ISSN 0006-2979, Biochemistry (Moscow), 2011, Vol. 76, No. 9, pp. 1022-1029. © Pleiades Publishing, Ltd., 2011.
Original Russian Text © D. V. Cherkashina, I. A. Sosimchik, O. A. Semenchenko, V. V. Volina, A. Yu. Petrenko, 2011, published in Biokhimiya, 2011, Vol. 76, No. 9, pp. 1254-1263.

Mitochondria-Targeted Plastoquinone Derivative SkQ_1 Decreases Ischemia–Reperfusion Injury during Liver Hypothermic Storage for Transplantation

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Received October 7, 2010
Revision received March 3, 2011

Abstract—The ability of the mitochondria-targeted plastoquinone derivative 10-(6′-plastoquinonyl)decyl triphenylphosphonium (SkQ_1) to decrease ischemia–reperfusion injury in isolated liver during hypothermic storage (HS) was studied. Rat liver was stored for 24 h at 4°C without or in the presence of 1 μM SkQ_1 with following reperfusion for 60 min at 37°C. The presence in the storage medium of SkQ_1 significantly decreased spontaneous production of reactive oxygen species and intensity of lipid peroxidation in the liver during HS and reperfusion. The GSH level after HS in solution with SkQ_1 was reliably higher, but reperfusion leveled this effect. At all stages of experiment the presence of SkQ_1 did not prevent the decrease of antioxidant enzyme activities such as catalase, GSH peroxidase, GSH reductase, and glucose-6-phosphate dehydrogenase. The addition of SkQ_1 to the storage medium improved energetic function of the liver, as was revealed in increased respiratory control index of mitochondria and ATP level. SkQ_1 exhibited positive effect on the liver secretory function and morphology after HS as revealed in enhanced bile flow rate during reperfusion and partial recovery of organ architectonics and state of liver sinusoids and hepatocytes. The data point to promising application of mitochondria-targeted antioxidants for correction of the ischemia–reperfusion injury of isolated liver during long-term cold storage before transplantation.

DOI: 10.1134/S0006297911090069

Key words: mitochondria-targeted antioxidant, SkQ_1, ischemia–reperfusion injury, hypothermic storage of isolated liver, free radical processes, respiratory activity of mitochondria

Ischemia–reperfusion injury and procedures for their amelioration are important problems of contemporary medicine and biology. In particular, such injuries are responsible for low adaptability of organs such as liver after transplantation. The procedure of liver preparation in most cases includes hypothermic storage (HS) accompanied by the cold-induced ischemia, while after transplantation, during return of the organ to normal temperature and resumption of circulation, its re-oxygenation takes place.

The search for efficient components of preservation solutions facilitating prolonged safe storage of isolated organs is a main direction of investigations in transplantation.

It is known that in conditions of cold ischemia, functioning of the mitochondrial respiratory chain, tricarboxylic acid cycle, and oxidative phosphorylation is disturbed, which results in inhibition of ATP synthesis [1]. The result of respiratory chain disturbance is the single-electron reduction of some oxygen in "parasitic" reactions with formation of reactive oxygen species (ROS) – O_2^-, H_2O_2, and $HO^\cdot$. The intensity of this process depends on the potential on the internal mitochondrial membrane [2, 3]. The hypothesis of "mild uncoupling" [4] was based on these data, according to which the decrease of "excessive" membrane potential should decrease ROS production. We have supposed, in turn, that the use of oxidative phosphorylation uncoupler 2,4-dinitrophenol (DNP) as a component of solution for HS will make it possible to lower oxidative injury of liver. In fact, this approach allowed us to decrease the level of accumulation of lipid peroxidation (LPO) products in liver, to prevent inhibition of antioxidant enzyme activities, and to improve the morpho-func-

Abbreviations: DHR 123, dihydrorhodamine 123; DNP, 2,4-dinitrophenol; G6PDH, glucose-6-phosphate dehydrogenase; HS, hypothermic storage; LPO, lipid peroxidation; MDA, malonic dialdehyde; NR, normothermic reperfusion; RCI, respiratory control index; ROS, reactive oxygen species; SkQ_1, 10-(6′-plastoquinonyl)decyl triphenylphosphonium; TBA, thiobarbituric acid.

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tional state of the organ after HS and following normothermic reperfusion (NR). The protective effect of DNP at the level of mitochondria involved decrease in respiration rate in state 4, increase in respiratory control index, and prevention of decrease in ATP level [5].

The encouraging results stimulated a search for compounds capable of selective regulation of ROS production by mitochondria. Studies by V. P. Skulachev et al., who elaborated and synthesized compounds called SkQ, attracted our attention. These compounds are antioxidant plastoquinone derivatives conjugated with lipophilic cations, which allows them to penetrate into mitochondria [6]. The efficiency of mitochondrial antioxidants of the SkQ group, and of SkQ_1 (10-(6′-plastoquinonyl)decyl triphenylphosphonium) in particular, was already shown on experimental models accompanied by ischemia–reperfusion injuries such as kidney and heart infarction and ischemic stroke [7]. However, the possibility of using SkQ as a component of solution for organ storage before transplantation was not studied.

We recently carried out experiments on determination of optimal SkQ_1 concentration in liver storage medium. The effect of SkQ_1 in final concentration 0.1-5 μM on basal level and rate of malonic dialdehyde (MDA) accumulation was estimated along with respiratory parameters of mitochondria on the model of 18-h-long HS in isolated rat liver. Dose-dependent effect of SkQ_1 was shown: the basal level of MDA decreases beginning from 1 μM, while the rate of induced LPO decreases already beginning from 0.5 μM; the extent of mitochondria coupling increases at concentrations 0.5 and 1 μM, and increase in the SkQ_1 concentration to 5 μM resulted in inhibition of respiratory activity of mitochondria [8]. Overall, the results allowed us to choose for further experiments the optimal final SkQ_1 concentration of 1 μM.

The aim of this work was to study the effect of 1 μM SkQ_1 as a component of preservation solution on pro-oxidant–antioxidant balance, respiratory activity of mitochondria, and secretory function and morphological state of isolated liver after 24 h HS and following reperfusion.

MATERIALS AND METHODS

SkQ_1 was provided through the courtesy of the Mitoengineering Center of Moscow State University.

Experimental model. Experiments were carried out on 200-250 g white mongrel female rats (n = 21). All manipulations with the animals were carried out according to requirements of the Institute Committee on Ethics coordinated with the Rules of the European Convention on Protection of Vertebrates Used for Experimental and Other Purposes (Strasbourg, 1986). Diethyl ether was used for narcosis.

The sucrose–saline solution developed at the Institute for Problems of Cryobiology and Cryomedicine was used as storage solution (250 mM sucrose, 1 mM $MgSO_4$, 0.5 mM $CaCl_2$, 15 mM Na_2HPO_4, 30 mM KH_2PO_4, 1% PEG 8000, pH 7.4) [5].

Isolated livers were divided to three groups (n = 7): (1) control, freshly isolated liver; (2) livers stored in the absence of SkQ_1; (3) the SkQ_1 group, livers were stored in the presence of 1 μM SkQ_1.

Abdominal cavities of anesthetized animals were lanced, ligature was applied under *v. porta*, and a needle was introduced and fixed in it. After the beginning of perfusion, the *v. cava inf.* was notched to provide for perfusate outflow from the liver. The blood was washed away from the liver with cooled physiological solution (30-50 ml) using a peristaltic pump. The livers were saturated with 50 ml storage solution cooled to 4°C. After saturation, *v. porta* and the common bile duct were cannulated using different diameter polyethylene catheters for reperfusion and bile collection. The liver was isolated and placed into a plastic weighing bottle with storage solution, containing SkQ_1 or without it, and stored at 0-4°C for 24 h. After HS, the liver was perfused in a recirculating system at 37°C for 60 min. For NR, Krebs–Ringer bicarbonate solution was used (120 mM NaCl, 4.8 mM KCl, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 2.6 mM $CaCl_2$, 10 mM HEPES, 4.5 mM glucose, pH 7.4), saturated for 5 min with CO_2. Flow rate was regulated in the range of 3.0-4.5 ml/min per mg tissue at hydrostatic pressure 30-40 mm H_2O [5, 9]. Before and after reperfusion, samples of liver tissue were taken for biochemical and histological analyses.

For histological investigations, liver tissue was fixed in 10% formaldehyde solution, and then the preparations were washed, dehydrated in a series of alcohol solutions of increasing concentrations, clarified in xylene, and embedded in paraffin. Tissue slices 5-7 μm thick obtained using an MPS-2 microtome were stained with hematoxylin and eosin. Microscopic analysis was carried out under a Carl Zeiss (Germany) microscope.

Polarographic measurement of respiration in liver homogenates. Respiratory parameters of mitochondria were determined in 20% (w/v) liver homogenates (homogenization medium consisted of 300 mM sucrose, 2 mM EDTA, 10 mM Tris-HCl, pH 7.4). The oxygen absorption rate was determined using a Clark-type platinum electrode in a thermostatically controlled chamber of 1 ml at 26°C on a Rank Brother model 20 polarograph (Great Britain) interfaced to a personal computer. The respiratory substrate was succinate (8 mM). Oxidative phosphorylation was initiated by addition of ADP (250 μM) for stimulation of respiratory activity in state 3 (V_3). Oxygen consumption rate at rest (V_4) was measured after exhaustion of the ADP. Respiration and phosphorylation were uncoupled (V_{3unc}) by DNP (100 μM). The measuring medium contained 200 mM mannitol, 50 mM sucrose, 1 mM EDTA, 10 mM KH_2PO_4, 30 mM Tris-HCl (pH 7.4) [9]. Oxygen consumption curves were calculated

according to Estabrook as described in [10]. Respiratory control index (RCI) was calculated as the ratio of V_3 to V_4.

Determination of spontaneous ROS production rate based on rate of dihydrorhodamine 123 oxidation. The rate of spontaneous ROS production in liver homogenates (prepared as described above) was estimated by oxidation rate of the fluorescent dye dihydrorhodamine 123 (DHR123) (Sigma, USA). The dye at final concentration 20 μM and mitochondrial suspension containing 0.2 mg protein were introduced into measuring medium containing 125 mM KCl, 10 mM HEPES, and 10 mM KH_2PO_4, pH 7.4. Measurements were carried out each 5 min during a 20-min period in a 96-well plate on a Tecan GENios spectrofluorimeter (Australia) at excitation and emission wavelengths 488 and 525 nm, respectively [11, 12].

Biochemical analysis. Biochemical analysis was carried out using a Cary spectrophotometer (Australia). Liver homogenates (20% w/v) were prepared in 50 mM Tris-HCl, 50 mM NaCl, pH 7.4.

The basal MDA level was estimated in liver homogenates using a method [13] based on colorimetric determination of absorption intensity of a complex of LPO secondary products with thiobarbituric acid (TBA). A 0.2-ml sample of homogenate was supplemented with 3 ml of 2% orthophosphoric acid, then 1 ml of 0.8% TBA was added. Samples were boiled in water bath for 45 min. After cooling, 4 ml of *n*-butanol was added, and the samples were mixed and centrifuged for 10 min at 3000 rpm. Absorption intensity of the butanol fraction was compared with that of a blank test at 535 nm.

Intensity of induced LPO was determined by the rate of MDA accumulation during 10 min incubation at 37°C of liver homogenates in pro-oxidant buffer containing 0.25 mM ascorbate and 12 μM $FeSO_4$ [14]. Suspension aliquots were taken at the beginning and end of incubation. The reaction was stopped by addition of 1 ml 30% TCA on ice. Then 0.5 ml H_2O was added, and the sample was centrifuged for 10 min at 3000 rpm. The supernatant was supplemented with 0.1 ml 5 M HCl and 1 ml 0.6% TBA, and the mixture was placed into a boiling water bath for 15 min. After cooling, absorption intensity was measured at 535 nm.

Activity of enzymes of the antioxidant defense system in liver homogenates was measured with continuous thermostatting and mixing. Determination conditions for each enzyme were as follows: for catalase – 0.01 M K-phosphate buffer, pH 7.4, 0.5 mM EDTA, 15 mM H_2O_2 (T = 37°C, 240 nm) [15]; for GSH peroxidase – 0.3 M KH_2PO_4, pH 7.0, 3 mM EDTA, 1.5 mM NaN_3, 32.5 mM GSH, 7.5 mM H_2O_2 (T = 23°C, 260 nm) [16]; for GSH reductase – 0.1 M K-phosphate buffer, pH 7.4, 0.1 mM NADPH, 0.5 mM EDTA, 1 mM GSSG (T = 37°C, 340 nm) [17]; for glucose-6-phosphate dehydrogenase (G6PDH) – 0.13 M Tris-HCl, pH 7.6, 80 mM $MgCl_2$, 3.5 mM $NADP^+$, 7.5 mM glucose-6-phosphate (T = 37°C, 340 nm) [18].

ATP content in liver homogenates was determined by the method of Adams using a Sigma express kit [19]. The reduced glutathione content in liver was estimated by intensity of its complex formation with alloxan having absorption maximum at 305 nm [20]. Protein content in liver homogenates was determined by the biuret method with BSA fraction V (Sigma) as standard.

The results were statistically processed using software packages Statistica v.5.5 and Origin 7.5. Data were estimated using Student's parametric (for mitochondrial respiratory activity) and non-parametric Mann–Whitney criteria expressed as $M \pm m$. Results at $p < 0.05$ were considered as significantly different.

RESULTS

Effect of SkQ_1 on free radical processes and the system of antioxidant protection in liver. Figure 1a shows data characterizing the rate of DHR 123 oxidation, which is indicative of the rate of spontaneous ROS production. Liver cold storage resulted in three-fold increase of this parameter compared to control, whereas following reperfusion caused its further increase. The presence of SkQ_1 in the storage medium decreased the rate of change of fluorescence intensity (ΔF/min) by 1.4 and 2 times compared to the group without antioxidant after HS and NR, respectively.

The basal level of MDA in liver increased 1.7-fold after HS and remained at this level after reperfusion. Addition of antioxidant to the storage solution had almost no effect on this parameter: after HS only a slight tendency to decrease was observed ($p = 0.057$), whereas after NR even this difference also disappeared (Fig. 1b).

The intensity of the Fe^{2+}-ascorbate-induced MDA accumulation more than doubled after HS and did not change after reperfusion. The presence of SkQ_1 in the medium completely prevented this increase, the parameter remaining at the control level both after HS and after NR (Fig. 1c).

The reduced glutathione level in liver decreased after HS and NR 1.6-2.0-fold compared to that in the control (Table 1). The addition of SkQ_1 to the solution restored the GSH content to the control level after HS, but this effect was leveled at the stage of reperfusion. The GSH peroxidase activity decreased 1.8-fold after HS, and subsequent NR had almost no further effect. The presence of SkQ_1 in the storage solution significantly decreased this parameter with regard to the group without antioxidant after both HS and NR. The GSH reductase activity decreased almost 2.5-fold after HS relative to the control and remained at the same low level after NR. The introduction of SkQ_1 to the storage solution also resulted in additional decrease of enzyme activity compared to the group without antioxidant, but after NR this parameter reached values of the latter. Cold storage resulted in decrease of catalase and G6PDH activities by 1.7- and 1.4-fold, respectively, while

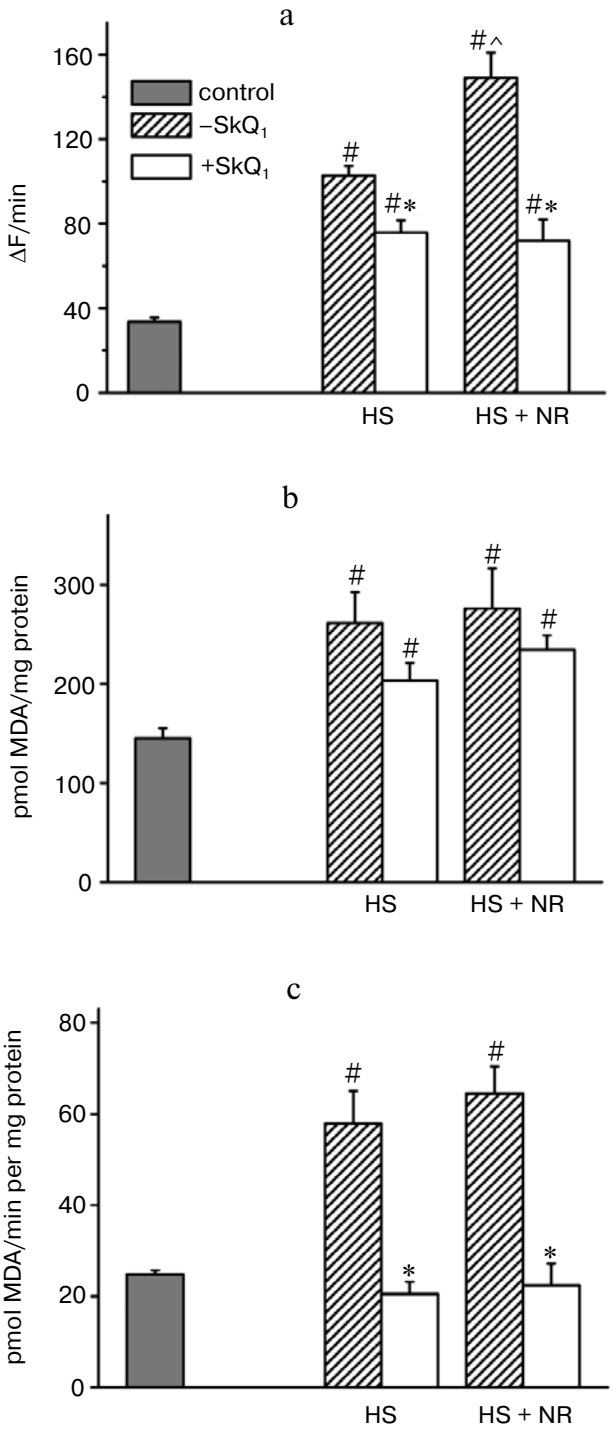

Fig. 1. Effect of SkQ$_1$ on spontaneous ROS production (a), basal MDA level (b), and intensity of induced LPO (c) in liver homogenates after hypothermic storage and normothermic reperfusion. # $p < 0.05$ compared to control; * $p < 0.05$ compared to group without SkQ$_1$; ^ $p < 0.05$ compared to hypothermic storage.

subsequent reperfusion did not cause additional changes in activities of these enzymes. The presence of SkQ$_1$ had no effect on catalase and G6PDH activities at all stages of the experiment (Table 1).

Effect of SkQ$_1$ on respiratory activity of mitochondria and ATP level in rat liver after HS and NR. HS of liver had no effect on O_2 consumption rates in state 3 and uncoupled respiration, but resulted in 1.7-fold increase of V_4 and corresponding decrease of RCI (Table 2). After reperfusion V_3 and V_{3unc} did not change, respiration rate V_4 increased by 1.4-fold compared to HS, while RCI decreased by 20%. The presence of SkQ$_1$ in the storage solution prevented V_4 increase after HS, which, in turn, resulted in restoration of RCI to the control level. After NR the situation changed: the rate in state 4 was as high as in the group without SkQ$_1$, but RCI was reliably higher owing to a 1.4-fold increase of V_3. Besides, an increase in the rate of uncoupled respiration was observed compared to that both in the group without antioxidant (1.7-fold) and in the control (2.2-fold).

In the control group the ATP level in liver was 5.18 ± 1.3 μmol/g tissue. Liver cold storage resulted in more than 50% decrease of this parameter, while subsequent perfusion caused its further halving (Fig. 2). With the addition of SkQ$_1$ to the storage solution, the ATP content increased 1.4-fold after HS and almost doubled after NR compared to the group without antioxidant, though in both cases it remained reliably lower than control values (Fig. 2).

Effect of SkQ$_1$ on secretory function and morphological state of liver after HS/NR. Secretory function of liver after storage was estimated by the rate of bile flow during reperfusion. For freshly isolated liver, this parameter was 16.0 ± 1.7 μl/h per g tissue (Fig. 3). HS of liver without SkQ$_1$ almost halved this parameter relative to control. The presence of the antioxidant in the medium almost completely prevented this decrease by 1.6-fold increase in the bile flow rate compared to the group without SkQ$_1$ (Fig. 3).

As seen in Fig. 4, in the control group the liver parenchyma had normal architectonics of hepatic lobes with somewhat dilated central veins, lumen of sinusoid

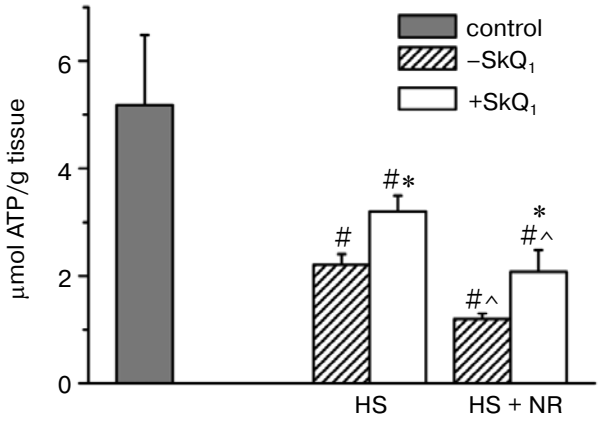

Fig. 2. Effect of SkQ$_1$ on ATP content in liver tissue after hypothermic storage and normothermic reperfusion. # $p < 0.05$ compared to control; * $p < 0.05$ compared to group without SkQ$_1$; ^ $p < 0.05$ compared to hypothermic storage.

Table 1. Effect of SkQ_1 on state of the antioxidant defense system in liver after hypothermic storage and normothermic reperfusion ($M \pm m$, $n = 7$)

Parameter	Control	HS		HS + NR	
		− SkQ_1	+ SkQ_1	− SkQ_1	+ SkQ_1
GSH content, μmol/g tissue	6.61 ± 1.2	4.15 ± 1.0#	6.77 ± 1.3*	3.15 ± 1.3#	4.14 ± 1.1#^
GSH peroxidase activity, μmol GSSG/min per mg protein	0.296 ± 0.05	0.165 ± 0.01#	0.110 ± 0.03#*	0.176 ± 0.01#	0.114 ± 0.01#*
GSH reductase activity, nmol NADPH/min per mg protein	21.6 ± 2.9	9.0 ± 3.7#	6.1 ± 1.7#*	8.8 ± 3.0#	8.4 ± 2.7#
Catalase activity, μmol H_2O_2/min per mg protein	123.2 ± 15.9	71.5 ± 16.1#	65.6 ± 12.2#	69.2 ± 9.6#	70.1 ± 7.6#
G6PDH activity, nmol NADPH/min per mg protein	18.1 ± 3	12.6 ± 1.7#	14.0 ± 2.5#	14.8 ± 3.3#	14.9 ± 2.1#

# $p < 0.05$ compared to control.
* $p < 0.05$ compared to group without SkQ_1.
^ $p < 0.05$ compared to hypothermic storage.

Table 2. Effect of SkQ_1 on respiratory activity of liver homogenates after hypothermic storage and normothermic reperfusion (nmol O_2/min per mg protein) ($M \pm m$, $n = 7$)

Respiration parameter	Control	HS		HS + NR	
		− SkQ_1	+ SkQ_1	− SkQ_1	+ SkQ_1
V_4	1.44 ± 0.1	2.45 ± 0.2#	1.89 ± 0.2*	3.37 ± 0.3#^	3.67 ± 0.4#^
V_3	7.02 ± 0.7	7.18 ± 0.5	8.33 ± 0.8	8.33 ± 0.9	11.65 ± 0.8*^
V_{3unc}	7.79 ± 0.4	9.08 ± 1.3	10.62 ± 1.1	9.68 ± 1.3	16.81 ± 1.5#*^
RCI	4.86 ± 0.1	2.98 ± 0.2#	4.46 ± 0.2*	2.46 ± 0.1#	3.56 ± 0.4#*

# $p < 0.05$ compared to control.
* $p < 0.05$ compared to group without SkQ_1.
^ $p < 0.05$ compared to hypothermic storage.

blood capillaries, and triad vessels. In hepatocytes the nuclei were light and euchromatic, and binuclear cells appeared as well.

After liver HS and NR, in the group without SkQ_1, the trabecular pattern in parenchyma was absent and there was no well-defined structure in sinusoids. Central veins were greatly dilated, and the sinusoidal capillaries were also dilated. The liver parenchyma represented a combination of hepatocytes with degenerative alterations: atrophic, small hepatic cells were determined, necrotic cells without nuclei or with sharp pycnotic nuclei were identified. In hepatocytes, hydropic dystrophy was prevalent, trabecular structure was observed in only 1/3 of lobes in a field of vision. Along with islets of cells with large nuclei, regions of necrosis with anuclear cells were revealed (Fig. 4).

After addition of SkQ_1 to the storage solution, the liver parenchyma returned to normal state manifested in organization of liver lobes, increase in the number of sinusoids and moderately dilated vein type vessels, more or less regular trabecular structure of parenchyma, as well as in

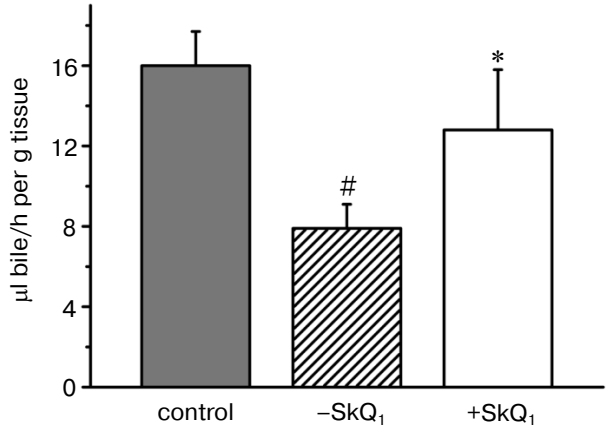

Fig. 3. Effect of SkQ_1 on bile flow rate during normothermic reperfusion after hypothermic storage of liver. # $p < 0.05$ compared to control; * $p < 0.05$ compared to group without SkQ_1.

tendency to correct organization of sinusoidal capillaries. Central veins were significantly dilated and for the most part they had clear lumen. In most cases hepatocytes were of equal size with well-outlined nuclei. No dystrophic alterations were identified in their cytoplasm (Fig. 4).

DISCUSSION

Our investigations have shown the efficiency of mitochondrial antioxidant SkQ_1 as a component of solution for HS of isolated liver. Decreased rates of spontaneous ROS production and intensity of induced LPO in liver both after 24 h HS and after NR are indicative of antioxidant activity of SkQ_1. It is necessary to stress that the model used by us suggests the saturation of the organ with preservation solution and following storage at temperatures close to zero. Thus, despite inhibition of all active metabolic and transport processes, we see that SkQ_1 efficiently enters liver cells. This positively characterizes this compound as a component of solutions for organ cold storage. In this case, upon restoration and re-oxygenation, in liver stored without SkQ_1 there is significant increase in spontaneous ROS production, unchanged in experimental group to the end of the experiment. SkQ_1 also stabilizes intensity of induced LPO, which characterizes reactivity and sensitivity of the pro-oxidant system. Although spontaneous ROS production remains above control values despite the presence of antioxidant, the rate of MDA accumulation completely returns to normal level. In this case we did not reveal a significant effect of SkQ_1 on basal level of secondary LPO products, which remained practically unchanged compared to the group without antioxidant, this possibly being indicative of significant contribution of non-mitochondrial free radical sources to oxidative stress development upon ischemia. It should be pointed out that in our earlier experiments the storage term was

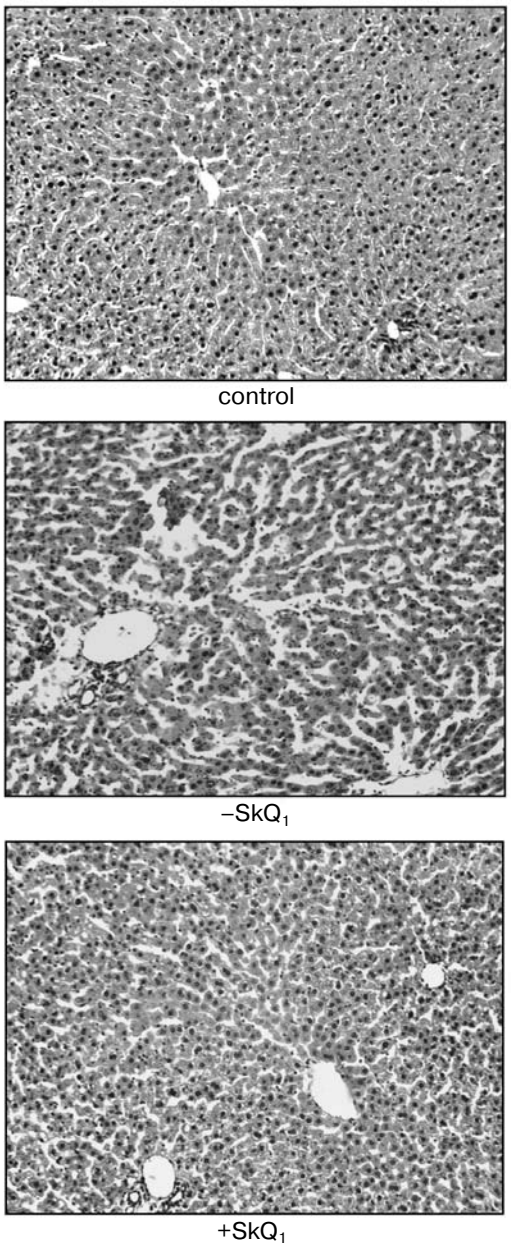

Fig. 4. Rat liver histology after 24 h hypothermic storage without or in the presence of SkQ_1 and following reperfusion. Hematoxylin-eosin staining (×200). Control. Normal architectonics of liver trabeculae, somewhat dilated lumens of sinusoidal blood capillaries, dilated central veins. Liver of SkQ_1-free group after storage and reperfusion. Necrosis regions with anuclear cells alternate with islets of cells with large nuclei, dilated central veins, trabecular pattern is absent, sinusoid organization is not identified, and their lumen is dilated. Liver of SkQ_1 group after storage and reperfusion. Regeneration of trabecular pattern and normalization of sinusoid organization are observed.

18 h rather than 24 h, and then we observed a decrease of basal MDA level at the same SkQ_1 concentration (1 μM) [8]. This discrepancy can be explained by a change in the ratio of contributions of different sources to total production of free radicals, because it is known that it depends on

the duration of cold ischemia: the longer it is, the larger the contribution of resident macrophages – Kupffer cells [21, 22]. In these cells mitochondria are not the main source of free radicals, but rather NADP oxidase localized on plasma membrane, because the data probably indirectly confirm the selectivity of effect of mitochondria-targeted antioxidants.

To our surprise, SkQ_1 mediated no decrease on spontaneous ROS production via the state of the liver antioxidant defense system. Moreover, in the experimental group there was an additional decrease of GSH reductase activity after HS and GSH peroxidase activity at all stages of the experiment. In most investigations, including those in our laboratory, on application of direct antioxidants or compounds potentially able to influence LPO processes, the decrease of intensity of free radical processes allows one to normalize or partially compensate inhibition of antioxidant enzyme activities [5, 23]. However, in this case only total content of GSH, which occupies an intermediate position between non-enzymatic and enzymatic units of the antioxidant system, in the presence of SkQ_1 remained at control level after HS and decreased only during reperfusion of the liver.

It should be noted that the main reason for development of ischemia–reperfusion injuries during HS/NR is a cooperative response of liver cells to stress. First periportal hepatocytes suffer, forming a necrosis zone after the disturbance of microcirculation [24]. Intercellular signaling results in mass release of GSH from hepatocytes for protection of liver sinusoid endothelial cells via sensitization of parenchyma cells to free radical effects [25], i.e. the hepatocyte injury in the case of HS/NR is of secondary character.

In this connection, the data collected can be interpreted as follows: in the group without antioxidant, GSH is spent for neutralizing ROS during HS, but due to inhibition of antioxidant enzyme activities upon cold ischemia [5, 23, 26, 27], its efficiency sharply decreases. In the SkQ_1 group, glutathione is not spent due to the presence of the antioxidant, as is confirmed by data concerning the ability of the SkQ group members to prevent exhaustion of the GSH pool [28]. It is necessary to stress again that this effect takes place at temperatures close to zero. Therefore, some increase in ROS production takes place also due to inactivation of antioxidant enzymes. It was shown that plastoquinone derivatives exhibit pronounced ability to "break" chain reactions of free radical formation, in particular by formation of hydroperoxides [29] utilized in mitochondria with involvement of the enzymatic GSH-dependent system. The latter is "too much" inhibited in the presence of SkQ_1. It is quite probable that just the depressor effect on the GSH utilizing systems is a mechanism of prevention of GSH pool exhaustion by plastoquinone derivatives. Owing to this, the detailed determination of the molecular mechanisms of the effect of SkQ_1 towards the GSH-dependent system undoubtedly requires further investigations.

It is found after normothermic reperfusion, despite increased ROS production, the GSH content in the absence of SkQ_1 remains unchanged compared with that during storage, while in the presence of the antioxidant it decreases to values corresponding to those in the group without SkQ_1. We suppose that in this case GSH is spent for support of endothelial cells of liver sinusoid, while SkQ_1 continues efficient restraint of spontaneous ROS production and stabilizes the liver pro-oxidant system as a whole. Keeping in mind that at the end of experiment GSH content in both groups was at the same level, we suppose that this is some "critical volume" of the glutathione pool, the loss of which can result in cell death.

Our earlier experiments [5] and data from the literature [30-32] suggest that decrease of ROS production by mitochondria, independently of approach, should positively influence functional condition of mitochondria. Actually, SkQ_1 prevented the increase in permeability of internal membrane, one of reasons for uncoupling of oxidative phosphorylation, and completely restored mitochondrial RCI. After reperfusion SkQ_1 also restored the coupling degree of respiration and phosphorylation, but this was not due to prevention of increased proton permeability. In the presence of SkQ_1 the rate of phosphorylating respiration increased, which can be due to increased activity of ATP-synthase complex (this is consistent with ATP content in liver tissue). However, along with increase in V_3 the rate of uncoupled respiration also increased, which is indicative of the maximal activity of the respiratory chain and is limited mainly by its kinetic parameters and activity of appropriate substrate carriers. This means that in the presence of SkQ_1 the activity of respiratory complexes is at a rather high level. This can be due to the antioxidant ability to normalize production of ROS, inhibiting activity of key enzymes of the Krebs cycle and respiratory chain, in particular, of NADH dehydrogenase and succinate dehydrogenase [33]. Besides, it is possible that prevention of decrease of activities of these enzyme results in enhanced formation of endogenous succinate, whose oxidation efficiency much exceeds that of succinate added to the measuring medium due to nearness to the enzyme active centers [34]. Prevention of uncoupling of oxidative phosphorylation and improvement of functional state of mitochondria after HS and NR after SkQ_1 addition to the solution, in turn, had a positive effect on the content of the main energy source in the cell – ATP.

The revealed alterations in redox and energy conditions of the liver also influenced its morphological and functional state. The rate of bile production is a very sensitive indicator of liver function under various stresses, including those in ischemia–reperfusion [35]. Besides, ROS and other free radicals play in hepatocytes the same important role in regulation of bile formation as in development of ischemia–reperfusion injuries and in cell death [35, 36]. We found that addition of SkQ_1 to the stor-

age solution almost completely normalizes the rate of bile formation after 24 h HS. This alteration can be considered as argument in favor of increase of general integrity of the organ as is also confirmed by improved hepatocyte morphology in the SkQ_1 group.

Morphological studies of the liver after 24 h HS and reperfusion confirmed, on the whole, the data of biochemical analysis. In the group without antioxidant there were vast foci of necrosis with anuclear cells, disturbance of organ architectonics, dilatation of central veins, and disorganization of sinusoids, but in the presence of SkQ_1 we observed restoration of the trabecular pattern and normalization of sinusoid organization, as well as improvement of morphology of parenchymatous cells against the background of practically complete absence of features of their dystrophy.

Thus the data show that the mitochondria-targeted antioxidant SkQ_1, used as a component of preservation solution, is capable of efficient maintenance of isolated liver during long-term cold storage owing to decreased spontaneous ROS production and reactivity of the prooxidant system, which has positive effect on respiratory activity of mitochondria and morphological state of the organ as a whole. In this case the SkQ_1 effect is realized both at temperatures close to zero and upon normothermic conditions during storage medium replacement by solution without antioxidant. This is indicative of its rather high ability to penetrate into liver cells independently of temperature.

Therefore, the model of cold ischemia and normothermic re-oxygenation of isolated liver is a suitable test system for screening the efficiency of compounds with the SkQ_1-like mechanism of action. Moreover, there is no doubt that the use of mitochondria-targeted antioxidants is promising for correction of ischemia–reperfusion injuries in isolated liver during long-term storage in the cold before transplantation.

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