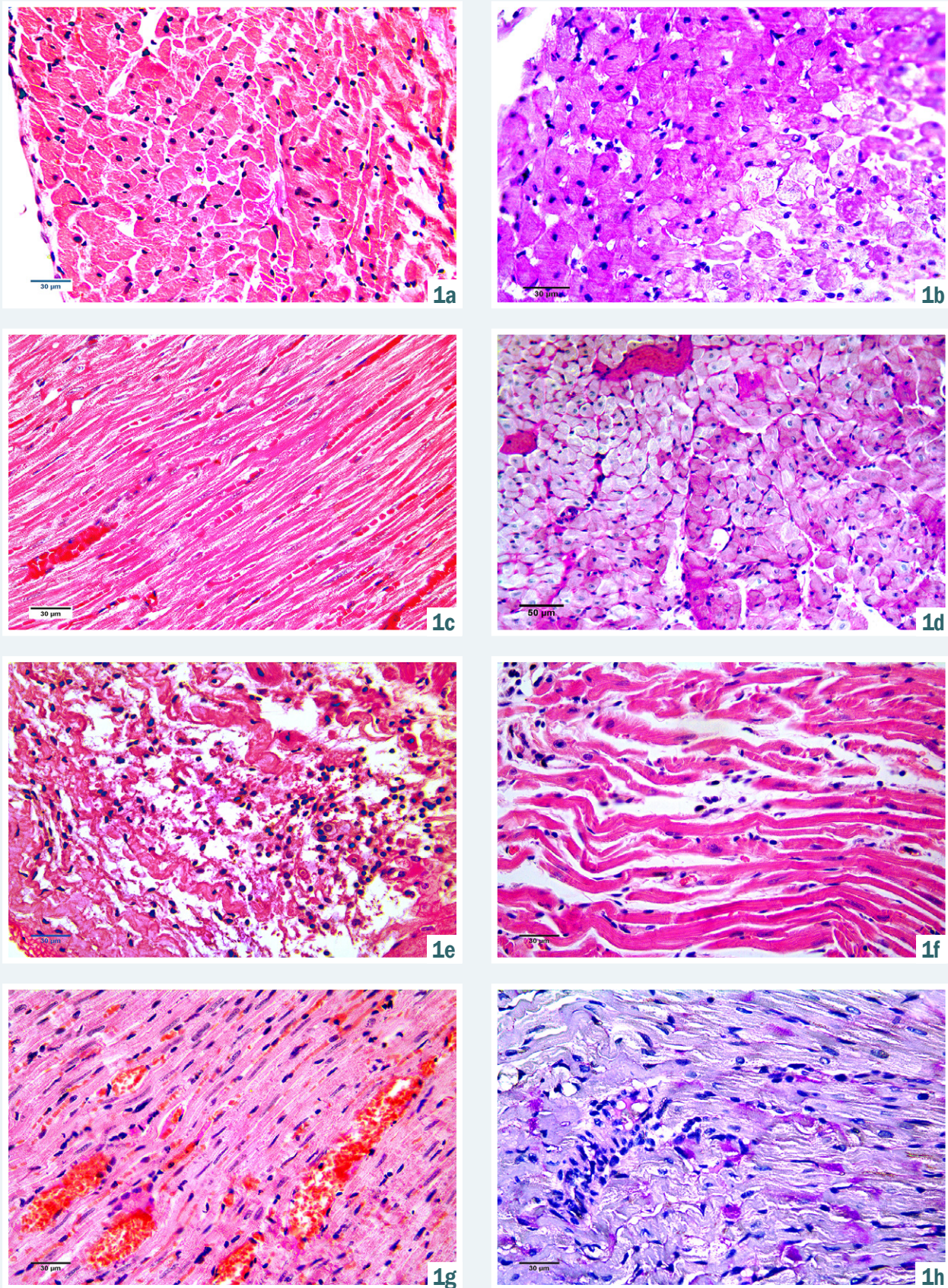


Fig. 1. Myocardium of intact animals (a, b). Increased microvascular blood flow (c) and low quantities of glycogen granules (d) in the myocardium of animals belonging to Group I on the 14th day of the experiment. Interstitial edema, lymphohistiocytic infiltration and lysis of single cardiomyocytes (e, f), red blood cell (RBC) aggregation (g), focal cardiosclerosis (h) on the 56th day of the experiment. Hematoxylin and eosin staining (a, c, e, f, g), periodic acid-Schiff (PAS) staining (b, d, h). Micrographs.

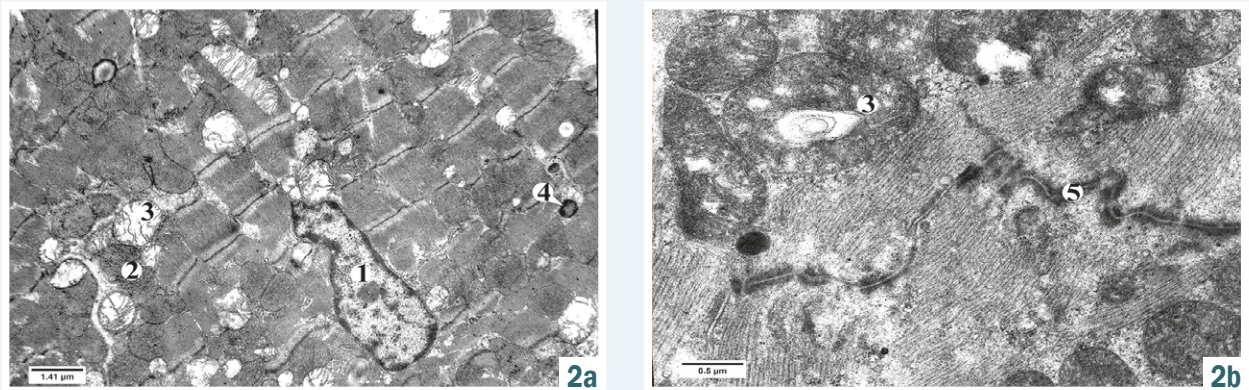


Fig. 2. Submicroscopic changes in left ventricular cardiomyocytes on the 14th day of simulating STZ-induced DM and CIS. Electron micrographs. **1:** cardiomyocyte nucleus; **2:** reserved mitochondria; **3:** mitochondria that underwent destructive changes; **4:** lipid droplet; **5:** intercalated disc.

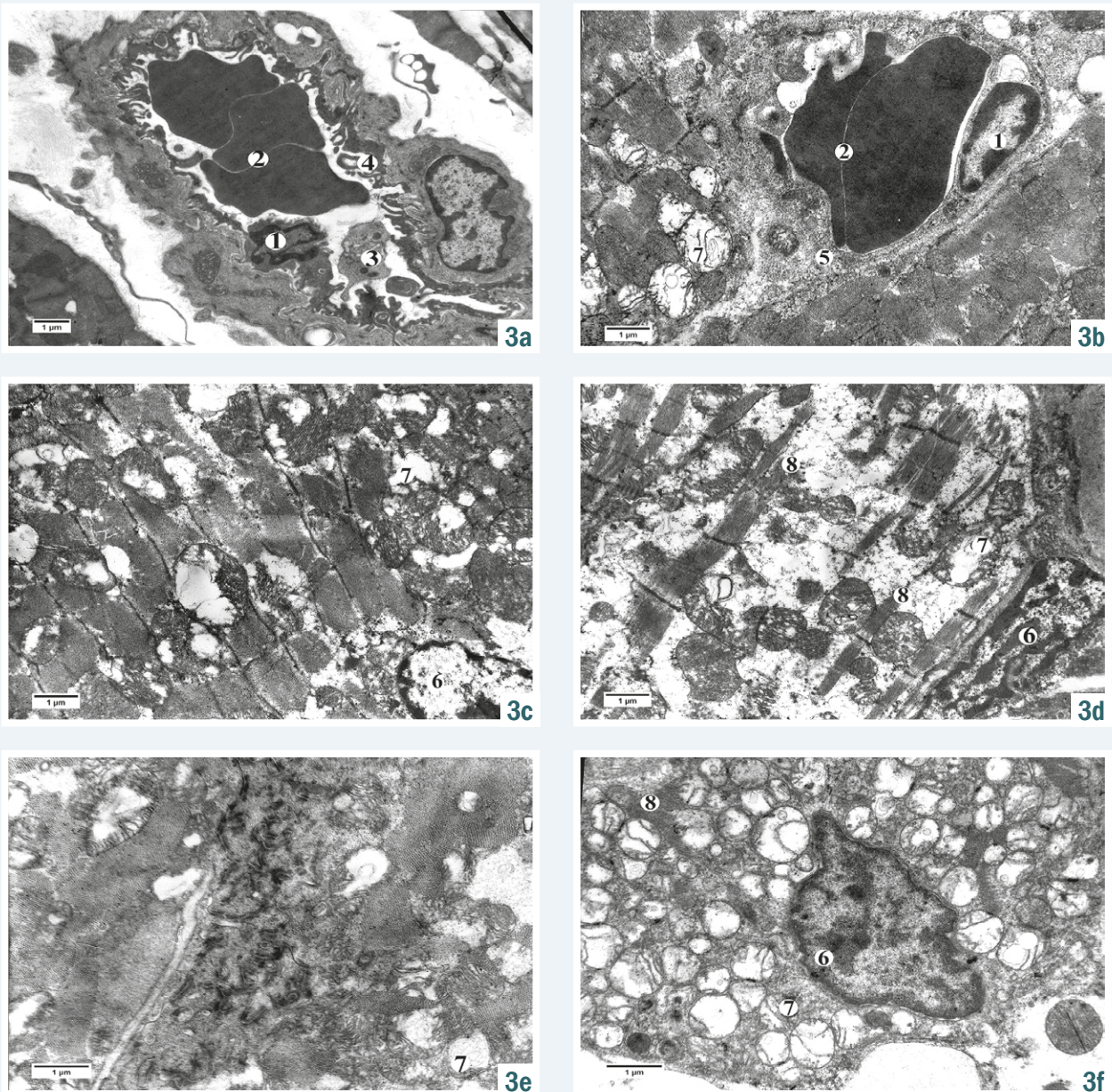


Fig. 3. Ultrastructure changes cardiomyocytes of myocardium on the 56th day of simulating STZ-induced DM and CIS. Electron micrographs. **1:** nucleus of endothelial cells; **2:** RBC aggregation; **3:** thrombocytes; **4:** microclasmatosis; **5:** basement membranes; **6:** cardiomyocyte nucleus; **7:** mitochondria that underwent destructive changes; **8:** lysis myofibrils.

Discussion

In the early stages of developing comorbid pathology and experimental DM on the background hyperglycemia and cortisolemia, the first signs of myocardial hypoxia due to impaired myocardial blood flow were observed. Arteriolar spasms in the microcirculation with partial or complete occlusion of the arteriolar lumen were first found. Hyperglycemia, hypercortisolemia, high blood levels of adrenocorticotropic hormone in rats with experimental DM on the 14th–28th days of the experiment have been described by other authors [8,9].

Impaired myocardial blood flow on the background of the initial signs of diabetic microangiopathy results in destructive mitochondrial changes and vacuolar degeneration which has been described earlier as well [10], however, for the first time ever, we have studied, described, and compared the state of the myocardium in case of a comorbidity. It worth noting that, during all the observation periods, changes were more pronounced in animals of Group I. Mitochondria were the very first organelles being damaged. In Group I and Group II, disorganization and destruction of the inner mitochondrial membrane was seen. In diabetic cardiomyopathy, cardiomyocytes undergo metabolic changes, including oxidative stress due to increased reactive oxygen species production by damaged mitochondria [11,12].

Sciarretta S. et al. have confirmed that stress-induced changes in mitochondrial cytochrome c oxidase can trigger a cascade of biochemical modifications in cardiomyocytes and lead to cardiomyocyte apoptosis [13]. In addition, in the early stages of simulation STZ-induced DM, cardiomyocyte surface area in different myocardial regions reduced, while the surface area of their nuclei remained almost unchanged as compared to intact animals, thereby leading to an increase in the N:C ratio.

According to Cagalinec M. et al., the surface area of cardiomyocytes reduced on the 7th day of STZ-induced DM due to their shrinkage [14]. Other researchers studying the effect of experimental DM on skeletal muscles postulate that the underlying cause is a reduction in glycogen content [15]. We hold the viewpoint that, in the early stages of developing STZ-induced DM, cardiomyocyte surface area reduces due to a reduction in glycogen content, as evidenced by the PAS-reaction and confirmed at the ultrastructural level. Glycogen granules filling the sarcoplasm between myofibrils and mitochondria in intact animals, were rarely seen.

On the 56th day of the experiment, animals of Group I and Group II developed diabetic cardiomyopathy. Cardiomyocyte atrophy, myolysis, focal fibrosis as well as interstitial and perivascular myocardial edema were observed. Cardiomyocytes were found to show vacuolar degeneration and apical ballooning, colliquative necrosis. Such changes occur due to the progression of insulin deficiency which reduces protein metabolism, provokes intracellular organelle degradation, and promotes increased autolysis in cardiomyocytes followed by a reduction in the content of actin that results in cardiomyocyte atrophy, fibrosis, and dilated cardiomyopathy [2,4].

We are of the opinion that the development of diabetic cardiomyopathy is associated with impaired glucose absorption due to insulin deficiency, impaired cardiomyocyte

metabolism, and developing microangiopathy. The latter progresses especially in the later stages of the experiment and results in impaired myocardial blood flow. Elevated cortisol levels in DM lead to the development of hyperergic inflammation in the vascular endothelium and increase fibrinogen synthesis [9,16,17]. The coagulation system is activated that results in hypercoagulation. Glucocorticoids affect collagen and mucopolysaccharide synthesis causing their excessive deposition in the vascular walls. We can explain the observed changes in the microcirculatory vessels by this very pathogenetic mechanism.

Conclusions

1. Experimental DM and its combination with CIS in the early stages of the experiment (day 14) led to a reduction in the surface area of ventricular cardiomyocytes in different myocardial regions and vacuolar degeneration. The initial signs of diabetic microangiopathy manifested as increased blood flow in the venules and veins, arteriolar spasm, RBC aggregation in the vascular lumen were found.

2. In the later stages of the experiment (day 56), interstitial edema, focal fibrosis, cardiomyocyte lysis, vacuolar degeneration, apical ballooning, colliquative necrosis were observed in the rat myocardium. Such changes occurred on the background of developing diabetic microangiopathy. The most pronounced myocardial changes were recorded in animals with a comorbidity.

Prospects for further research. Future studies of changes in the myocardium of the heart in experimental diabetes mellitus and its correction of various antidiabetic agent are promising. This will allow to create the new pathogenetic antidiabetic treatments for the correction of diabetic cardiomyopathies.

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