IN VIVO EFFECT OF BETULONIC ACID ON THE STATE OF LIVER TISSUE AND FUNCTIONAL ACTIVITY OF LIVER MITOCHONDRIA

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Abstract. This work shows the in vivo effect of the bioactive triterpenoid betulonic acid on the liver of C57BL/10 mice and the functioning of the mitochondria of this organ. We have found that betulonic acid has no significant effect on the histological parameters of the mouse liver, as well as on the biochemical parameters of the blood serum of the studied animals. At the same time, betulonic acid has demonstrated mitochondrial targeting. Betulonic acid has shown a decrease in the functional activity of mitochondria, especially in the case of their energization with succinate, a substrate of complex II of the respiratory chain of organelles. Treatment with betulonic acid has no effect on the resistance of mouse liver mitochondria to the induction of a calcium-dependent MPT pore. On the other hand, we have revealed the antioxidant effect of betulonic acid associated with a decrease in the rate of H2O2 generation in the mouse liver mitochondria. The paper discusses the possible use of betulonic acid as a mitochondria-targeting agent.

Keywords: liver, mitochondria, betulonic acid, oxidative phosphorylation, calcium-dependent pore, reactive oxygen species.

List of Abbreviations

AST – aspartate aminotransferase
ALT – alanine aminotransferase
EGTA – ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
HEPES – 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
ADP – adenosine diphosphate
ATP – adenosine triphosphate
MPT – mitochondrial permeability transition pore

Introduction

Substances of plant origin are widely used as Substances of plant origin are widely used as dietary supplements and medicines. Recently, the attention of specialists to plant triterpenoids, combining their availability with valuable biological activity, has been increasing. The outer bark of various birch species is the richest in extractives, the predominant components of which are pentacyclic triterpenoids of the lupane and β-amyrin series, with betulin being the main component. Betulin and its derivatives exhibit a wide spectrum of biological activity (antiviral, antiulcer, antitumor, capillary strengthening, etc.). Purposeful chemical modification of betulin and similar compounds leads in some cases to the production of substances that have a broader spectrum of action and low toxicity (Tolstikov, 2005; Spivak *et al.*, 2021; Dubinin *et al.*, 2021).

Pure betulin and its closest derivatives are of interest for medicine as a basis for the development of new therapeutic agents. They are inhibitors of the polio virus, febrile and respiratory diseases, as well as tuberculosis (Tolstikov, 2005).

Since betulin is extremely poorly soluble in water and alcohols, the use of its more soluble derivatives is promising. Analysis of the literature data showed that many acyl derivatives of betulin have a broad spectrum of biological activity, but since not all betulin derivatives have been studied, it is necessary to expand the range of acyl derivatives of betulin available for research in order to identify new representatives of this class with biological activity (Kogai, 2008). One of these derivatives is betulonic acid.

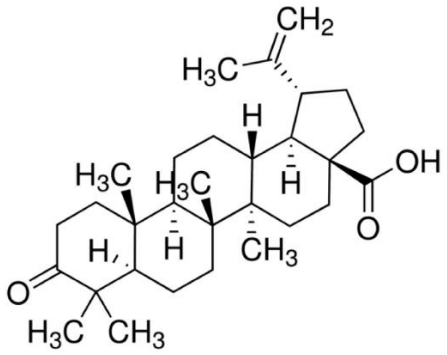

Fig. 1. Structural formula of betulonic acid

Betulonic acid is a representative of the pentacyclic triterpenoid of the lupane series (Fig. 1), it is found in small amounts in the bark and fruits of some plants. The acid is easily obtained synthetically - by oxidation of betulin extracted from birch bark. Betulonic acid and its derivatives have been found to possess several medicinal properties, such as anti-viral (Pavlova *et al.*, 2003), antimicrobial (Haque *et al.*, 2014), anti-Human cytomegalovirus (Dinh Ngoc *et al.*, 2014), anti-inflammatory (Vasilevsky et al., 2009), antioxidant (Sorokina *et al.*, 2004), hepatoprotective (Vasilevsky *et al.*, 2009), immunostimulant (Anikina *et al.*, 2010), and anticancer effects (Kazakova *et al.*, 2015). Betulonic acid is an example of a natural compound that was found to be a promising candidate as an antitumor agent since it can inhibit the growth of different types of tumor cell lines (Yang *et al.*, 2013). It should be noted that in vivo derivatives of betulonic acid are also capable of inhibiting the development of tumors (Saxena *et al.*, 2006; Zhukova *et al.*, 2010), but the molecular mechanism of this action of triterpene is not known.

We have previously shown that mitochondria of eukaryotic cells can be the target of the effect of betulonic acid. Indeed, we have established that under in vitro conditions, betulonic acid is capable of significantly modifying the functioning of liver mitochondria, inhibiting the respiration of organelles and activating the production of reactive oxygen species (Dubinin *et al.*, 2020). However, it is still unknown what effect this triterpenoid will have in vivo. Therefore, in this work we investigated the effect of intragastric administration of this agent on the state of the liver and the functional activity of the mitochondria of this organ in laboratory mice of the C57BL/10 line.

Materials and Methods

Betulonic acid administration. Mature male laboratory mice of the C57BL/10 line weighing 28–31 g were randomly divided into two groups, each group consisted of ten animals. Group 1 (control): the mice of this group were injected intragastrically using a gastric tube with a placebo (a solution consisting of saline, ethanol and tween 20, in a ratio of 4:1:1) daily for 4 weeks. Group 2 (betulonic acid): mice of this group received a solution of betulonic acid in saline, with the addition of ethanol and tween 20 (50 μg/kg body weight) daily for 4 weeks. After 4 weeks, the animals were withdrawn from the experiment. Immediately after dissection of the animals, blood samples were taken for biochemical studies, as well as liver samples for histological examination and isolation of mitochondria. All manipulations with animals were performed in accordance with the European Convention for the Protection of Vertebrates used for experimental and other purposes (1986) and the principles of the Helsinki Declaration (2000). All the protocols were approved by the Mari State University Ethics Committee, decision number: 12/2020 on 12.12.2020.

Histological examination. Samples of liver were fixed in neutral buffered 10% formalin, embedded in paraffin using standard procedures. For histological examination, sections were made with a thickness of 5 μm on a microtome MC-2. Tissue staining was carried out according to the standard method of hematoxylin and eosin. For examination of fibrosis or other connective tissue disorders and measurement of the bleeding areas, part of the preparations was stained according to Van Gieson's and Pearls methods. Examination of slides was carried out using a EVOS M5000 imaging system (Thermo Fisher Scientific, Waltham, MA, USA) and free program ImageJ.

Analysis of biochemical parameters of blood serum. We have evaluated some biochemical parameters of the blood serum of experimental animals. Whole blood from each animal was collected in tubes containing heparin and centrifuged for 15 min at 10,000 rpm. The resulting serum without traces of hemolysis was taken and biochemical studies were carried out. We evaluated the activity of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and total bilirubin. The activity of the enzymes AST and ALT was assessed by the endpoint method using the diagnostic kits «AST-Olvex» and «ALT-Olvex», respectively (Olvex Diagnosticum, Russia). The total bilirubin content was assessed by the Jendrassik-Grof method and the Olvex bilirubin diagnostic kit (Olvex Diagnosticum, Russia). Measurement of biochemical parameters was carried out in a 0.2 ml cuvette on a MultiscanGo plate spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

Isolation of mice liver mitochondria. Mitochondria were isolated from the liver of experimental mice using a convenient technique of differential centrifugation (Dubinin *et al.*, 2018). The isolation medium contained 250 mM sucrose, 1 mM EGTA and 5 mM Hepes/KOH buffer (pH 7.4). The mitochondrial protein concentration was determined by the biuret method with bovine serum albumin (BSA) used as standard.

Determination of mitochondrial respiration and oxidative phosphorylation. Mitochondrial respiration was recorded by a polarographic method at 25 °C in a 0.5 mL thermostatted cell using a Clarke type oxygen electrode and an Oxygraph Plus setup (Hansatech Instruments, Great Britain). The incubation medium contained 200 mM sucrose, 20 mM KCl, 5 mM KH2PO4, 0.5 mM EGTA, 2 mM MgCl2, 10 mM Hepes-KOH (pH 7.4). The following concentrations of substrates and other reagents were used: 2.5 mM potassium malate, 2.5 mM potassium glutamate, 5 mM succinic acid, 0.2 mM ADP, 50 μM 2,4-dinitrophenol, and 1 μM rotenone. The concentration of mitochondrial protein in the cuvette was ~ 1.0 mg/mL. Estimated were the mitochondrial respiration in resting state (i.e., basal mitochondrial respiration in the presence of exogenous substrates or state 2), in state 3 (in the presence of exogenous substrates and ADP), in state 4 (when all added ADP is converted to ATP), the respiration rate of mitochondria in state 3UDNP (in the presence of the protonophore uncoupler 2,4-dinitrophenol (DNP) at a concentration of 50 μM causing the maximal stimulation of respiration). The rates of substrate oxidation were expressed as nmol O_2 × min−1 × mg−1 mitochondrial protein. Respiratory control ratio (RC = state 3/state 4).

Determination of Ca^{2+} retention capacity of liver mitochondria. The transport of Ca^{2+} across the inner mitochondrial membrane was monitored spectrophotometrically with an arsenazo III (2,2′-(1,8-Dihydroxy-3,6-disulfonaphthylene-2,7-bisazo)bisbenzenearsonic acid, 2,7-Bis(2-arsonophenylazo)chromotropic acid) indicator at 675–685 nm using a plate reader Multiskan GO (Thermo, USA) at 25 °C under constant stirring. The incubation medium contained 210 mM mannitol, 70 mM sucrose, 1 mM KH2PO4, 50 μM arsenazo III, 10 μM EGTA, 10 mM Hepes-KOH, pH 7.4. The concentrations of substrates and other reagents were as follows: 2.5 mM potassium malate, 2.5 mM potassium glutamate, 5 mM succinic acid, 1 μM rotenone. The concentration of mitochondrial protein in the cell was ~ 0.5 mg/mL.

Estimation of H_2O_2 production by liver mitochondria. The rate of H_2O_2 production was measured using a test system including a fluorescent indicator Amplex Red and horseradish peroxidase on a Fluorat-02-Panorama spectrofluorometer (Lumex Instruments, Russia) at 560 nm excitation and 590 nm emission wavelengths (Dubinin *et al.*, 2019). The incubation medium contained 210 mM mannitol, 70 mM sucrose, 1 mM KH2PO4, 10 μM EGTA, 10 mM HEPES–KOH, 10 μM Amplex Red and horseradish peroxidase (1 a.u./mL), pH 7.4. The concentrations of substrates and other reagents were as follows: 2.5 mM potassium malate,

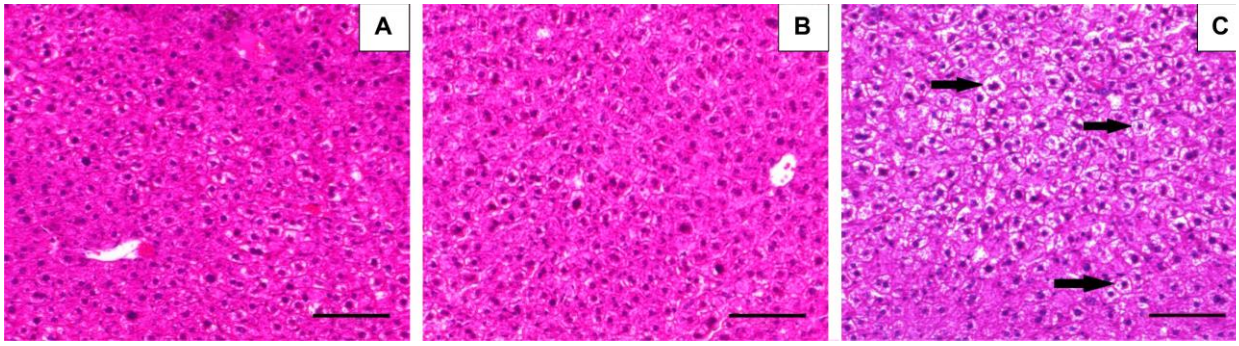

Fig. 2. Photomicrographs of hematoxylin-eosin stained mice liver sections of control group (A), and betulonic-acid-treated group (B). Panel C shows mobilization of glycogen in cytoplasm of liver cell of mice from betulonic-acid-treated group. Kraevsky cells are marked by arrows. Typical images are presented in five sets for each experimental condition. Scale bar is 100 μm

Table 1

Levels of serum biochemical parameters

Animals (*n*=10)	Control	Betulonic acid
ALT	50±1	49±1
AST	111±3	117±7
Total bilirubin	3,9±0,4	4,1±0,1

The results are presented as mean values ±SEM (*n* = 10)

2.5 mM potassium glutamate, 5 mM succinic acid, 1 μM rotenone. The concentration of mitochondrial protein in the cuvette was 0.15 mg/mL. The measurements were carried out at 37 °C and constant stirring. The amount of the resulting hydrogen peroxide was calculated from the calibration curve. A standard H2O2 solution was prepared on the day of experiment; its concentration was determined using the molar absorption coefficient E240 = = 43.6 $M^{-1} \times cm^{-1}$.

Statistical analysis. The data were analyzed using GraphPad Prism 8 and Excel software and were presented as means ± SEM. Statistical differences between the means were determined by a Mann–Whitney U test, where $p < 0.05$ was considered to be statistically significant.

Results

At the first stage of the study, to assess the toxic effect of betulonic acid, we studied its effect on the biochemical parameters of the blood serum of experimental animals, an also evaluated the histological changes in the liver tissue.

We have evaluated some biochemical parameters of the blood serum of experimental animals, in particular the level of activity of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and the concentration of total bilirubin. The presented indicators are generally accepted biochemical criteria for assessing the state of the liver (Friedman and Young, 1997; Tietz, 1994).

Table 1 shows that the biochemical parameters of the two groups of experimental animals do not differ. One could assume that betulonic acid is not capable of causing the appearance of biochemical markers of liver damage in the blood serum, which means that it does not induce the destruction of hepatocytes.

Histological examination of liver samples of experimental mice treated with betulonic acid showed a picture of uneven blood filling of sinusoid capillaries, which varies from low to weak fullness (Fig. 2). There are dystrophic changes in hepatocytes of the type of glucidosis

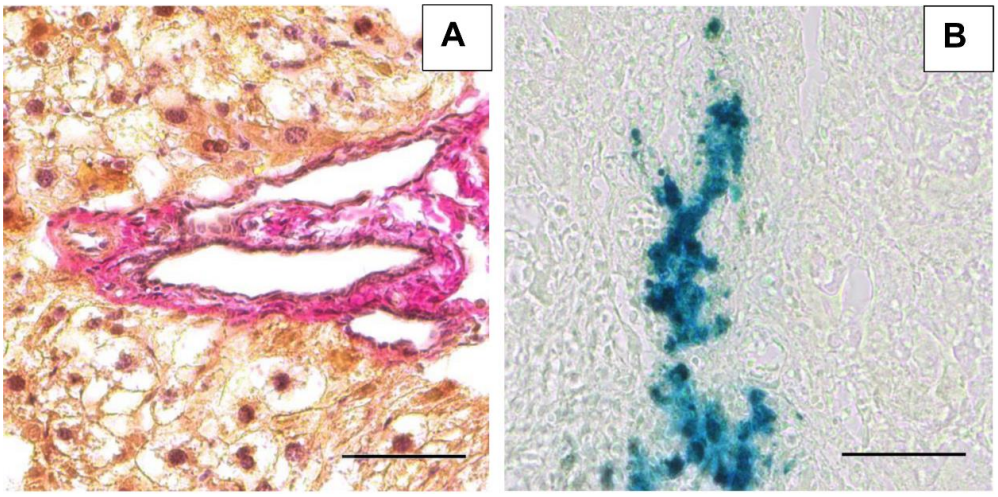

Fig. 3. Photomicrographs demonstrating: (A) perivascular sclerosis in liver (reddish color), Van Gieson and (B) Hemorrhages in liver (blue color), Perls. Scale bar is 50 μm

Table 2

The area of connective tissue and hemorrhages in the liver of mice

Animals (*n*=10)	Connective tissue, %	Hemorrhages, %
Control	2,42±0,41	–
Betulonic acid	5,55±1,46	0,83±0,72

The results are presented as mean values ± SEM (n = 10)

Table 3

Effect of betulonic acid on the respiration of liver mitochondria fueled by glutamate and malate

Animals	State 2	State 3	State 4	State 3U_{DNP}	RC
	nmol O_2 ∗ min^{-1} ∗ mg^{-1} protein				rel. un.
Control	3.24±0.13	22.43±0.66	4.36±0.14	25.04±1.20	5.16±0.13
Betulonic acid	2.88±0.23	20.58±0.66*	4.46±0.16	22.62±1.11	4.65±0.21

Respiration of mitochondria was fueled by 2.5 mM glutamate and 2.5 mM malate. The results are presented as means ± SEM (n = 5). * $p < 0.05$ (vs control group, Mann–Whitney U test)

and the formation of Kraevsky cells, which indicates the loss of glycogen from the cytoplasm of these cells (Fig. 2C). The lumen of the central veins is mostly empty, there are pronounced sclerotic changes in the walls of large vessels, erythrostasis and dilation of the perisinusoidal space of Disse (Fig. 3A). The control group of mice also had the above changes, but they were less pronounced. The presented pathological changes may be a manifestation of a stressful reaction of the body and acute shock on death by decapitation.

Moreover, a histochemical study of the structure of connective tissue and liver hemorrhages was carried out (Fig. 3B), followed by a quantitative assessment of the results. The ratio of the area of the studied structure to the histology slides area was considered for an objective assessment of the results. The results are shown in the Table 2.

Significant differences in the content of connective tissue and the intensity of hemorrhages in the liver of experimental and control animals could not be detected. However, a noteworthy

Table 4

Effect of betulonic acid on the succinate-fueled respiration of liver mitochondria

Animals	State 2	State 3	State 4	State $3U_{DNP}$	RC
	nmol O_2 ∗ min^{-1} ∗ mg^{-1} protein				rel. un.
Control	8.46±0.23	49.43±1.13	9.64±0.40	50.68±0.92	5.17±0.14
Betulonic acid	7.52±0.25*	43.43±1.49*	9.56±0.32	44.85±1.51*	4.57±0.16*

Respiration of mitochondria was fueled by 5 mM succinate. The results are presented as means ± SEM (n = 5). * $p < 0.05$ (vs control group, Mann–Whitney U test)

detail is that the variance of the studied signs is a more pronounced in animals treated with a betulonic acid, which may indicate an increase in the norm of reaction.

Taking into account the previously identified mitochondrial targeting of the effects of betulonic acid, in the next part of the work we evaluated its in vivo effect on the functional activity of mouse liver mitochondria. One of the main functions of these organelles is cellular respiration and ATP synthesis; therefore, we evaluated the effect of this agent on oxygen consumption by mitochondria in the presence of various substrates.

Table 3 shows the rate of glutamate/malate-dependent respiration of liver mitochondria in animals treated with betulonic acid and in the control group. One can see that long-term administration of betulonic acid does not affect the rate of oxygen consumption by mitochondria in states 2 and 4. However, liver mitochondria from the betulonic acid-treated group of animals showed a 1.1-fold decrease in the rate of ADP-stimulated respiration (state 3). In this case, the respiratory control ration, which is an indicator of the effectiveness of oxidative phosphorylation, showed a tendency to decrease in animals receiving betulonic acid. However, the differences in this parameter were statistically insignificant.

In the case of studying the effect of betulonic acid on succinate-dependent respiration of liver mitochondria, the effects were more pronounced. One can see that animals receiving betulonic acid are characterized by a significant decrease in the respiration rate in state 2, as well as a 1.1-fold decrease in the rate of ADP-stimulated respiration, compared with the mitochondria of animals from the control group (Table 4). In addition, we noted a decrease in the rate of mitochondrial respiration in state 3 and, accordingly, a decrease in the respiratory control ratio (by 1.1 times), compared with the control group.

Thus, the results obtained indicate that prolonged use of betulonic acid in mice leads to a decrease in the functional activity of organelles, which is most pronounced in succinate-fueled mitochondria.

Previously, a similar effect of betulonic acid on the functioning of liver mitochondria was shown in vitro as well. It was found that the suppression of the work of organelles by this triterpene may be due to inhibition of the redox activity of the complexes of the respiratory chain of liver mitochondria and, first of all, complexes III and IV, as well as the total activity of complexes II and III (Dubinin *et al.*, 2020).

We have previously shown that betulonic acid is able to significantly modify the surface properties of liver mitochondrial membranes in in vitro experiments. (Dubinin et al, 2020). This led to membrane aggregation and facilitated their permeabilization. It is known that a change in the functional properties of mitochondrial membranes can be accompanied by a change in their resistance to the induction of a calcium-dependent pore (MPT-pore). MPT-pore plays an important role in pathophysiological processes, participating in the induction of cell death by apoptosis and necrosis (Belosludtsev

Table 5

Calcium capacity of liver mitochondria (nmol Ca^{2+} ∗ mg^{-1} protein)

Animals	Glutamate/malate	Succinate/rotenone
Control	78,1 ± 1,3	104,1 ± 4,3
Betulonic acid	79,5 ± 2,3	107,8 ± 2,4

2.5 mM glutamate + 2.5 mM malate or 5 mM succinate + 1 μM rotenone were used as respiratory substrates. The results are presented as mean values ± SEM (*n*=5)

Table 6

Effect of betulonic acid-treatment on the rate of H_2O_2 production by mouse liver mitochondria (H_2O_2 ∗ min^{-1} ∗ mg^{-1} protein)

	Glutamate/malate	Succinate/rotenone
Control	480 ± 34	437 ± 19
Betulonic acid	307 ± 34*	282 ± 34*

2.5 mM glutamate + 2.5 mM malate or 5 mM succinate + 1 μM rotenone were used as respiratory substrates. The results are presented as mean values ± SEM (*n*=5). * $p < 0.05$ (vs control group, Mann–Whitney U test)

et al., 2019). We evaluated the ability of Ca^{2+} to induce pore opening in mitochondria, expressed quantitatively as the calcium capacity of mitochondria, i.e. that is the maximum amount of Ca^{2+} that can be accumulated in the matrix of organelles without subsequent opening of the pore. Table 5 shows that the liver mitochondria of mice treated with betulonic acid do not differ from the mitochondria of control animals in terms of calcium capacity in the presence of glutamate/malate and succinate/rotenone.

In previous studies, it was shown that triterpenoids and, in particular, betulonic acid, can have a significant effect on the production of hydrogen peroxide by mitochondria (Dubinin *et al.*, 2020), which can be used to regulate the redox status of organelles and the intensity of oxidative stress in the cell. Here, we also evaluated the in vivo effect of betulonic acid on the production of hydrogen peroxide by the mitochondria of the mouse liver. As can be seen from the data in Table 6, the liver mitochondria of mice treated with betulonic acid are characterized by a decrease in the intensity of hydrogen peroxide production compared to control animals. When organelles are energized with glutamate and malate, the rate of H2O2 generation decreases 1.6 times, while in the presence of succinate, this effect is less pronounced (1.5 times decrease in comparison with the control).

Discussion

Plant triterpenoids are a large group of substances with great pharmacological potential. One of the most promising representatives of triterpenoids is betulonic acid exhibiting a wide range of biological effects (Lombrea et al., 2021). In particular, we have previously shown that mitochondria can be the target of betulonic acid action in eukaryotic cells (Dubinin et al., 2020). Indeed, this agent significantly modified the surface properties of the membranes of these intracellular organelles, which led to the suppression of the intensity of oxidative phosphorylation and overproduction of reactive oxygen species. The aim of this study was to confirm the mitochondria-targeted action of this triterpenoid in vivo. In this regard, we intragastrically treated C57BL/10 mice with betulonic acid daily for 4 weeks.

We have found that betulonic acid does not significantly affect the state of the liver at the tissue level. Indeed, we did not find differences in the main parameters of the blood serum of

control and experimental animals (ALT, AST, total bilirubin), reflecting the state of this organ (Table 1). Histological examination also revealed no differences in the structure of the liver of control and experimental animals (Fig. 2 and 3, Table 2).

However, an assessment of the state of liver mitochondria in experimental animals revealed a noticeable suppression of the functional activity of organelles in mice treated with betulonic acid. The administration of betulonic acid led to a significant decrease in the rate of respiration and oxidative phosphorylation of mitochondria (Tables 3 and 4), which is most likely due to the previously revealed ability of this triterpenoid to reduce the redox activity of complexes of the respiratory chain of organelles (Dubinin et al., 2020). This effect of betulonic acid was more pronounced in the case of using succinate as an oxidation substrate, which may be due to an additional decrease in the rate of electron transport from succinate dehydrogenase (Dubinin et al., 2020). Along with this, it was found that, despite the known membranotropic effect of this agent, the administration of betulonic acid does not affect the resistance of liver mitochondria to the induction of a calcium-dependent MPT-pore (Table 5). On the other hand, liver mitochondria from mice treated with betulonic acid showed a decrease in hydrogen peroxide production compared to control animals (Table 6), which may indicate a possible antioxidant effect of this triterpenoid. One could assume that this effect of betulonic acid is also associated with a decrease in the rate of oxygen consumption by mitochondria and suppression of the functioning of the complexes of the respiratory chain of organelles that are capable of generating reactive oxygen species.

We hypothesize that this effect of betulonic acid can be used to regulate the conditions associated with the overproduction of reactive oxygen species in cells under conditions of oxidative stress. Further studies are needed to elucidate the in vivo mechanism of action of this agent in order to find out whether the mitochondria-directed effects of betulonic acid are a consequence of the direct action of the triterpenoid on organelles or associated with the triggering of specific mitochondria-associated signaling pathways.

Acknowledgments

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