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# Effect of Dapagliflozin on the Functioning of Rat Liver Mitochondria *In Vitro*

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We studied the effect of a new hypoglycemic compound dapagliflozin on the functioning of rat liver mitochondria. Dapagliflozin in concentrations of 10-20 μM had no effect on the parameters of respiration and oxidative phosphorylation of rat liver mitochondria. Increasing dapagliflozin concentration to 50 μM led to a significant inhibition of mitochondrial respiration in states 3 and $3U_{DNP}$. Dapagliflozin in this concentration significantly reduced calcium retention capacity of rat liver mitochondria. These findings indicate a decline in the resistance of rat liver mitochondria to induction of $Ca^{2+}$-dependent mitochondrial permeability transition pore. In a concentration of 10 μM, dapagliflozin significantly decreases the rate of $H_2O_2$ formation in rat liver mitochondria, which attested to an antioxidant effect of this compound. Possible mitochondrion-related mechanisms of the protective action of dapagliflozin on liver cells are discussed.

**Key Words:** *dapagliflozin; mitochondria; mitochondrial permeability transition pore; mitochondrial respiration; reactive oxygen species*

Diabetes mellitus associated with either impaired insulin secretion or cell resistance to the action of this hormone (type I and type II diabetes, respectively) is one of the most common metabolic diseases in the world. In both cases, a common pathological change is an increase in blood glucose, hyperglycemia, which eventually leads to serious damage to organs, including the liver (fatty liver dystrophy along with inflammation and hepatocytes injury). Diabetes is now recognized as one of the concomitant diseases that determine severe course of viral pneumonia caused by COVID-19 [2,5,14].

Gliflozins are a novel class of hypoglycemic compounds used for mono- or combination therapy in the treatment of type II diabetes mellitus. The action of the compounds is mediated by inhibition of the sodium glucose co-transporter 2 (SGLT2). It is known that SGLT2 is expressed in S1 and S2 segments of the renal proximal tubules and is responsible for reabsorption of urine glucose into the blood. Gliflozins exhibits an insulin-independent hypoglycemic action by blocking SGLT2-mediated glucose reabsorption in the kidney, which leads to enhanced excretion of glucose with the urine. By now, three SGLT2 selective inhibitors (canagliflozin, empagliflozin, and dapagliflozin) have undergone clinical trials and have been approved for the use in patients with diabetes mellitus [12].

Despite specific action, canagliflozin, and to a lesser extent dapagliflozin, could be nephrotoxic in patients and their use is associated with increased risk of diabetic ketoacidosis [7]. In case of canagliflozin, the cytotoxic effect can be associated with induction of mitochondrial dysfunction in animal tissues. Canagliflozin has been shown to inhibit both complex I of the mitochondrial electron transport chain and glutamate dehydrogenase [13]. However, other studies indicate a possible protective effect of gliflozins against mitochondrial dysfunction associated with diabetes. For instance, the use of empagliflozin and dapagliflozin normalized the structure of mitochondria and modulates mitochondrial dynamics in the myocardium of diabetic animals [9,15].

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Due to the inconsistency of the available data on the effect of dapagliflozin on mitochondria and its potential toxic effect on intracellular structures, thorough study the effect of different concentrations of this compound on the mitochondrial function is required.

Our aim was to study the effect of dapagliflozin on the main functional parameters of mitochondria isolated from rat liver: respiration and oxidative phosphorylation, $H_2O_2$ generation, and induction of $Ca^{2+}$-dependent pore in mitochondria.

## MATERIALS AND METHODS

The experiments were conducted on adult male Wistar rats (210-230 g) in accordance with the principles of European Convention for the Protection of Vertebrates Used for Experimental and Other Purposes (Strasbourg, 1986). The study protocols were approved by the Ethics Committees of the Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences (Protocol No. 6/2021, February 8, 2021).

In the experiments, a freshly prepared stock solution of dapagliflozin (Apex Biotech) in ethanol with a concentration of 10 mM was used. The final concentration of the solvent in samples did not exceed 1%. Other reagents were purchased from Sigma-Aldrich.

Mitochondria were isolated from rat liver by differential centrifugation as described earlier [4]. The homogenization buffer contained (in mM): 210 mannitol, 70 sucrose, 1 EDTA, and 10 HEPES-KOH buffer (pH 7.4). The concentration of mitochondrial protein in the final suspensions was 80-100 mg/ml (measured by the method of Lowry).

Mitochondrial respiration was recorded by the polarographic method with an Oxygraph-2k respirometer (Oroboros). The incubation medium contained 130 mM KCl, 5 mM $NaH_2PO_4$, 5 mM succinate, 5 μM EGTA, 1 μM rotenone, and 10 mM HEPES-KOH (pH 7.4). The concentration of mitochondrial protein was 0.5 mg/ml. Mitochondrial respiration in different metabolic states was assessed as described earlier [3].

The amount of $Ca^{2+}$ required for the mitochondrial permeability transition (MPT) pore opening (defined as $Ca^{2+}$ capacity) was measured after massive loading of mitochondria with $CaCl_2$ [4]. $CaCl_2$ solution (25 μM) was added in fractional manner to the mitochondrial suspension every 60 sec. After several additions, external $Ca^{2+}$ concentration increased indicating massive calcium release from the organelles due to opening of the MPT pore in the inner mitochondrial membrane. $Ca^{2+}$ concentration was measured using a Record-4 potentiometric system with a $Ca^{2+}$-selective electrode (Niko-Analit). The mitochondria were incubated in a medium containing 150 mM mannitol, 50 mM KCl, 2 mM $KH_2PO_4$, 5 mM succinate, 6 μM EGTA, 1 μM rotenone, and 10 mM HEPES-KOH buffer (pH 7.4). The concentration of mitochondrial protein in the cuvette was 3 mg/ml.

The rate of $H_2O_2$ production in mitochondria was measured using Amplex Red fluorescent indicator ($\lambda_{ex}$=560 nm; $\lambda_{em}$=590 nm) in a Tecan Spark 10M plate reader under thermostating (at 37°C) and constant stirring [3]. The incubation medium contained 210 mM mannitol, 70 mM sucrose, 5mM succinate, 1 mM $KH_2PO_4$, 10 μM EGTA, 1 μM rotenone, and 10 mM HEPES-KOH (pH 7.4). At the beginning of measurements, horseradish peroxidase (1 U/ml) and 10 μM Amplex Red were added to the incubation medium. The concentration of mitochondrial protein in the cuvette was 0.15 mg/ml.

The data were processed statistically by the Student's *t* test using GraphPad Prism 7 software. The results were presented as *M*±*SEM*, because the data fit the normal distribution. Statistical differences between the means were considered significant at $p<0.05$.

## RESULTS

**Effect of dapagliflozin on bioenergetic parameters of rat liver mitochondria.** We studied the effect of dapagliflozin on the parameters of mitochondrial respiration in different functional states and coupling of respiration with ATP synthesis in the presence of succinate as the oxidation substrate. In concentrations 10-20 μM, dapagliflozin had little effect on the rates of mitochondrial respiration in all the studied states (Table 1). Increasing the dapagliflozin concentration to 50 μM resulted in inhibition of mitochondrial respiration in the ATP stimulated and uncoupled states (state 3 and $3U_{DNP}$, respectively) by 20% relative to the control. At the same time, the efficiency of ATP synthesis estimated by the parameter of respiratory control and ADP/O ratio significantly decreased (1.90±0.03 and 1.63±0.09 in the absence and in the presence of 50 μM dapagliflozin, respectively) (Fig. 1, *a*, *b*). The time of phosphorylation increased by 1.3 times, when 50 μM dapagliflozin was added (Fig. 1, *c*). These findings indicate that dapagliflozin in high concentrations suppresses respiration and synthesis of ATP in rat liver mitochondria. It can be assumed that the effect of dapagliflozin is mediated by inhibition of the activity of complexes of the respiratory chain.

**Effect of dapagliflozin on the rate of $H_2O_2$ production by rat liver mitochondria.** According to published data, administration of dapagliflozin to diabetic animals suppresses the development of oxidative stress in tissues [10]. In this regard, we studied the effect of this compound on $H_2O_2$ production by mitochondria. Dapagliflozin in a concentration of 10 μM

**TABLE 1.** Effect of Dapagliflozin on the Respiration Rates of Rat Liver Mitochondria

| Dapagliflozin, μM | Rate of mitochondrial respiration, nmol $O_2$×min$^{-1}$×mg$^{-1}$ protein | | | |
|---|---|---|---|---|
| | state 2 | state 3 | state 4 | state $3U_{DNP}$ |
| 0 | 11.2±1.6 | 43.0±5.7 | 9.5±2.8 | 43.8±4.4 |
| 10 | 10.4±1.2 | 38.5±8.1 | 8.7±1.6 | 37.3±7.1 |
| 20 | 10.9±0.7 | 37.3±6.5 | 9.2±1.5 | 36.5±8.1 |
| 50 | 11.9±1.0 | 34.8±4.6* | 9.6±1.6 | 34.4±4.2* |

**Note.** Mitochondrial respiration in state 3 was induced by adding 200 μM ADP; mitochondrial respiration in state $3U_{DNP}$ was triggered by adding 50 μM 2,4-dinitrophenol (DNP). *$p$<0.05 in comparison with the control (in the absence of dapagliflozin). The mean values of 6 experiments are presented.

significantly decreased the rate of $H_2O_2$ production by mitochondria (Fig. 2). Further increase in dapagliflozin concentration restored the rate of $H_2O_2$ generation to almost the control level. Thus, we can assume that dapagliflozin at low concentrations can exhibit antioxidant properties and reduce oxidative damages to mitochondria in hepatocytes.

**Effect of dapagliflozin on $Ca^{2+}$-dependent permeabilization of rat liver mitochondria.** MPT pore is a mega-channel formed by the conglomerate of proteins of the inner and outer mitochondrial membranes. According to modern views, ATP synthase and adenylate translocator are considered to be proteins that supposedly form the MPT pore channel. Currently, regulatory protein cyclophilin D, the target of pore inhibitor cyclosporin A, is the only established component of the MPT pore structure. The formation of $Ca^{2+}$-dependent pores in the mitochondrial membrane is believed to be the key process in induction of cell death, mediated by the release of proapoptotic proteins

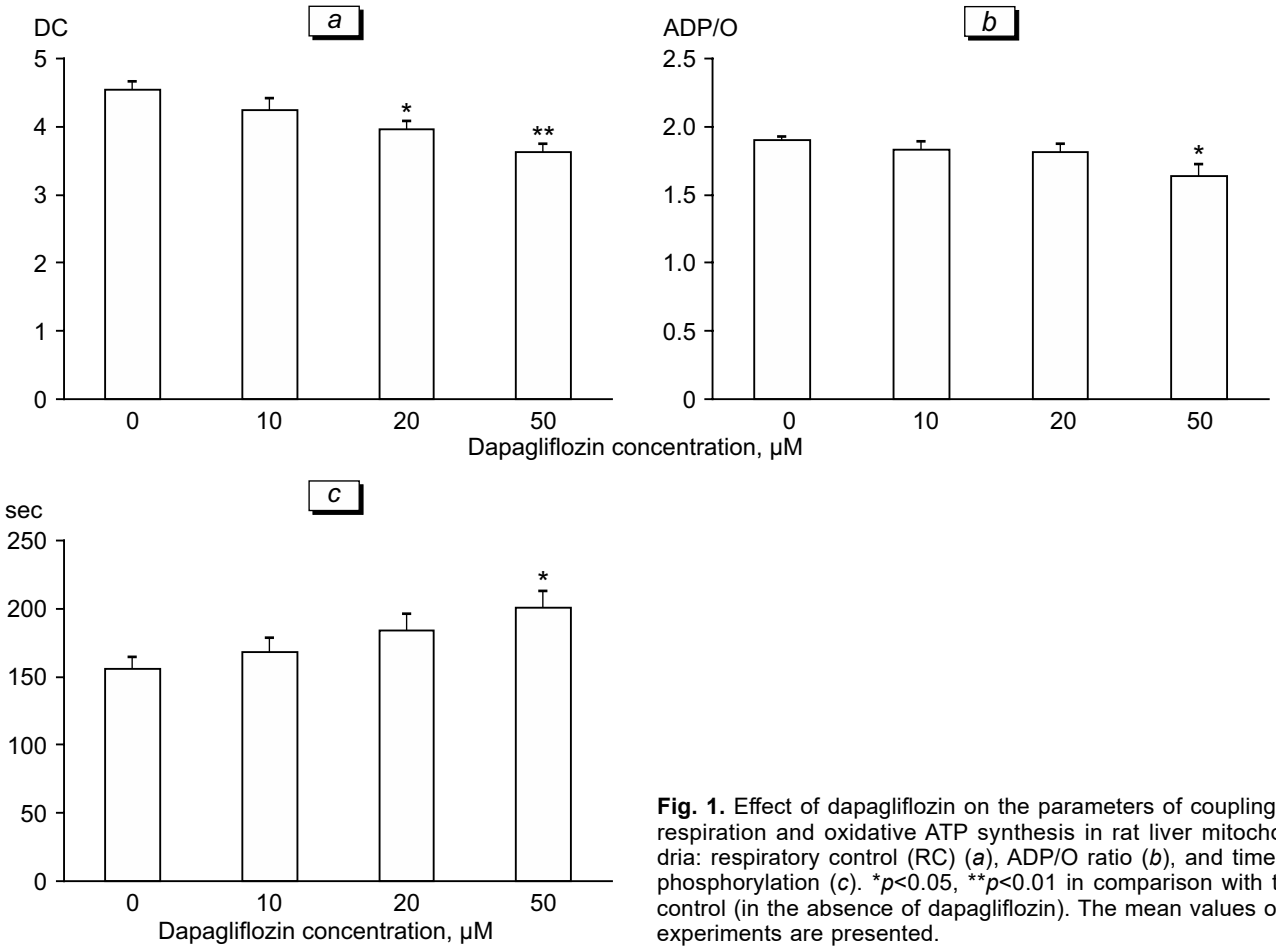


**Fig. 1.** Effect of dapagliflozin on the parameters of coupling of respiration and oxidative ATP synthesis in rat liver mitochondria: respiratory control (RC) (*a*), ADP/O ratio (*b*), and time of phosphorylation (*c*). *$p$<0.05, **$p$<0.01 in comparison with the control (in the absence of dapagliflozin). The mean values of 6 experiments are presented.

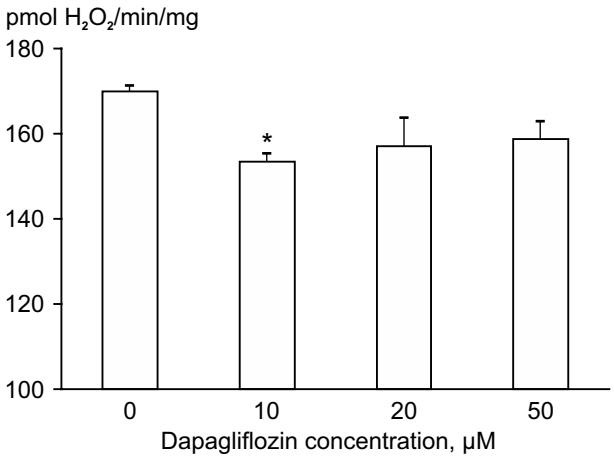


**Fig. 2.** Rate of $H_2O_2$ production by rat liver mitochondria in the presence of dapagliflozin in different concentrations. *$p$<0.05 in comparison with the control (in the absence of dapagliflozin). The mean values of 5 experiments are presented.

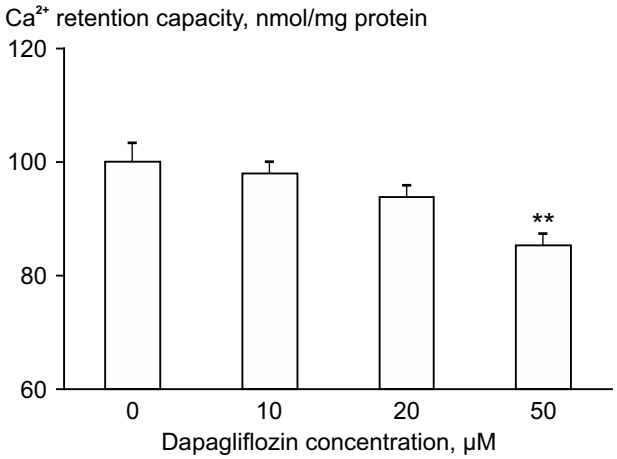


**Fig. 3.** Calcium retention capacity of rat liver mitochondria in the presence of dapagliflozin in different concentrations. **$p$<0.01 in comparison with the control (in the absence of dapagliflozin). The mean values of 5 experiments are presented.

from the organelles (cytochrome C, apoptosis-inducing factor, *etc*.) [1].

Dapagliflozin did not affect calcium capacity of rat liver mitochondria in concentrations of 10-20 μM, but significantly lowered this parameter in a concentration to 50 μM (Fig. 3). This suggests that dapagliflozin in high concentrations decreases the resistance of mitochondria to the opening of MPT pores and can increase their sensitivity to $Ca^{2+}$ accumulation.

Thus, our findings suggest that dapagliflozin in moderate concentrations (10-20 μM) does not affect the functioning of rat liver mitochondria. Moreover, it exhibits antioxidant properties in these concentrations. At the same time, increasing the concentration of dapagliflozin to 50 μM led to negative effects on mitochondria: inhibition of both respiration and oxidative phosphorylation and induction of $Ca^{2+}$-dependent MPT pore. It should be noted that the concentrations of dapagliflozin used in the work lie within the maximum clinical concentration of gliflozins ($C_{max}$=10 μM) and the concentration used in experiments on laboratory animals *in vivo* (~65 μM) [11,13]. It is also known that effective concentration of gliflozins selected for the treatment of diabetic patients with chronic kidney disease or elderly patients with polypharmacy can be significantly higher than the recommended dose [6,8]. Thus, the observed effects of high concentrations of dapagliflozin can underlie its toxic effect on cells and tissues in humans and animals during the treatment of severe pathologies. All the data suggest the need to select effective concentrations of these drugs more carefully in the therapy of diabetes mellitus with varying degrees of disease severity.

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