INTRODUCTION

The model of experimental, streptozotocin-induced diabetes mellitus (STZ-induced DM) (1, 2) has been shown to exhibit signs of endogenous protection developing already during the acute phase and leading to unexpectedly high resistance of the heart to adverse external stimuli such as calcium overload and ischemia. Further studies performed on this model led to findings similar to those reported in research using animal models of ischemic preconditioning. It has been hypothesized that the heart, as a vital organ, has a number of compensatory mechanisms that become activated in an attempt to accommodate energy demands in DM condition and maintain cardiac function in response to pathological stimulus represented by ischemic reperfusion (I/R) damage (3, 4). In its early stages DM has all main attributes of metabolic preconditioning, thereby providing the opportunity to monitor the initiation and course of endogenous protection mechanisms in the acute phase of the disease (5-8). The experimental model of STZ-induced DM has been reported as representing type I diabetes where STZ is transported into the pancreatic beta cells - the islets of Langerhans - through a glucose transporter GLUT-2, where it accumulates and through a variety of mechanisms causes their destruction and the onset of type I diabetes (9, 10). This sort of metabolic preconditioning has been described in studies that documented remodeling of subcellular membrane system in the myocardium with consequences which are not necessarily pathological, but rather they reflect a specific endogenous form of cardioprotection (11-13).

Intrinsic cardioprotective mechanisms become activated in the early diabetes mellitus (DM) and this may protect the heart from ischemia/reperfusion (I/R) similarly as in case of ischemic preconditioning. However, this protection may be blunted in the presence of cardiovascular risk factors. Assuming that hypercholesterolemia (HCH) frequently accompanies DM, this study extends findings from separate models of DM and HCH by investigation the impact of HCH on DM-induced changes, including those of compensatory nature, in rat heart and its mitochondria. We used a factorial design with all combinations of treatment factors DM and HCH: control rats (C) and streptozotocin-treated rats (DM), both on standard chow (C and DM) and fed fat-cholesterol diet (HCH and DM-HCH). Isolated, Langendorff perfused hearts were subjected to 30 min global ischemia followed by reperfusion. Significantly increased levels of cholesterol in DM-HCH after I/R injury abrogated compensatory fluidization characteristic of DM mitochondria membranes. Concomitantly, the mitochondrial Mg2+-ATPase activity in DM-HCH was depressed. In comparison with DM, which showed significantly reduced size of myocardial infarction with simultaneously improved recovery of contractile function due to conditioning, DM-HCH hearts exhibited attenuated resistance to I/R injury. Taken together, cholesterol-enriched diet was associated with inflicting damage and has been implicated in the mechanisms leading to suppression of cardiac protection presented in diabetic group. Apparently, DM and HCH are factors which are not additive in their effects, therefore, caution should be exercised, when interpreting findings from studies considering these factors in isolation. Our findings suggest that this complex condition could accelerate the development of late diabetic complications.

Key words: myocardial hypercholesterolemia, acute diabetes mellitus, heart mitochondria, ischemia/reperfusion injury, cardioprotection
adaptation (14). The degree of adaptation can be quantified, first, by physiological measures that assess cardiac function under various experimental conditions such as infarct size (IS), ventricular tachycardia, severity of arrhythmia or recovery of left ventricular developed pressure (LVDP) (15-17), and second, by measures that assess the structural and functional properties of heart mitochondria (6, 18, 19).

Recently, several research groups have begun to explore the active role of cardiac mitochondria in the processes of endogenous protection. Commonly used models of preconditioning have yielded results pointing to the mitochondria that appear to be a key and effective end-effector responsible for myocardial energy maintenance under I/R injury (20). Although abnormal glucose metabolism defines DM and accounts for many of its symptoms and associated adverse and compensatory events, efforts to understand these processes are increasingly focused on disordered lipid metabolism. Recent studies have found that anti-contractile effects exerted byperivascular adipose tissue on the vasculature are reduced in hypertension and obesity (21). Many animal and human studies have been published that explore the mechanistic links between disorders of fatty acid-/lipid metabolism and insulin resistance (22), however, to our knowledge, none of the reviewed studies addressed the issue whether or not the cholesterol levels, which are naturally increased in DM, can affect developing compensatory responses. Here we present findings from a study designed to investigate individual and combined effects of the STZ-induced DM and cholesterol-enriched diet. We chose a model of the acute DM condition in order to better control experimental conditions, since disordered lipid metabolism would reflect effect of the both factors, developing diabetes and the cholesterol-enriched diet. The outcome - the cardiovascular adaptation (14). The degree of adaptation can be quantified, first, by physiological measures that assess cardiac function under various experimental conditions such as infarct size (IS), ventricular tachycardia, severity of arrhythmia or recovery of left ventricular developed pressure (LVDP) (15-17), and second, by measures that assess the structural and functional properties of heart mitochondria (6, 18, 19).

Moreover, enhanced resistance to I/R in the early stage of experimental DM has been found to share several molecular mechanisms of protection conferred by ischemic preconditioning (IPC) (24, 25). Endogenous cardioprotective mechanisms become activated in early stage of DM and this may protect the heart from I/R injury through enhancement of endothelial nitric oxide synthase (nNOS) expression, NO formation, activation of cell survival signals, and decreased oxidative stress, which are the mechanisms responsible for cardioprotective action of IPC as well (24). However, it has been well documented that cardiovascular risk factors may interfere with cardioprotective interventions such as IPC (26). Similarly, under different experimental conditions it has been observed that the cardioprotective mechanisms present in experimental early DM are blunted or even abolished in both conditions, the advanced stages of DM and the combination of early DM with hypercholesterolemia (HCH) (15, 27, 28).

Changes in mitochondrial membrane lipid composition and content modify all lipid-dependent processes that take place in the membrane (29, 30), e.g. mitochondrial respiration (31, 32) oxidative phosphorylation, and other mitochondrial processes such as generation of reactive oxygen species (ROS) (33) and Ca\(^{2+}\)-induced mitochondrial permeability transition (30, 33). In this respect especially the cholesterol content is of importance. Although plasma cholesterol correlates with membrane cholesterol in a non-trivial manner, in vivo exposure to hypercholesterolemic environment increases incorporation of cholesterol into the plasma membranes as well as in subcellular membranes thus influencing their physicochemical properties (34-36). Cholesterol consists of four hydrocarbon rings, which are strongly hydrophobic - however, the hydroxyl (OH) group attached to one end of cholesterol is weakly hydrophilic, meaning that cholesterol is an amphipathic molecule. Thus, the optimal membrane location of cholesterol is with its backbone embedded in the hydrocarbon core, roughly along the membrane normal, and with the OH group protruding into the polar headgroup region of the membrane (37). Cholesterol molecules interact with phospholipid molecules within one monolayer of a lipid bilayer (Fig. 1) (38) and, unlike phospholipids which have a rigid structure, it is extremely low, cholesterol can move easily from one leaflet of the bilayer to the other (39). The intercalation of cholesterol leads to straightening of the phospholipid acyl tails and prevents the molecules from diffusing. All these events result in rigidization (stiffening) of the membranes (40) which is more intense, when cholesterol is present in an oxidized form (38).

High cholesterol concentrations most often lead to a decrease in membrane fluidity and thickness thereby hindering

![Fig. 1. The cholesterol molecule inserts itself in the membrane with the same orientation as the phospholipid molecules. Note that the polar head of the cholesterol is aligned with the polar head of the phospholipids. The arrow depicts spontaneous ‘flip-flop’ of the cholesterol molecule from one leaflet of a bilayer to the other. Processed according to Wolfe (38).](image-url)
the mobility of the intermembrane components (41, 42). Although the amount of cholesterol found in the inner membrane is low in comparison with that in the outer membrane, the incorporated amount of cholesterol is sufficient to influence both, the activity of mitochondrial ATP synthase (43) and the fluidity of inner mitochondrial membrane (34). Lateral displacement of membrane proteins due to cholesterol incorporation results in the formation of aggregates in the mitochondrial outer membrane owing to an increase in the membrane cholesterol (45). However, when it is present in high concentrations, cholesterol can substantially disrupt the ability of the phospholipids to interact among themselves, which increases fluidity and lowers the gel-sol transition temperature (39). The above mentioned makes the dependence of fluidity on the amount of membrane cholesterol more elaborate, thus quantitative measurements of membrane fluidity should always be interpreted with respect to the technique and the reference values in the experimental setup. In the light of the above, using a spatially and time-averaged (i.e. steady-state) fluorescence anisotropy (r) may be a good choice in situations where scientists are interested in distinguishing between various experimental conditions. Since animal cell plasma membranes can contain substantial quantities of cholesterol, this issue may be significant in some pathologies, such as familiar hypercholesterolemia (46), atherosclerosis and coronary heart disease (47), hypertension, diabetes and others cardiovascular and metabolic diseases (48). Hyperlipidemia has been found to attenuate the cardioprotective effect induced by IPC as a form of cardioprotection (49, 50). This finding is also supported by other studies confirming the interference between cardioprotective effect of ischemic preconditioning and HCH (16, 51, 52). HCH accelerates the evolution of myocardial ischemia, delays recovery on reperfusion, and deteriorates the anti-ischemic effect of preconditioning in humans (53). All these observations raise concern that HCH might have a negative influence on adaptation, presented in the experimental model of acute STZ-induced DM, which is the reflection of the cardioprotection (16, 18). We focused on verifying whether the HCH induced by a cholesterol-enriched diet would influence the degree of cardioprotection induced by acute STZ-induced DM. Specifically, we aimed on investigating the impact of HCH on mitochondrial membrane fluidity, mitochondrial energetics and related pathophysiological changes in myocardial injury and function.

MATERIALS AND METHODS

All animal experiments were performed in strict accordance with the rules issued by the State Veterinary Administration of the Slovak Republic, legislation No 289/2003 and with the regulations of the Animal Research and Care Committee of Institute for Heart Research.

Experimental animal models

Male Wistar rats 10 – 12 weeks aged (229 ± 20 g b.w.) were kept under conditions of standard light regimen (D:L, 12:12) at 22 ± 2°C in cages (max. 5 animals per cage) with free access to water. We used control healthy rats and STZ-induced early diabetic rats, both with normal chow and fed with fat-cholesterol diet. Control animals were fed with standard chow (C rats; n = 8 per group) and with fat-cholesterol diet (HCH rats; n = 8 per group). HCH was induced by cholesterol-enriched diet consisting of 1% cholesterol, 1% coconut oil, 20 g/day (HCH rats; n = 8 per group). Acute experimental DM was

Fig. 2. Experimental design of myocardial ischemic-reperfusion injury induced in control group, cholesterol-enriched diet group (HCH group), streptozotocin induced acute diabetic group (DM group), and combination of cholesterol-enriched diet and streptozotocin induced acute diabetic group (DM-HCH group).
induced by a single dose of streptozotocin (STZ) (80 mg kg\(^{-1}\); intraperitoneally) dissolved in 0.1 mol L\(^{-1}\) citrate buffer, pH 4.0 (DM rats; \(n = 8\) per group). Animals with simultaneously induced diabetes mellitus and hypercholesterolemia for five days were used for the induction of the diabetic-hypercholesterolemic state (DM-HCH rats; \(n = 8\) per group).

The DM and DM-HCH rats had been receiving 5% glucose solution per os during the first 24 h after STZ application to prevent transient hypoglycaemia (15, 51). A modified double-disease model of the DM-HCH rats were developed by Jiao et al. and Kusunoki et al. (54, 55). Progress of disease was being monitored during five days by measuring of glycosuria using Gluko PHAN strips (Erba-Lachema, Brno, Czech Republic). Experimental protocol is depicted in Fig. 2.

**Anesthesia**

Prior to heart excision the experimental animals were anesthetized with thiopental (50 – 60 mg kg\(^{-1}\); intraperitoneally) administered together with heparin (500 IU; intraperitoneally).

**Determination of metabolic status of animals**

The samples of blood for estimation were collected from the abdominal aorta of the animals from all groups. Metabolic state was evaluated by determination of glucose and triacylglycerol contents, total cholesterol content, low-density lipoprotein cholesterol (LDL), high-density lipoprotein cholesterol (HDL), very low-density lipoprotein (VLDL), glycohemoglobin by using test strips of Multicare and LUX multiparameter system, (Biochemical system internation, Florence, Italy) in the blood (blood sample volume was 10 – 15 µL) as well as insulin in the serum (RIA kit, Linco Research USA).

**Table 1. Determination of metabolic status of animals. Effect of the hypercholesterolemia induced by cholesterol-enriched diet and experimental model of streptozotocin-induced diabetes mellitus on the metabolic parameters.**

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>HCH</th>
<th>DM</th>
<th>DM-HCH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose [mmol L(^{-1})]</td>
<td>5.33 ± 0.16</td>
<td>5.99 ± 0.23</td>
<td>17.80 ± 0.89**</td>
<td>20.89 ± 1.57**</td>
</tr>
<tr>
<td>Triacylglycerol [g L(^{-1})]</td>
<td>1.25 ± 0.10</td>
<td>1.33 ± 0.13</td>
<td>4.63 ± 0.32**</td>
<td>4.90 ± 0.42**</td>
</tr>
<tr>
<td>Cholesterol [mmol L(^{-1})]</td>
<td>1.76 ± 0.12</td>
<td>1.79 ± 0.12</td>
<td>2.40 ± 0.15*</td>
<td>3.03 ± 0.56**</td>
</tr>
<tr>
<td>LDL cholesterol [mmol L(^{-1})]</td>
<td>0.24 ± 0.10</td>
<td>0.32 ± 0.05</td>
<td>0.59 ± 0.08*</td>
<td>1.38 ± 0.39**</td>
</tr>
<tr>
<td>HDL cholesterol [mmol L(^{-1})]</td>
<td>0.72 ± 0.11</td>
<td>0.80 ± 0.12</td>
<td>0.81 ± 0.11</td>
<td>0.86 ± 0.15</td>
</tr>
<tr>
<td>VLDL cholesterol [mmol L(^{-1})]</td>
<td>0.28 ± 0.05</td>
<td>0.37 ± 0.09</td>
<td>0.31 ± 0.10</td>
<td>0.63 ± 0.13*</td>
</tr>
<tr>
<td>Glycohemoglobin [%Hb]</td>
<td>4.05 ± 0.13</td>
<td>4.21 ± 0.33</td>
<td>7.68 ± 1.02**</td>
<td>7.81 ± 1.11**</td>
</tr>
<tr>
<td>Insulin [ng mL(^{-1})]</td>
<td>1.04 ± 0.15</td>
<td>1.02 ± 0.15</td>
<td>0.49 ± 0.09**</td>
<td>0.48 ± 0.12**</td>
</tr>
</tbody>
</table>

**Langendorff perfusion**

Perfusion protocol was performed as previously described (15, 51). The hearts of anesthetized animals were rapidly excised and perfused at 37°C in the Langendorff mode at a constant perfusion pressure of 73 mmHg. The perfusion solution was a modified Krebs-Henseleit buffer infused with 95% O\(_2\) and 5% CO\(_2\) (pH 7.4) containing (mmol L\(^{-1}\)): 118 NaCl, 3.2 KCl, 1.2 MgSO\(_4\), 25 NaHCO\(_3\), 1.18 KH\(_2\)PO\(_4\), 2.5 CaCl\(_2\) and 5.5 glucose. An epicardial electrogram was recorded by two electrodes attached to the apex of the heart and the aorta. Left ventricular (LV) pressure was measured by means of a nonelastic balloon inserted into the LV cavity (water-filled to obtain end-diastolic pressure of 5 – 7 mmHg and connected to a pressure transducer (MLP844 (ADInstruments, Germany)). LV systolic pressure (LVSP); LV diastolic pressure (LVDiP); LV developed pressure (LVDP [systolic minus diastolic pressure]), maximal rates of pressure development and fall \([dP/dt]_{\text{max}}\), respectively) as the indexes of contraction and relaxation, heart rate (HR) and coronary flow (CF) were measured during a preischemic stabilization period and were continuously recorded until the end of 40 min of reperfusion using PowerLab/8SP Chart 7 software (ADInstruments, Germany).

**Protocols of global ischemia**

The hearts of all rats were assigned to the following protocol (\(n = 8\) per group). After a 20 min stabilization period in Langendorff mode, the hearts were subjected to 30 min of global ischemia followed by 40 min of reperfusion by clamping and unclamping of aortic inflow for the evaluation of post-ischemic contractile dysfunction (myocardial stunning). Recovery of LVDP at the end of 40 min reperfusion served as the end point of injury.
**Isolation of mitochondria**

Each excised heart was cut into small pieces by scissors in the presence of small volume of ice-cold isolated solution (containing in mmol.L⁻¹): 180 KCl, 4 EDTA, 1% bovine serum albumin, pH 7.4. Minced heart tissue was suffused with 20 mL of ice-cold isolated solution without protease and centrifuged at 1000 g for 10 min. Resulting supernatant was centrifuged down at 6200 g for 10 min and pellet containing mitochondria was then again re-suspended in an albumin-free isolation solution (containing in mmol.L⁻¹: 180 KCl, 4 EDTA). It was centrifuged down at 6200 g for 10 min and subsequently used for estimation of protein concentration as well as for further biophysical and biochemical investigations.

**Determination of Mg²⁺-ATPase activity**

The Mg²⁺-ATPase activity represents the activity of mitochondrial ATP synthase working in the presence of Mg²⁺ cations in a reverse direction, i.e. splitting ATP molecule to ADP and inorganic phosphate \( P_i \). The activity was determined in 1 mL of incubation medium containing: 200 µL imidazole buffer (250 mmol.L⁻¹), 100 µL MgCl₂ (40 mmol.L⁻¹), 100 µL ATP-Tris (40 mmol.L⁻¹) and 50 µg of mitochondrial fraction with concentration (1 µg.µL⁻¹) in the presence and absence of 100 µL DNP (0.1 mmol.L⁻¹). The addition of DNP results in the disruption of all mitochondrial membranes, the influx of Mg²⁺ cations into the mitochondria, which allows measuring the total activity of mitochondrial Mg²⁺-ATPase. The reaction was started by the addition of ATP after 10 min pre-incubation with 50 µg of mitochondrial fraction at 37ºC. After 20 min the reaction was terminated by 1 mL of ice-cold isolated solution containing 180 mmol.L⁻¹ KCl and 4 mmol.L⁻¹ Na₂EDTA adjusted to pH 7.4 by Tris-HCl. Lipids were extracted from 500 µl of membrane suspension with 1000 µl of chloroform:methanol (1:2 v/v) under vortexing for 30 s. Subsequently, 500 µl of chloroform was added and vortexed again for 30 s. Extraction was terminated by addition of 500 µl of 15 mmol.L⁻¹ Na₂EDTA containing 4% NaCl and spinning down for 10 min at 1900 g. Then 600 µl of the lipid-containing lower layer of chloroform:methanol was transferred to a separate test tube and carefully evaporated at room temperature under a continuous stream of nitrogen to prevent oxidation. Beginning from this point, all the procedure was performed in a nitrogen atmosphere. The dry lipids were then dissolved in 3 mL of cyclohexane, vortexed for 30 s, and used spectrophotometrically at 700 nm as the amount of inorganic phosphate (\( P_i \)) liberated by ATP splitting per unit of the time (µmol.P.g⁻¹.h⁻¹).

**Determination of mitochondrial membrane fluidity**

Mitochondrial membrane fluidity was determined in terms of fluorescence anisotropy of the lipophilic fluorescence probe 1,6-diphenyl-1,3,5-hexatriene (DPH). Fluorescence anisotropy measurements are widely used as sensitive indicators of cell membrane fluidity. The DPH probe incorporates into the membrane in parallel to the acyl chains of lipid bilayer and ‘senses’ movement of lipids in the membrane. Increased rate of its movement is associated with a decrease in the fluorescence anisotropy. Isolated mitochondrial fraction was resuspended to a 0.5 mg.mL⁻¹ of protein in the solution containing: 180 mmol.L⁻¹ NaCl, 4 mmol.L⁻¹ EDTA (pH 7.4) and were marked by 0.25 μL of DPH solution resuspended in a mixture of acetone and water (1:250). Samples were incubated at 22 ± 1°C. The fluorescence was determined at an excitation wavelength of 360 nm and at an emission wavelength of 425 nm according to Shinitzky (56).

**Determination of conjugated dienes content**

The content of conjugated dienes in the lipids from mitochondrial membranes was assessed by the method (57) adapted to the estimation of conjugated dienes in the membranes of heart mitochondria. Isolated heart mitochondria were suspended at a concentration of 1 mg.mL⁻¹ in a solution containing 180 mmol.L⁻¹ KCl and 4 mmol.L⁻¹ Na₂EDTA adjusted to pH 7.4 by Tris-HCl. Lipids were extracted from 500 µl of the membrane suspension with 1000 µl of chloroform:methanol (1:2 v/v) under vortexing for 30 s. Subsequently, 500 µl of chloroform was added and vortexed again for 30 s. Extraction was terminated by addition of 500 µl of 15 mmol.L⁻¹ Na₂EDTA containing 4% NaCl and spinning down for 10 min at 1900 g. Then 600 µl of the lipid-containing lower layer of chloroform:methanol was transferred to a separate test tube and carefully evaporated at room temperature under a continuous stream of nitrogen to prevent oxidation. Beginning from this point, all the procedure was performed in a nitrogen atmosphere. The dry lipids were then dissolved in 3 mL of cyclohexane, vortexed for 30 s, and used spectrophotometrically at 232 nm. Determination of conjugated dienes content was performed in a nitrogen atmosphere. The dry lipids were then dissolved in 3 mL of cyclohexane, vortexed for 30 s, and used.

Fig. 3. Effect of the hypercholesterolemia induced by cholesterol-enriched diet and experimental model of streptozotocin-induced diabetes mellitus on the size of myocardial infarction. C, control group; DM, group with streptozotocin-induced diabetes mellitus; HCH, group with hypercholesterolemia induced by cholesterol-enriched diet; DM-HCH, diabetic-hypercholesterolaemic group (simultaneously streptozotocin-induced diabetes mellitus with hypercholesterolemia induced by cholesterol-enriched diet); IS/AR, infarct size/area at risk. Data are means ± SEM; n = 8 per each group; *P < 0.05 versus DM, #P < 0.05 versus control.
directly for spectrophotometric determination of the conjugated dienes at $\lambda = 233$ nm, $\varepsilon = 29,000$ L.mol$^{-1}$.cm$^{-1}$.

**Determination of myocardial infarction**

Separate set of animals was used to determine the size of myocardial infarction. Langendorff perfused hearts of all experimental groups were exhibited to 30 min global ischemia followed by 40 min (with balloon) plus 80 min (without balloon). After this protocol the size of the infarcted area and the area at risk (AR) size were delineated by staining with 2,3,5-triphenyltetrazolium chloride (TTC) and determined by a computerized planimetric method as previously described (58). Since the AR represents the entire area of the LV in the global ischemia protocol, IS was expressed as a percentage of LV size.

**Statistical analysis**

Descriptive and univariate analyses were performed on all selected animals’ characteristics. Mean ± SEM (standard error of mean) is given for the normally distributed variables or a median and interquartile range if data showed substantial deviations from normality. Categorical variables are presented as relative counts and percentages.

We used a one-way analysis of variance (ANOVA) to analyze between-group differences in metabolic parameters, and a two-way ANOVA to analyze data from factorial experiments: treatment effect (cholesterol-enriched diet as the first main factor) in rats which were grouped by presence of diabetes condition (DM as the second main factor). Two-way ANOVA with repeated measures was used in case of time-dependent recordings with a pre-planned post-hoc comparison at the end of reperfusion (40. min).

Level for statistical significance for testing between-group differences was set at $\alpha = 0.05$ and that for testing interaction at less conservative $\alpha' = 0.15$. All statistical tests were two-sided.

**RESULTS**

**Development of diabetes and hypercholesterolemia**

Effect of the HCH induced by cholesterol-enriched diet and experimental model of streptozotocin-induced DM on the metabolic parameters are shown in Table 1. Rats in the DM groups exhibited significant elevated levels of glucose ($P < 0.01$), triacylglycerols ($P < 0.01$), total cholesterol ($P < 0.05$), LDL cholesterol ($P < 0.01$), glycohemoglobin ($P < 0.01$), decreased level of insulin ($P < 0.01$), non-significant increased levels of HDL cholesterol and VLDL cholesterol on the 5th day after the application, thus confirming the development of diabetes. The increase in the mentioned parameters was more pronounced in the DM-HCH group on comparison with control group HCH group did not show any significant effect of metabolic parameters on comparison with control rats (Table 1).

**Characteristic of functional parameters and lethal injury in isolated rat hearts**

Preischemic stabilization parameters (LVSP, LVDiP, LVDP, $+\frac{dP}{dt}_{max}$, HR and CF) exhibited no significant changes among the groups and these data are summarized in Table 2. Hearts from DM rats on the standard diet showed a significantly reduced size of myocardial infarction in comparison with control hearts (Fig. 3). Accordingly, recovery of contractile function (LVDP) after I/R was improved in the diabetic animals. Similarly hearts from DM rats on the standard diet exhibited a significantly better recovery of LVDiP, $+\frac{dP}{dt}_{max}$ as well as $-\frac{dP}{dt}_{max}$ compare to control.

**Table 2.** Preischemic values of parameters of myocardial function in Langendorff-perfused rat hearts.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>C</th>
<th>DM</th>
<th>HCH</th>
<th>DM-HCH</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR [beats/min]</td>
<td>255 ± 6</td>
<td>249 ± 8</td>
<td>259 ± 9</td>
<td>248 ± 9</td>
</tr>
<tr>
<td>CF [ml/min]</td>
<td>10.8 ± 1.0</td>
<td>11.3 ± 1.2</td>
<td>12.5 ± 0.9</td>
<td>12.2 ± 09</td>
</tr>
<tr>
<td>LVSP [mmHg]</td>
<td>82.3 ± 5.2</td>
<td>88.4 ± 7</td>
<td>85.0 ± 5.6</td>
<td>87.0 ± 5.3</td>
</tr>
<tr>
<td>LVDiP [mmHg]</td>
<td>6.0 ± 1.0</td>
<td>6.7 ± 1.5</td>
<td>5.9 ± 0.8</td>
<td>6.1 ± 0.9</td>
</tr>
<tr>
<td>LVDP [mmHg]</td>
<td>76.8 ± 2.3</td>
<td>82.6 ± 5.9</td>
<td>78.2 ± 8.7</td>
<td>80.9 ± 8.2</td>
</tr>
<tr>
<td>$+\frac{dP}{dt}_{max}$ [mmHg/s]</td>
<td>2295 ± 123</td>
<td>2214 ± 187</td>
<td>2168 ± 203</td>
<td>2183 ± 152</td>
</tr>
<tr>
<td>$-\frac{dP}{dt}_{max}$ [mmHg/s]</td>
<td>1425 ± 118</td>
<td>1368 ± 123</td>
<td>1325 ± 99</td>
<td>1359 ± 160</td>
</tr>
</tbody>
</table>

Statistical analyses were performed using StatsDirect 3.0.191 software (Stats Direct Ltd., Cheshire, UK) and GraphPad Prism 7.0 (GraphPad Software, Inc., USA).
control hearts (Table 3). A one-week HCH diet did not affect response to I/R in the control hearts. However, it reduced the ability of diabetic hearts to resist I/R, since diabetic rats on the cholesterol-enriched diet exhibited significantly increased size of infarction (Fig. 3) and depressed functional recovery (LVDP, LVDiP, +dP/dtmax) when compared with the DM group (Fig. 4, Table 3). After 40 min reperfusion, HR tended to decrease similarly in all groups by 12% to 20%, and CF ranged between 73% and 83% of baseline values. There were no significant changes in values of HR and CF among the groups after 40 min of reperfusion (data not shown).

Alteration in mitochondrial membrane properties in diabetes mellitus condition and high cholesterol diet

In order to find out whether cholesterol-enriched diet after I/R injury might have modulated cardioprotection due to membrane remodeling induced by diabetic condition and evaluated in terms of the markers of mitochondrial membrane structure, function and damage such as fluidity, DNP-stimulated Mg2+-ATPase activity, and conjugated dienes as a marker of oxidative damage, we have chosen a factorial experiment, and have investigated whether the strength of relationship differed between DM animals and the controls. To do so, we also tested the interaction between both factors - DM condition and the cholesterol-enriched diet in order to determine what proportion of variation in the above parameters of mitochondrial structure and function could be ascribed to DM when adjusting for adverse effect of cholesterol-enriched diet. Two-way ANOVA showed that both main factors, DM and HCH, had statistically significant effect on mitochondrial membrane fluidity (Fig. 5), which means that diabetic condition increased fluidity (ignoring cholesterol-enriched diet) and cholesterol-enriched diet decreased fluidity (again, irrespective of DM status). DNP-stimulated Mg2+-ATPase activity was significantly increased in DM; the factor cholesterol-enriched diet non-significantly decreased the activity across DM status levels (Fig. 6). Cholesterol-enriched diet also significantly increased levels of conjugated dienes with non-significant contribution of the diabetic condition (Fig. 7).

Table 3. Parameters of myocardial function in Langendorff-perfused rat hearts after I/R injury. C, control group; DM, group with streptozotocin-induced diabetes mellitus; HCH, hypercholesterolemia group (hypercholesterolemia induced by cholesterol-enriched diet); DM-HCH, diabetic hypercholesterolemic group (simultaneously streptozotocin-induced diabetes mellitus with hypercholesterolemia induced by cholesterol-enriched diet); HR, heart rate; CF, coronary flow; LVSP, left ventricular systolic pressure; LVDiP, left ventricular diastolic pressure; LVDP, left ventricular developed pressure; +dP/dtmax and –dP/dtmax, maximum rates of pressure development and fall, respectively. Data are means ± SEM; n = 8 per each group; *P < 0.05 versus DM group, #P < 0.05 versus control.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>C</th>
<th>DM</th>
<th>HCH</th>
<th>DM-HCH</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVSP [mmHg]</td>
<td>52.7 ± 4.8</td>
<td>65.8 ± 5.3</td>
<td>53.0 ± 6.0</td>
<td>59.8 ± 6.2</td>
</tr>
<tr>
<td>LVDiP [mmHg]</td>
<td>30.6 ± 5.9</td>
<td>17.9 ± 3.6</td>
<td>26.2 ± 6.5</td>
<td>29.2 ± 6.8*</td>
</tr>
<tr>
<td>+dP/dtmax [mmHg/s]</td>
<td>860 ± 186</td>
<td>1392 ± 228</td>
<td>911 ± 165</td>
<td>874 ± 147*</td>
</tr>
<tr>
<td>–dP/dtmax [mmHg/s]</td>
<td>492 ± 118</td>
<td>784 ± 305</td>
<td>587 ± 109</td>
<td>558 ± 207</td>
</tr>
</tbody>
</table>
However, we observed significant interaction effects in all the variables (all P < 0.15). The presence of these interactions can be seen in Fig. 8, when comparing lines connecting the respective means. Apparently, the lines are non-parallel in all the above mentioned characteristics, which means that the factors were not additive in the resulting effect, but the effect size of one factor depended on the level of the second factor. Thus, the main factors, DM and HCH, should not be considered in isolation.

Significant differences in the simple mean effects of DM and HCH are marked in Figs. 5-7. When compared with C group mitochondrial membrane fluidity in DM group was increased as reflected by significantly reduced rigidity index (Fig. 5). This

![Index of membrane rigidity](image)

**Fig. 5.** Effect of the hypercholesterolemia induced by cholesterol-enriched diet and experimental model of streptozotocin-induced diabetes mellitus on the mitochondrial fluorescence anisotropy of DPH in the rat heart. C, control group; DM, group with streptozotocin-induced diabetes mellitus; HCH, group with hypercholesterolemia induced by cholesterol-enriched diet; DM-HCH, diabetic-hypercholesterolaemic group (simultaneously streptozotocin-induced diabetes mellitus with hypercholesterolemia induced by cholesterol-enriched diet). Data are means ± SEM; n = 8 per each group; *P < 0.05 versus DM, #P < 0.05 versus control.

![Mitochondrial Mg²⁺-ATPase activity](image)

**Fig. 6.** Changes in mitochondrial DNP-stimulated Mg²⁺-ATPase activity induced by hypercholesterolemia induced by cholesterol-enriched diet and experimental model of streptozotocin-induced diabetes mellitus. C, control group; DM, group with streptozotocin-induced diabetes mellitus; HCH, group with hypercholesterolemia induced by cholesterol-enriched diet; DM-HCH, diabetic-hypercholesterolaemic group (simultaneously streptozotocin-induced diabetes mellitus with hypercholesterolemia induced by cholesterol-enriched diet). Data are means ± SEM; n = 8 per each group; *P < 0.05 versus control.

![Conjugated dienes](image)

**Fig. 7.** Effect of the hypercholesterolemia induced by cholesterol-enriched diet and experimental model of streptozotocin-induced diabetes mellitus on the concentration of conjugated dienes in the heart mitochondria. C, control group; DM, group with streptozotocin-induced diabetes mellitus; HCH, group with hypercholesterolemia induced by cholesterol-enriched diet; DM-HCH, diabetic-hypercholesterolaemic group (simultaneously streptozotocin-induced diabetes mellitus with hypercholesterolemia induced by cholesterol-enriched diet). Data are means ± SEM; n = 8 per each group.
finding was paralleled by a significant increase in mitochondrial DNP-stimulated Mg\(^{2+}\)-ATPase activity (Fig. 6) in the DM group in comparison with the control group. The positive effect of increased mitochondrial membrane fluidity and DNP-stimulated Mg\(^{2+}\)-ATPase activity was not found in the group on the cholesterol-enriched diet in which the diet significantly reduced fluidity (Figs. 5 and 6). In both DM groups on standard and cholesterol-enriched diet the membrane fluidity was comparable to that observed in control group (Fig. 5). The content of conjugated dienes was not significantly different among experimental groups (Fig. 7).

**DISCUSSION**

The enhanced tolerance to I/R injury observed in the STZ-DM hearts can be considered as a form of intrinsic cardioprotection
analogous to that induced by a short-term adaptive phenomenon of ischemic preconditioning in the normal heart, where numerous metabolic stimuli, in particular those related to both, oxidative damage and increased intracellular calcium signaling, can trigger protection against I/R (59). Early period after onset of DM is associated with activation of adaptive mechanisms which successfully counteract metabolic disorders leading to irreversible cell damage and arrhythmias. Similar efficacy against reperfusion-induced arrythmias as presented in early phase of DM can be observed with the use of Na+/H+ exchanger inhibitors as well as Na+/Ca2+ exchanger inhibitors (60). Several mechanisms have been proposed to explain a lower sensitivity to I/R in the diabetic heart. The alterations in the intracellular pH, a decreased clearance of protons via Na+/H+ exchanger, and decreased rate of glycolysis in the diabetic myocardium, may represent pivotal factors attenuated response to I/R injury (23). Accordingly, our results showed a decreased susceptibility of DM hearts to I/R injury represented by significantly improved recovery of contractile function after I/R as well as decreased size of myocardial injury. Duration of the diabetic state plays a crucial role in the myocardial response to ischemia and in IPC-like effects, thus initially increased resistance to I/R injury declines in the chronic phase of diabetes (61). Improved postischemic contractile recovery in the diabetic myocardium is strongly dependent on the movement of the protein molecule in the membrane lipid environment. Adaptive changes in the physicochemical properties underlying membrane fluidity allow the membrane proteins, including ATP synthase, to keep working under suboptimal conditions (65).

Particularly, changes in fluidity of mitochondrial membranes are considered to be an adaptive response with a positive impact on physiological/functional performance of the membranes. In this regard, it can be attributed to endogenous protective mechanisms. Our finding of increased mitochondrial membrane fluidity in the diabetic group accompanied with enhanced ATPase activity (62) is in agreement with previously observed cardioprotective effects induced by ischemic preconditioning (63).

Further results have proved that the condition of acute DM leads also to increased levels of lipids, lipoproteins and glycoproteins (64), to remodeling of mitochondrial membranes with a direct impact on the membrane proteins (5). It has been shown that activity of mitochondrial ATP synthase is essentially increased and mitochondrial function compromised when rats were exposed to both factors, DM and HCH. Since HCH diet itself caused no alteration either in mitochondrial function or in cardioprotective mechanisms, which was reflected in attenuation of protective signaling pathways upstream of glycogen synthase kinase-3β (GSK-3β) and in the opening of mitochondrial permeability transition pores (mPTP) (71). Elevated formation of mPTP is one of possible explanations of mitochondrial membrane fluidization. Enhanced formation and mPTP opening during acute STZ-DM occur in mitochondrial membrane fluidization and facilitation of ATP transport without lethal damage (72). Hypercholesterolemia-exacerbated myocardial I/R injury may relate to enhanced oxidation due to disturbed regulatory systems and antioxidant network required to minimize ROS production, further, to attenuation of PI3K/Akt pathways, and induction of mitochondrial permeability transition pore (mPTP) opening (73, 74). In addition, other experimental and clinical studies have revealed that hypercholesterolemia aggravates the protective effect of preconditioning and postconditioning (75-77).

In addition to the effects found in preconditioning HCH is considered to impair the cardioprotective effect of ischemic postconditioning by altering of nitrosative stress signal and by increasing the production of several oxidants such as peroxynitrite and lipid peroxidation compounds (75). Delineating the impact of various factors leading to structural and functional remodeling of heart helps identify signaling and regulatory pathways involved in adaptive and maladaptive processes. All this might have significant implications for seeking ways how to delay or prevent the transition to heart failure. Although it is widely accepted fact that DM adversely affects heart structure and function independently of hypercholesterolemia, caution should be used, when interpreting findings from studies considering these factors in isolation. The reason is that ignoring combination of these conditions (DM and HCH) could confound results by exacerbating or weakening the obtained relationship between the intended factor and the selected outcome variable. In this study significantly increased level of cholesterol registered in the DM-HCH group antagonized the endogenous protective mechanisms by increasing the rigidity of mitochondria membranes. Consequently, the mitochondrial Mg2+-ATPase activity in DM-HCH group became depressed.

On the organ level evaluated functional heart recovery (LVDP) after I/R was also significantly decreased in DM-HCH when compared to DM group, which may be partially linked to the functional changes observed on level of heart mitochondria. Depressed LVDP recovery together with increased size of myocardial infarction observed in DM-HCH group in comparison to DM group suggest that protection conferred by acute DM was blunted or even abolished if HCH diet was applied. Taken together, HCH seems to interfere with cardioprotective mechanisms, which was reflected in attenuation of adaptive responses that would otherwise be developed in diabetes. As a result, vulnerability of the heart to I/R was increased and mitochondrial function compromised when rats were exposed to both factors, DM and HCH. Since HCH diet itself caused no alteration either in mitochondrial function or in cardioprotective mechanisms, which was reflected in attenuation of adaptive responses that would otherwise be developed in diabetes, the presence of a specific interaction between hypercholesterolemia and acute diabetic condition should be taken into consideration.

**Conclusion**

Although the short-term administration of cholesterol-enriched diet did not cause any significant differences in the monitored parameters, in diabetic hearts it was associated with significant alterations in the functional and structural properties of mitochondria as well as in myocardial structure and function. Altered composition and structure of the mitochondrial membranes both have been proved to contribute to increased resistance of diabetic heart to experimentally applied ischemic insult, which has been attributed to compensatory adaptation. In our present study, we have shown that the applied cholesterol-enriched diet induces adverse remodeling which negatively affect mitochondrial function in diabetic animals. This remodeling may be related to distortion of the mitochondrial membrane protein-
l lipid interactions due to incorporation of cholesterol molecules. Altogether, our results show that the application of cholesterol-enriched diet led to inhibition of compensatory, endogenously initiated cardioprotective mechanisms, observed in diabetic group of animals.

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