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## The evaluation of 2.3-diazepine influence on tissue respiration of the liver and its exocrine function in rats with a rotenone model of Parkinson's disease

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Parkinson's disease (PD) is a progressive neurodegenerative disorder, characterized by the loss of dopaminergic neurons in the substantia nigra pars compacta. The causes of PD are not fully understood; however, increasing evidence implicates disturbed respiratory function of the mitochondria and a lack of energy in cells. **Aim.** To study the effects of 2.3-diazepine (2.3-DP), a new derivative of benzodiazepine, on liver tissue respiration (LTR) and energy dependent processes of bile and bile acids (BAs) production in a rat model of PD. **Methods.** PD was induced by intraperitoneal injections of rotenone (ROT). LTR (the intensity of the oxygen uptake) was assessed using the polarograph LP-9 (Czech Republic). Secreted bile was collected during 1 hour of the experiment through the polyethylene catheter inserted into the common bile duct. BAs were separated by the method of thin layer chromatography. **Results.** ROT diminished the index of liver oxygen consumption by 34 % ( $p < 0.001$ ), reduced bile flow by 33.8 % ( $p < 0.001$ ) and disturbed the conjugation of cholic acid with amino acids taurine and glycine reducing the index of conjugation by 29.2 % ( $p < 0.001$ ). ROT also increased by 25.6 % ( $p < 0.001$ ) the part of acidic pathway in the biosynthesis of BAs. The application of 2.3-DP results in full or partial recovery of LTR, bile flow, concentrations of BAs and their ratio in the bile of rats with PD. **Conclusion.** 2.3-DP markedly affected function of the liver parenchyma in a rat model of PD. This drug changed the intensity of LTR, bile flow and notably modified bile chemical compositions.

**Key words:** Parkinson's disease, 2.3-diazepine, tissue respiration, bile, bile acids.

## Introduction

Parkinson's disease (PD) is a progressive neurodegenerative disorder, characterized by the selective loss of dopaminergic neurons in *nigra pars compacta* of the *midbrain* leading to a significant reduction in dopamine levels in the striatum [1]. The factors causing selective neuronal cell death have not been clearly defined. To date, more than 15 genes have been identified which are associated with the hereditary forms of primary parkinsonism. The most studied PARK1  $\alpha$ -synuclein plays an important role in synaptic vesicular transport and storage of neurotransmitters. Mutations (hereditary or due to the effects of exogenous neurotoxic factors) in the  $\alpha$ -synuclein gene lead to a change in the structure of the protein, its accumulation in the neuron and aggregation with the formation of Levi's bodies. Currently,  $\alpha$ -synuclein is considered as a key molecular marker of pathology of neurons and modulation of neurodegeneration of Parkinson's type [2]. Numerous studies suggest that an inhibition of mitochondrial complex I and a mitochondrial dysfunction leading to respiration disorder and energy deficit in the neurons might play an important role in neurodegeneration during progression of PD [3]. The cardinal manifestation of PD is severe abnormalities in motor function, namely rigidity, tremor in rest, bradykinesia, and the loss of postural reflexes as well as non-motor features [1, 4]. Previous studies have shown that in PD, altered metabolic pathways may be associated with the exchange of certain compounds, in particular bile acids (BAs) [5]. Rotenone (ROT), an inhibitor of mitochondrial complex I, is usually used to reproduce the pathological features of PD in animal mo-

dels [6, 7]. The modeling of PD in rats by systemic administration of ROT revealed the symptoms similar to human pathology, namely nigrostriatal degeneration and dysfunction of mitochondria [8]. Nowadays, pharmacological treatment of PD is performed by dopamine and levodopa (Ldopa) agonists. Dopamine agonists are the medicines that activate the dopamine receptor. They mimic or copy the function of dopamine in the brain but cause several serious undesirable effects, including nausea, vomiting, hypotension, and, in the long run, motor complications [9]. In this context, the drugs with the ability to modulate mitochondrial dynamics, function and biogenesis may have important clinical applications in the future treatment of PD. L.A. Fonseca *et al.* showed that benzodiazepine- derivative substances protect the neuronal mitochondria against ROT-induced damage in rats model of PD [10]. Ukrainian scientists have developed the chemical compounds of the class of 2.3-benzodiazepines, which are characterized by the activity in relation to CNS. The clinical success of tofizopam, one of these drugs, initiated further researches in order to obtain new derivatives of 2.3-benzodiazepine. As an inhibitor of the  $\gamma$ -secretase enzyme, which plays an important role in the investigation of Alzheimer's disease (AD), a derivative of the 2.3-benzodiazepine-1.4-dione containing the diamond fragment 1.106 was proposed [11]. In the late 90s of the last century the first publications appeared devoted to the biological activity of condensed 2.3-benzodiazepines containing annelated heterocycles in the position of 3-4. Thus, the derivatives of triazolo [4.5-c] imidazo [1.2-c]

[2.3] diazepine 1.104 showed a high anticonvulsant activity of the Talampanel drug.

The liver is an organ with intense metabolism and in a healthy person, it consumes about 20 % of the total amount of oxygen in the body [12]. In turn, LTR is provided by mitochondria. The functioning of the mitochondria in neurons and hepatocytes is similar, therefore it was reasonable to establish the involvement of hepatocyte mitochondria in this pathology. The oxygen-dependent biosynthetic processes in the liver are closely related to the energy metabolism. Consequently, the consumption of oxygen by the liver may additionally indicate the functional status of the mitochondria.

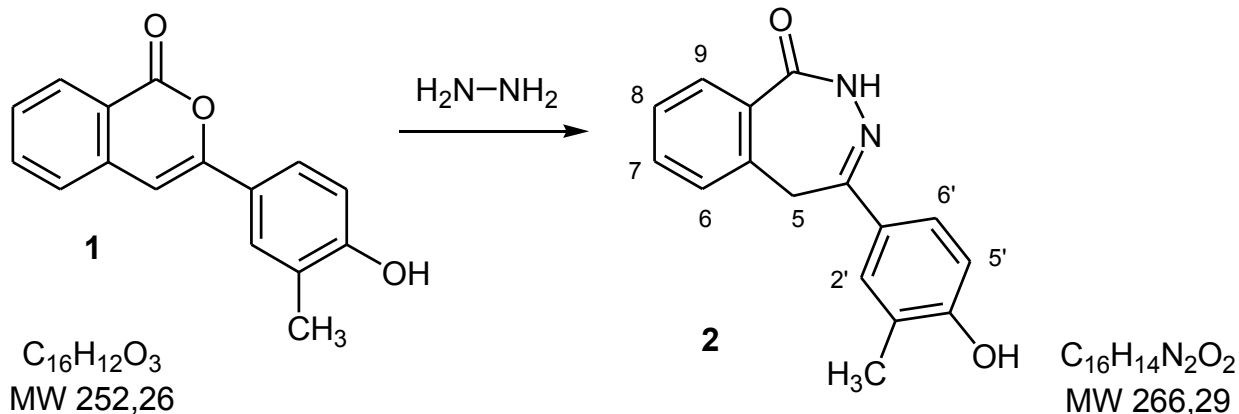
The present study therefore was aimed to investigate: 1) the features of liver function in rats with ROT-induced PD, in particular, tissue respiration of this organ, the rate of bile flow,

as well as the biliary content of BAs, the synthesis of which directly depends on the state of energy metabolism in hepatocytes; 2) the effect of the synthetic agent of 2.3-DP on the parameters of LTR, the rate of bile secretion and the content and ratio of BAs in the bile of rats with experimental ROT model of PD.

## Materials and Methods

*Compounds and reagents.* ROT, dimethyl sulfoxide (DMSO) and urethane were purchased from Sigma (St. Louis, MO, USA). Thiopentalum natrium was produced in Ukraine (OAO "Kyivmedpreparat").

*Elemental analysis of 2.3-DP* was performed in the analytical laboratory of V.P. Kukhar Institute of Bioorganic Chemistry and Petrochemistry, NAS of Ukraine, Kyiv, Ukraine: (*E*)-4-(4-Hydroxy-3-methylphenyl)-2*H*-benzo[*d*][1.2]diazepin-1(*5H*)-one



To a suspension of 1.26 g (0.005 mol) of 3-(4-hydroxy-3-methylphenyl)-1*H*-isochroman-1-one (**1**) in 25 ml of isopropyl alcohol 0.9 ml (0.015 mol) of 80 % hydrazine hydrate was added and the mixture was refluxed 3–4 hours (during the reaction, isocoumarin

slowly dissolved, the conversion was controlled by TLC). The resulting solution of product **2** was evaporated using a rotary evaporator to half-volume and cooled. The crystal solid was separated by filtration and recrystallized from isopropyl alcohol. Yield 85 %. Mp 256–258 °C.

$^1\text{H}$ ,  $^{13}\text{C}$  NMR spectra were recorded at Bruker Avance DRX500 NMR operating at 500 MHz frequency for  $^1\text{H}$  and 125 MHz for  $^{13}\text{C}$  experiments. NMR chemical shifts are reported in ppm, in the  $\delta$  scale and are referenced using TMS as internal standard. LC-MS data were acquired on Agilent 1100 HPLC system equipped with diode matrix and Agilent LC/MSD SL mass-selective detector, with chemical ionization at atmospheric pressure (APCI).

$^1\text{H}$  NMR (500 MHz, DMSO- $d_6$ ,  $\delta$ , ppm,  $J$ , Hz): 2.14 (3H, s,  $\text{CH}_3$ -3 $\phi$ ), 3.99 (2H, s,  $\text{CH}_2$ -5), 6.83 (1H, d,  $J = 8.4$ , H-5 $\phi$ ), 7.38 (1H, t,  $J = 7.3$ , H-8), 7.48–7.56 (2H, m, H-6,7), 7.62 (1H, br. d,  $J = 8.3$ , H-6 $\phi$ ), 7.67 (1H, s, H-2 $\phi$ ), 7.78 (1H, d,  $J = 7.6$ , H-9), 9.83 (1H, br. s, OH), 11.01 (1H, NH).

$^{13}\text{C}$  NMR (125 MHz, DMSO- $d_6$ ,  $\delta$ , ppm): 16.5, 34.3, 115.1, 124.8, 126.0, 126.5, 127.7, 127.8, 129.8, 130.1, 132.6, 133.3, 138.4, 158.2, 160.2, 167.2.

LCMS,  $m/z$ : 267 [M+1] $^+$ .

Found, %: C, 72.24; H, 5.26; N, 10.53. Calc. for  $\text{C}_{16}\text{H}_{14}\text{N}_2\text{O}_2$ , %: C, 72.16; H, 5.30; N, 10.52.

*Experimental animals.* Study has been done in acute experiments on 60 male mature Wistar rats (obtained from Institute of Toxicology, Academy of Medical Sciences of Ukraine (Kyiv, Ukraine)), weighing 230–270 g. The animals were housed under standard conditions of humidity (55–60 %), in a temperature-controlled environment ( $22 \pm 2^\circ\text{C}$ ) with a 12 h light/dark cycle, with unlimited access to filtered water and commercial food throughout the experimental period. Animal housing care and the application of experimental procedures were in accordance with the existing international requirements and norms of humane attitude towards animals (Strasbourg, 1986, Law

of Ukraine dated February 21, 2006, No. 3447-IV) and to the decision held by Biological Ethics Committee, Faculty of Biology, National Taras Shevchenko University of Kyiv (protocol No. 3 from April 9, 2009). This work has been done in accordance with Declaration of Helsinki (World medical assembly, 1964), Declaration of Principles on Tolerance (28th session of UNESCO, 1995), Universal Declaration on Bioethics and Human Rights related to introduction of new biomedical technologies, accepted in 1997 in the city of Oviedo (Spain) and signed by parliament of Ukraine in 2002, Law of Ukraine No. 3447 IV "About animals protection from brutal behavior".

*Experimental design.* Rats were injected with either ROT (2.0 mg/kg, intraperitoneally (i.p.)) suspended in sunflower oil (1 ml/kg) (ROT vehicle) or vehicle alone [13], daily for 28 consecutive days (from 10:00 a.m to 12:00 a.m.). After one week, the rats were randomly divided into six groups: (1) control group (n=10), rats received an injection of sunflower oil (1 ml/kg of body weight) that did not include ROT; (2) ROT group (n=9), ROT (2.0 mg/kg); (3) ROT+1 % solution of DMSO group (n=8), ROT (2.0 mg/kg)+1 % DMSO (1 ml/kg); (4) ROT+2.3-DP group (n=8), ROT (2.0 mg/kg)+2.3-DP (0.5 mg/kg); (5) ROT+ 2.3-DP group (n=8), ROT (2.0 mg/kg)+2.3-DP (1.0 mg/kg); (6) ROT+2.3-DP group (n=8), ROT (2.0 mg/kg)+2.3-DP (2.0 mg/kg). Immediately before use, 2.3-DP was freshly suspended in a 1 % DMSO solution. 2.3-DP (0.5, 1.0 and 2.0 mg/kg) or 1 % DMSO (1 ml/kg), were administered daily, i.p. as a single dose, for 28 days, starting immediately after the last injection of ROT. The dose had been determined

based on the lowest dose of other neuroprotective factors shown to be effective. During the experimental study period, animals from each group were weighed every seven days.

*Behavioral Analysis.* In the ROT-treated groups, rat fur became yellow and dirty; the rats showed reduced and slow movement, tremor, rigor and an unstable gait, which were identified as PD-like symptoms [13].

*Locomotor Activity.* The open-field test can be used to evaluate the spontaneous activity of rats. Twenty nine days following treatment rats were placed in an open field square box (100 cm long, 100 cm wide, and 40 cm high), and the test area is well illuminated. The experiment was carried out in a quiet environment. Rats were video-recorded (Fotocam, Canon) for 6 min, 1 min for habituation and 5 min for behavioral analyzes. The animal was placed into the center of the bottom of the box and turn on the camera to observe for 5 min. Two motor parameters were quantified throughout this test: locomotion frequency (number of squares crossings, defined as the number of quadrant crossings with the four paws) and rearing frequency (times the animal rise for at least 2s on their rear paws in the air or against the walls). Before the test, 70 % ethanol was used to thoroughly wipe the inner wall and bottom surface of the box, so as not to affect the results of the next test. Replace the animals and continue the experiment [14].

*Study of LTR.* The intensity of oxygen absorption by the liver tissues was assessed by the rate of decrease in  $pO_2$  in the liver parenchyma with half a minute occlusion of the portal vein and the hepatic artery. Before the experiment, the rats were anesthetized with urethane (1 g/kg, 1 ml/kg, intraperitoneally

(i.p.)). Oxygen tension ( $pO_2$ ) in the liver parenchyma was recorded using an LP-9 polarograph (Czech Republic). An open platinum microelectrode in glass insulation ( $d = 0.3$  mm) with an operating voltage of 0.65 V was used as an indicator. A standard calomel electrode was used as a reference electrode. Several platinum electrodes were inserted into the liver, and the electrical signals from them were sent to the polarograph. To calculate the level of  $pO_2$  in the liver tissue, the method of the electrodes calibration in an environment with a known level of  $pO_2$  was applied [15].  $pO_2$  in the liver was calculated according to the equation:  $pO_2 = pO_{2atm} \cdot Ae \cdot Se / (Ac \cdot Sc)$ , where:  $pO_{2atm}$  is the oxygen tension in a calibrated saline solution, balanced by an atmospheric pressure of  $O_2$  — 150 mm Hg; Ac, Ae is the amplitude of the calibration and experimental signals (in mm of record); Sc and Se are the sensitivity of the polarograph scales during calibration and experiment. The rate of oxygen consumption by the liver tissue was determined by the curve of the drop in  $pO_2$  during asphyxia of the animal or occlusion of the afferent liver vessels [16]. The coefficient of oxygen consumption by the liver was calculated from this curve:  $K = \lg(I_1 / I_2) / 0,43 \cdot (t_2 - t_1)$ , where:  $I_1$  and  $I_2$  are the polarogram current values corresponding to  $pO_2$  at times  $t_1$  and  $t_2$  from the onset of asphyxia or occlusion of the liver afferent vessels. The coefficient K was determined in the interval from the 10th to the 30th second of asphyxia or vascular occlusion.

*Determination of the bile flow and the bile concentration of different BAs.* Every rat was anesthetized with thiopentalum natrium (4 mg/100 g rat b.w., i.p.). Common bile duct was cannulated with polyethylene catheters and

secreted bile was collected every 30 minutes during 1 hour of the experiment by micropipette connected to cannula located in the bile duct. Bile flow was calculated by  $\mu\text{l/g}$  of rat body weight. All manipulations were performed after stabilization of the bile flow (30 minutes).

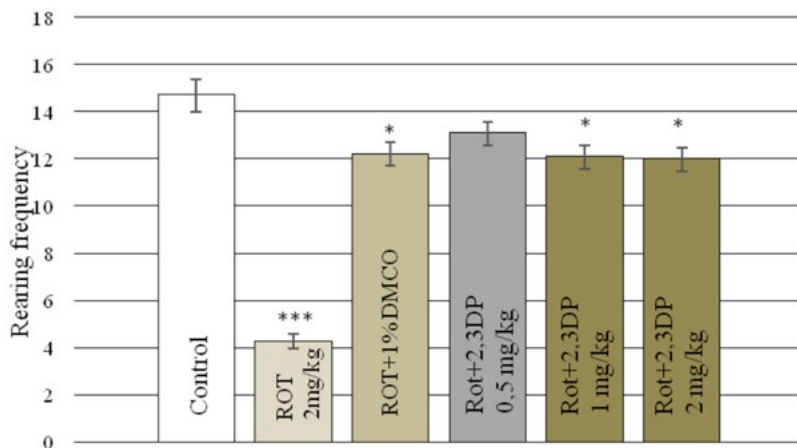
Free and conjugated BAs were divided by thin layer chromatography method [17]. For this purpose, 0.1 ml of bile was added to 1.9 ml of cold, extracted mixture of ethanol and acetone (1 : 3). Samples were kept cool ( $-10$  to  $0^{\circ}\text{C}$ ) in an ice chamber for 25–30 minutes and then centrifuged for 10–12 minutes at 3000–4000 r.p.m. The extracts were then poured in conoid glass test tubes and dried at  $37$ – $40^{\circ}\text{C}$  to get dry remainders. Dry remainders were dissolved in 50–100  $\mu\text{l}$  of ethanol-water mixture (6 : 4). 5–10  $\mu\text{l}$  of samples were inflicted on the preliminary washed and marked chromatography plates ( $15 \times 15$  cm, silica gel plates on aluminium back, Kavalier, Czech). Free and conjugated BAs deviation was carried out in the system containing amyl ester acetic acid, toluole, butanole, acetic acid and water (3:1:1:3:1, respectively), in glass chromatography chambers. Chromatograms were dyed after five times sprinkling from a glass fine-disperser pulveriser with the dye stuff (15 ml icy acetic acid, 1 g phosphomolybdic acid, 1 ml sulphuric acid and 5 ml of 50 % trichloroacetic acid solution). Chromatograms were put at  $60$ – $70^{\circ}\text{C}$  during 5 minutes. Quantitative determination of BA content was performed by densitometer GP-920 (Shimadzu, Japan) under reflected light ( $\lambda$  620 nm). This method allowed dividing mixture of BAs into following fractions: TCA, TCDCA+TDCA, GCA, GCDCA+GDCA, CA, CDCA+DCA. BAs content was calculated by mg per 100 ml of bile (mg%).

*Statistical analysis.* The results were assessed by: the parametric one-way ANOVA method and also in combination with the Tukey test for pairwise comparison and  $p$  values less than 0.05 were considered significant; the non-parametric Kruskal-Wallis ANOVA method and also in combination with the Mann-Whitney test for pairwise comparison with *Bonferroni* correction and  $p$  values less than 0.01 were considered significant. Statistical analysis was performed by Origin Pro 8.0, and the charts were drawn by Excel software.

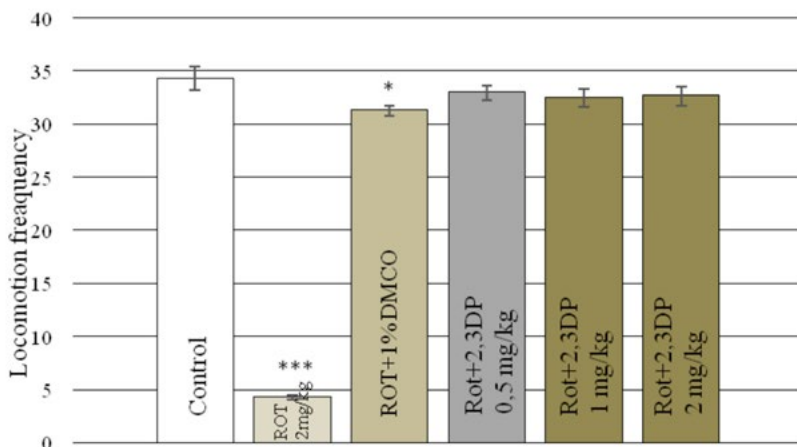
**Results.** The present results revealed a significant decrease in rearing frequency (by 71 %,  $p < 0.001$ ) and in locomotion frequency (by 92 %,  $p < 0.001$ ) in ROT group as compared to the control group. 2,3-DP treatment improved behavioral performance of rats with PD (Fig. 1, 2).

The current data revealed that during 56 days of the experiment in the control group of rats, body weight increased by 47 % compared to baseline. In the ROT+DMSO group for the same period of time, the weight gain was only 19.1 % ( $p < 0.001$ ). In contrast to the previous group, the use of 2,3-DP in doses of 0.5 and 1.0 mg/kg improved the weight gain of rats poisoned with ROT by 30.8 % ( $p < 0.001$ ) and 23 % ( $p < 0.001$ ), respectively (Fig. 3).

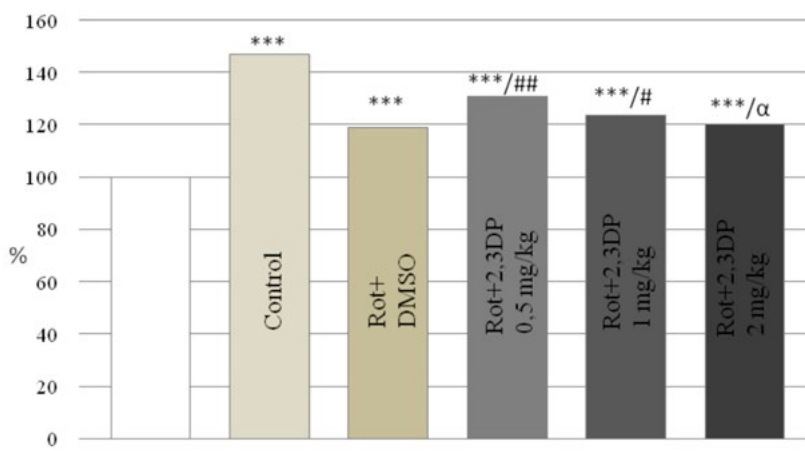
The administration of ROT to rats caused a significant inhibition of LTR, as evidenced by a decrease in the oxygen consumption (K) compared with the control group by 34 %,  $p < 0.001$  (Table 1). In rats with a ROT model of PD which were treated with 1 % DMSO solution during 28 days, LTR recovered up to 73.4 % of control level whereas in animals treated with 2,3-DP at doses of 0.5 and 1 mg/kg, LTR was restored to control levels ( $K = 1.96 \pm 0.15$  and  $1.98 \pm 0.17$ , respectively).



**Fig. 1.** Change in rearing frequency in rats with ROT-induced PD treated with 2,3-DP (open field test was used). The data are presented as the means ± S.E.M. \* —  $p < 0.05$ , \*\*\* —  $p < 0.001$  versus control group.



**Fig. 2.** The effect of 2,3-DP treatment on the locomotion frequency in rats with ROT-induced PD (open field test was used). The data are presented as the means ± S.E.M. \* —  $p < 0.05$ , \*\*\* —  $p < 0.001$  versus control group.



**Fig. 3.** Effects of 2,3-DP treatment on the weight gain in ROT-poisoned rats. \*\*\* —  $p < 0.001$  versus control group; the data are presented as the means ± S.E.M. # —  $p < 0.05$ , ## —  $p < 0.01$  versus ROT+DMCO group; α —  $p < 0.05$  in comparison with the ROT+2,3-DP 0.5 mg/kg group.

Under the effect of 2.3 DP no significant changes were obtained in the group ROT+2.3-DP 2 mg/kg.

In ROT treatment the rats significantly reduced the level of bile secretion as evidenced by a decrease in bile volume in the group on average by 33.8 % ( $p < 0.001$ ). In rats of the ROT+DMSO and ROT+2.3 DP 2.0 groups, bile flow was lower on average by 24.3 % ( $p < 0.01$ ) than in control rats. In 2.3-DP-treated rats (0.5 and 1 mg/kg), the value of the bile flow did not differ from the control level (Table 2).

The biochemical analysis of bile showed that bile levels of TCA and GCA in ROT-treated rats were decreased by 18.2 % ( $p < 0.001$ ) and by 17.0 % ( $p < 0.01$ ), respectively as compared with vehicle group. In ROT+2.3-DP group, the levels of TCA in the bile were found to be significantly increased following drug administration for 28 days. Without applying 2.3-DP, the concentration of TCA in bile did not return to normal level reaching 84 % of the control. On the contrary, administration of 2.3-DP did not affect the reduced content of GCA. Meanwhile,

the concentration of TCDCA+TDCA in rat bile did not change in all experimental groups in comparison with control data (Table 3) whereas the concentrations of both GCDCA+GDCA and CA increased by 19.5 % ( $p < 0.05$ ) and by 18.8 % ( $p < 0.05$ ), respectively. In 2.3-DP-treated group the content of GCDCA+GDCA remained elevated in the ROT+2.3-DP 2 mg/kg group, but CA concentration recovered to the control level in all 2.3-DP-treated groups of rats.

Under the effect of ROT, the content of CDCA+DCA in bile was modified. This index was significantly increased as compared with control data. The stimulatory effect of ROT on CDCA+DCA level in rat bile was about 58.3 % ( $p < 0.001$ ) when compared with vehicle (control) group. After the application of 2.3-DP, this indicator completely returned to the control level only in the ROT+2.3-DP 0.5 mg/kg group (Table 3).

The current data revealed that the total BAs concentrations in ROT and ROT+DMSO groups were less as compared with control data by 9.7 % ( $p < 0.05$ ) and by 9.1 % ( $p < 0.01$ ), respectively. Under the influence of 2.3-DP at

**Table 1. The effects of 2,3-DP on ROT-induced decrease in liver tissue oxygenation**

| Animals group                    | Liver oxygen consumption (x100) |
|----------------------------------|---------------------------------|
| Control                          | 1.92±0.03                       |
| ROT 2 mg / kg                    | 1.27±0.08 ***                   |
| ROT 2 mg / kg + 1 % DMSO         | 1.41±0.12***                    |
| ROT 2 mg / kg + 2.3-DP 0.5 mg/kg | 1.96±0.15 ##                    |
| ROT 2 mg / kg + 2.3-DP 1 mg/kg   | 1.98±0.17 ##                    |
| ROT 2 mg / kg + 2.3-DP 2 mg/kg   | 1.5±0.18                        |

The data are presented as the means ± S.E.M. Different letters:  $p < 0.01$ ;  $n = 9$ . \*\*\* —  $p < 0.001$  compared with the control group; ## —  $p < 0.01$  compared with the ROT+DMSO group.

**Table 2. The influence of 2,3-DP on bile flow in rats (µl/g) with ROT-induced PD**

| Animal group                   | The volume of secreted bile |
|--------------------------------|-----------------------------|
| Control                        | 2.7±0.08                    |
| ROT 2 mg/kg                    | 1.8±0.08***                 |
| ROT 2 mg/kg + 1 % DMSO         | 2.1±0.1***                  |
| ROT 2 mg/kg + 2.3-DP 0.5 mg/kg | 2.4±0.09###                 |
| ROT 2 mg/kg + 2.3-DP 1 mg/kg   | 2.3±0.08###                 |
| ROT 2 mg/kg + 2.3-DP 2 mg/kg   | 2.1±0.07 **                 |

The data are presented as the means ± S.E.M. Different letters:  $p < 0.01$ ;  $n = 8$ . \*\* —  $p < 0.01$ ; \*\*\* —  $p < 0.001$  compared with the control group; ### —  $p < 0.001$  compared with the ROT group.



**Table 3. The effect of 2.3-DP on the concentration of BAs in the bile of rats with PD**

| Animal group                      | TCA<br>mg/%           | TCDCa+TDCA<br>mg/% | GCA<br>mg/%     | GCDCA+GDCA<br>mg/% | CA<br>mg/%      | CDCA+ DCA<br>mg/%  |
|-----------------------------------|-----------------------|--------------------|-----------------|--------------------|-----------------|--------------------|
| Control                           | 219.2±5.2             | 106.7±3.7          | 121.2±3.8       | 22.5±1.05          | 15.4±0.7        | 13.2±0.5           |
| ROT 2 mg/kg                       | 179.3±6.0<br>***      | 117.3±3.1          | 107.5±2.7<br>** | 26.9±0.9*          | 18.3±0.8<br>*** | 20.9±0.4<br>***    |
| ROT 2 mg/kg +1 %<br>DMSO          | 184.0±5.1<br>***      | 97.1±2.8<br>###    | 95.9±2.7<br>*** | 21.4±1.0           | 17.5±0.55<br>*  | 16.5±0.4<br>***, # |
| ROT 2 mg/kg<br>+ 2.3-DP 0.5 mg/kg | 219.7±5.3<br>###, aaa | 97.3±2.9<br>###    | 104.2±3.3<br>** | 23.2±1.0           | 15.4±0.4<br>##  | 13.0±0.6<br>###    |
| ROT 2 mg/kg + 2.3-DP<br>1 mg/kg   | 217.7±3.7<br>###, aaa | 111.8±3.0<br>α, £  | 92.5±3.6<br>*** | 24.2±1.2           | 16.3±1.0        | 10.8±0.7 *<br>###  |
| ROT 2 mg/kg + 2.3-DP<br>2 mg/kg   | 204.1±4.5<br>##       | 99.8±3.3<br>##     | 99.7±1.9<br>*** | 27.5±0.9<br>*      | 16.8±0.6<br>##  | 11.2±0.6<br>*, ### |

Values are presented as the means ± S.E.M. \* —  $p < 0.05$ , \*\* —  $p < 0.01$ ; \*\*\* —  $p < 0.001$  vs. the vehicle control group; # —  $p < 0.05$ , ## —  $p < 0.01$ , ### —  $p < 0.001$  vs. the ROT group; α —  $p < 0.05$ , aaa —  $p < 0.001$  vs. the ROT+ 1 % DMSO group; £ —  $p < 0.05$  vs. the ROT+2.3-DP 0.5 mg/kg group.

doses of 0.5 and 1 mg/kg, the concentration of total BAs were equal to the control level. Similar differences were observed in the changes of total conjugated BAs content in the bile of rats with PD. This index was decreased by 12.2 % ( $p < 0.001$ ) and by 17.3 % ( $p < 0.001$ ) in both ROT and ROT+DMSO groups, respectively. ROT-treated rats exhibited an increase in biliary total conjugated BAs level relatively to the control data after applying 2.3-DP doses of 0.5 and 1 mg/kg. In the ROT+DMSO and ROT+2.3 DP groups, the bile concentration of total conjugated BAs remained lower than the control indicators by 17.3 % ( $p < 0.001$ ) and by 10.6 % ( $p < 0.01$ ), respectively. The content of total free BAs, in response to ROT administration, was higher than in the control rats by 31.3 % ( $p < 0.01$ ). On the contrary, after 2.3 DP-treatment the total free BAs content diminished to the initial level in all experimental groups. In ROT+DMSO group when compared with ROT-treated rats no statistical difference was observed. Accordingly, the ratio

between total conjugated and total free BAs diminished in this groups of rats by 26.7 % ( $p < 0.01$ ) and by 29.2 % ( $p < 0.001$ ), respectively compared to control values (Table 4).

The obtained results indicate that in rats with ROT model of PD the statistically significant decrease of the content of total trihydroxycholanic BAs was 12.2 % ( $p < 0.001$ ), and the level of total dihydroxycholanic BAs, on the contrary, increased by 16.2 % ( $p < 0.01$ ). Accordingly, the coefficient of hydroxylation decreased by 16 % ( $p < 0.001$ ). All rats of the ROT+DMSO group exhibited somewhat reduced bile concentration (by 16.5 %,  $p < 0.001$ ) of total trihydroxycholanic BAs. The similar result was observed in ROT+2.3-DP 0.5 and ROT+2.3-DP 1.0 groups except for ROT+2.3-DP 2.0 group, the indicators of which were as in the control group. Conversely, the content of dihydroxycholanic BAs in the bile was restored to [the] control level in all groups of rats with modeled PD both treated by 1 % DMSO and by 2.3-DP in different doses. Thus,

**Table 4. Changes in the concentration of total, total conjugated and total free BAs in the bile of rats with ROT-induced PD treated by 2,3-DP**

| Animal group                   | Total BAs mg/%         | Total conjugated BAs mg/% | Total free BAs mg/%          | Ratio between total conjugated and free BAs |
|--------------------------------|------------------------|---------------------------|------------------------------|---|
| Control                        | 511.3±9.2              | 482.0±8.7                 | 29.4±1.0                     | 16.1±0.7                                    |
| ROT 2 mg/kg                    | 472.5±12.9*            | 423.2±10.7***             | 38.6±0.8**                   | 11.5±0.6***                                 |
| ROT 2 mg/kg+1 % DMSO           | 432.5±7.5***, #        | 398.6±7.7 ***             | 33.9±0.3***                  | 11.8±0.3***                                 |
| ROT 2 mg/kg + 2,3-DP 0.5 mg/kg | 476.5±5.7 <sup>a</sup> | 448.1±5.5 <sup>aa</sup>   | 28.4±0.7###, <sup>aaa</sup>  | 15.2±0.4###, <sup>aaa</sup>                 |
| ROT 2 mg/kg+2,3-DP 1 mg/kg     | 473.4±9.4 <sup>a</sup> | 446.2±8.7 <sup>aa</sup>   | 27.2±0.8###, <sup>aaa</sup>  | 15.8±0.45###, <sup>aa</sup>                 |
| ROT 2 mg/kg+2,3-DP 2 mg/kg     | 459.1±8.8**            | 431.1±8.7**               | 28.0±0.45###, <sup>aaa</sup> | 15.4±0.3###, <sup>aaa</sup>                 |

The data are presented as the means ± S.E.M. Different symbols: \* — p < 0.05; \*\* — p < 0.01; \*\*\* — p < 0.001 as compared with control rats; # — p < 0.05, ### — p < 0.01, #### — p < 0.001 relative to ROT+DMSO group; <sup>a</sup> — p < 0.05, <sup>aa</sup> — p < 0.01, <sup>aaa</sup> — p < 0.001 vs. the ROT+DMSO group. Secreted bile was collected during 1 hour of the experiment.

the ratio between total trihydroxycholanic and total dihydroxycholanic BAs in these groups of rats also corresponded to the control values (Table 5).

**Discussion.** PD is accompanied not only by motor disorders, but also by non-motor symptoms, such as weight loss, constipation, loss of the sense of smell, sleep disturbances, cognitive deficits and depression [4]. Available diagnostic approaches still lead to a high proportion of false diagnoses, in particular, at the early stages of PD, so biochemical markers are required to inform the pathogenesis of parkinsonism. Here, we applied the thin layer chromatography method to determine the profiles

of bile metabolites. Characteristics of the metabolites indicate perturbations in the BAs metabolism in PD, which underscores the power of metabolomic approaches.

ROT, a mitochondrial complex I inhibitor, was reported to reproduce a number of neuropathological features of PD, including loss of dopaminergic neurons [6, 8]. We predicted that this substance may induce a significant inhibition of LTR. Indeed, in the present study, ROT application resulted in a decreased coefficient of oxygen consumption by the liver tissue by 34.3 %. 2,3-DP in doses of 0.5 and 1 mg/kg completely restored this index in rats with experimental model of PD. These

**Table 5. The ratio between total trihydroxycholanic and total dihydroxycholanic BAs within bile of rats with PD under the influence of 2,3-DP**

| Animal group           | Total trihydroxycholanic BAs, mg/% | Total dihydroxycholanic BAs, mg/% | Hydroxylation factor |
|------------------------|------------------------------------|-----------------------------------|----------------------|
| Control                | 356.1±9.6                          | 142.2±4.3                         | 2.5±0.03             |
| ROT 2 mg / kg          | 307.2±9.2***                       | 165.3±4.7**                       | 1.86±0.06***         |
| ROT +1 % DMSO          | 297.35±6.5***                      | 135.2±4.6##                       | 2.2±0.08##           |
| ROT+2,3-DP 0.5 mg/kg   | 324.5±5.5 *                        | 133.5±2.9                         | 2.4±0.06##           |
| Rot + 2,3-DP 1.0 mg kg | 312.2±4.1***                       | 146.8±3.5                         | 2.3±0.08##           |
| Rot + 2,3-DP 2 mg kg   | 320.7±4.3                          | 138.5±4.1##                       | 2.2±0.04##           |

Means ± S.E.M. \* — p < 0.05; \*\* — p < 0.01; \*\*\* — p < 0.001 as compared with control group; ## — p < 0.01 as compared with ROT group.

results suggest that the LTR is an adequate indicator in the drug effectiveness research that may be involved in the correction of liver functions disturbed by PD. In rats with PD induced by ROT, a disturbance in the functions of hepatocyte mitochondria presumably takes place, which resulted in the reduction of the intensity of tissue respiration and oxygen dependent biosynthetic processes in the liver. Thus, on the one hand the volume of produced bile decreased, the conjugation of CA with the amino acids taurine and glycine reduced (the conjugation factor reduced by 29.2 %), but on the other hand, the portion of acidic pathway in the biosynthesis of BAs increased, enhancing the hydrophobicity of BAs pool. Our results demonstrated that 2.3-DP protects against ROT-induced disorders of liver functions in rats with PD promoting the conjugation of CA with taurine to increase the concentration of TCA. In the presented study, the metabolic changes in the bile of the rats with ROT-simulated PD, were mainly related to the functional state of hepatocyte mitochondria as well as the exchange of BAs. Our results demonstrated that in the rats with PD the total secreted bile volume during overall time of the experiment decreased by 33 % compared to the control subjects. The animals with PD treated by 2.3-DP, exhibited improved bile flow.

The bile formation is a unique function of the liver which is vital for survival of the organism. BAs, as well as their salts, determine the basic properties of bile as a digestive secretion. The intensity of bile secretion is closely related to LTR. As previously has been shown, the reduction in the oxygenation of the liver tissue steadily leads to a decrease in bile volu-

me, formed for a certain period of time. It was concluded that the hepatic tissue oxygenation affects hepatic energy metabolism, thus modifying the rate of bile flow in the liver [18]. The unique detergent properties of BAs are essential for the digestion and absorption of hydrophobic nutrients in the small intestine, including dietary fats, fat-soluble vitamins. They also modulate biliary and lipid secretion, regulate the activity of key enzymes involved in the cholesterol homeostasis and prevent cholesterol precipitation [19]. Taking into account the regulatory importance of bile and BAs, we can assume that disorders in both the formation of bile flow and the exchange of BAs may cause a derangement of many complex physiological and biochemical processes in the body. To assess the functional state of the liver, namely the quality of secreted bile, the ratio between individual groups of BAs is important. Therefore, the ratio between total BAs conjugated with amino acids taurine and glycine and total free BAs, the so-called conjugation factor, was studied as well as the ratio between bile trihydro- and dihydrocholates. The findings of this work showed that ROT with long-term (during 28 days) administration significantly reduced the rate of conjugation of CA with glycine (11.3 %), and especially with taurine (by 18.2 %), whereas the concentration of free CA within bile increased (by 19.0 %). As a result, the rate of total conjugated BAs significantly decreased and the number of total free BAs, on the contrary, increased.

The results of our experiments agree with the findings of Govorukha *et al.* [20]. They found that liver tissue hypoxia caused by various methods, increased the level of free CA

in bile. These data confirm the inhibitory effect of ROT on the activity of enzymes that provide conjugation of free CA with amino acids. Under the influence of 2.3-DP in doses of 0.5 and 1 mg/kg, the bile content of TCA returned to the control levels. On the contrary, the bile content of GCA in all groups of animals remained lower than in the control group. Both amino acids taurine and glycine, interact with CoA-ether of the corresponding BA during conjugation. The catalysts of this reaction are microsomal CoA-ligase and cytosolic N-acetyltransferase, which operate with the energy consumption and in the presence of NAD, AMF,  $Mg^{2+}$ , CoA. The energy deficit in the cell inhibits the conjugation of CA with amino acids. 2.3-DP improved the conjugation of free CA with taurine in ROT-treated rats, probably affecting one of the components of this complex process. Concurrently with the above-mentioned events, in the bile of rats with PD, the level of free CDCA+DCA increased markedly (by 58.3 %) with a partial enhance in their conjugation with glycine. The tested drug also improved this parameter in all 2.3-DP-treated groups of rats, reducing it to the control level. The reduction of conjugation factor in ROT-treated group in general indicates a markedly decrease (by 29.3 %) in the activity of enzymes involved in this process. Under the effect of 2.3-DP, this index was completely restored in rats with a ROT model of PD.

Decreased BAs secretion leads to defective micellar solubilization of dietary lipids, and this contributes to lipid malabsorption in these patients. The ability of the bile to emulsify the fats and promote their absorption in the intestine depends on the content of the conjugated

BAs [21]. Based on the latter, it can be assumed that 2.3-DP improves these properties of bile. Noteworthy, in addition to the above-mentioned we also found that the ROT reduced the content of trihydroxycholanolic BAs, but caused an increase in the level of dihydroxycholanolic BAs. The ratio of trihydroxy- to dihydroxycholanolic BAs indicates an increase of the role of the "alternative (acidic) pathway" in the biosynthesis of BAs in rats with PD. This synthesis is carried out with the participation of mitochondrial enzymes and is confirmed by a significant increase in bile levels of CDCA and DCA. As is known, BAs are formed by hepatocytes with cholesterol in two pathways: neutral (DCA and CDCA in equal parts) and acidic (predominantly CDCA). The classic pathway involving cholesterol 7 $\alpha$ -hydroxylase is the major pathway in BAs biosynthesis because its contribution to total BAs synthesis is ~90 % in humans and ~75 % in mice [22]. The ratio between these components of the bile is determined as the coefficient of hydroxylation. This factor shows both the hydrophobicity of BAs pool and the degree of emulsification of fats in the duodenum. According to the obtained results, the coefficient of hydroxylation of BAs was significantly decreased in rats with PD however, 2.3-DP improved this rate. On the other hand noteworthy, the hydroxylation factor returned to the control level also in the group of rats, treated with 1 % DMSO solution only. Consequently, the using of 2.3-DP was not of fundamental importance in this case, since the body had enough resources for providing homeostasis. Recently, Rosa A, *et al.* have shown in mice that some BAs, in particular tauroursodeoxycholic acid, act as mitochondrial sta-

bilizer and anti-apoptotic agent in a number of models of neurodegenerative diseases, including PD. This substance prevented a decrease of dopaminergic fibers and ATP levels, mitochondrial dysfunction and neuroinflammation [23].

In our study we observed that ROT-treated rats showed a tendency to weight loss and increased mortality rate. Possible causes related to this decline in body weight could be caused to a gastrointestinal dysfunction in PD [24], in particular with a delay in gastric emptying and the damage to gastrointestinal neurons, occurred during ROT intoxication [25]. We also observed that ROT-treated animals administered with 2.3-DP were less prone to lose weight and to die, arguing in favor of the protective effects of tested drug.

Earlier it was found that severe defects in the complex I activity induce mitochondria depolarization and  $\text{Ca}^{2+}$  dysregulation [26]. The excessive mitochondrial  $\text{Ca}^{2+}$  uptake can cause non-selective permeabilization of the inner mitochondrial membrane possibly leading to swelling the mitochondria and dissipation of the membrane potential from massive proton leakage. Fonseca-Fonseca LA and co-authors support this mechanism. They found that mitochondria isolated from brain of rats with PD, induced by ROT, spontaneously lost their membrane potential and were more prone to membrane permeability/swelling than the vehicle group [10]. Here we observed a decrease in the intensity of LTR in rats following ROT administration. The improvement of this indicator after application of 2.3-DP shows the restoration of mitochondrial functions in hepatocytes of the liver under the influence of the tested substance. The improvement of the

liver choleric function, the biochemical composition of bile, which we observed in our experiments, also indicates an increase in the energy status of hepatocytes under the influence of 2.3-DP.

**Conclusions** Our results demonstrate that 2.3-DP protects against ROT-induced disorders in PD by promoting the LTR and especially conjugation of CA with taurine to enhance bile concentration of TCA. Additionally, the tested drug normalizes the ratio between the acidic and the neutral pathways in the biosynthesis of BAs, attenuating the hydrophobicity of bile. Probably, the improvement of liver secretory function in rats with PD, which we observed, is associated with the restoration of energy homeostasis of the liver under the influence of 2.3-DP. Considering the complexity of molecular and neurological systems, further studies are required to characterize 2.3-DP as a new possible drug against PD.

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### Оцінка впливу 2,3-діазепіну на тканинне дихання і зовнішньосекреторну функцію печінки щурів з ротеновою моделлю хвороби Паркінсона

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Хвороба Паркінсона (ХП) — це прогресуюче нейродегенеративне захворювання, що характеризується втратою дофамінергічних нейронів у субстанції *nigra pars compacta*. Причини ХП не повністю зрозумілі, проте, все більше даних свідчить про порушення дихальної функції мітохондрій і нестачі енергії в клітинах. **Мета.** Вивчити вплив 2,3-діазепіну (2,3-ДП), нового похідного бензодіазепіну, на тканинне дихання печінки (ТДП) і енергетично залежні процеси продукції жовчі й жовчних кислот (ЖК) у щурів з ХП, модельованою ротеноном (РОТ). **Методи.** ХП була викликана у щурів внутрішньоочеревинними ін'єкціями РОТ. ТДП (інтенсивність поглинання кисню) оцінювали за допомогою полярографа LP-9 (Чеська Республіка). Секретовану жовч збирали протягом 1 години експерименту через поліетиленовий катетер, вставлений в загальний жовчний протік. ЖК розділяли методом тонкошарової хроматографії. **Результати.** РОТ зменшував коефіцієнт споживання  $O_2$  в печінці на 34 % ( $p < 0.001$ ), знижував відтік жовчі на 33.8 % ( $p < 0.001$ ) і порушував кон'югацію холевої кислоти з амінокислотами таурином і гліцином, зменшуючи коефіцієнт кон'югації на 29.2 % ( $p < 0.001$ ). РОТ також збільшував на 25.6 % ( $p < 0.001$ ) частку кислого шляху в біосинтезі ЖК. Застосування 2,3-ДП призводило до повного відновлення ТДП, або часткового — відтоку жовчі, концентрацій ЖК і їх співвідношення в жовчі щурів з ХП. **Висновок.** 2,3-ДП істотно впливає на функцію паренхіми печінки у щурів з РОТ моделлю ХП. Цей препарат відновлює ТДП, покращує відтік жовчі і помітно поліпшує її хімічний склад, порушений при застосуванні РОТ.

**Ключові слова:** хвороба Паркінсона, 2,3-діазепін, тканинне дихання печінки, відтік жовчі, жовчні кислоти.

### Оценка влияния 2,3-диазепина на тканевое дыхание и внешнесекреторную функцию печени крыс с ротеновой моделью болезни Паркинсона

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Болезнь Паркинсона (БП) — это прогрессирующее нейродегенеративное заболевание, характеризующееся потерей дофаминергических нейронов в субстанции *nigra pars compacta*. Причины БП не полностью поняты, тем не менее, все больше данных свидетельствует о нарушении дыхательной функции митохондрий и недостатке энергии в клетках. **Цель.** Изучить влияние 2,3-диазепина (2,3-ДП), нового производного бензодиазепина, на тканевое дыхание печени (ТДП) и энергетически зависимые процессы продукции желчи и желчных кислот (ЖК) у крыс с БП, моделированной ротеноном (РОТ). **Методы.** БП была вызвана у крыс внутрибрюшинными инъекциями РОТ. ТДП (интенсивность поглощения кислорода) оценивали с помощью полярографа LP-9 (Чешская Республика). Секретированную желчь собирали в течение 1 часа эксперимента через полиэтиленовый катетер, вставленный в общий желчный проток. ЖК были разделены методом тонкослойной хроматографии. **Результаты.** РОТ уменьшал коэффициент потребления  $O_2$  в печени на 34 % ( $p < 0.001$ ), снижал отток желчи на 33.8 % ( $p < 0.001$ ) и нарушал конъюгацию холевой кислоты с аминокислотами таурином и глицинем, уменьшив коэффициент конъюгации на 29.2 % ( $p < 0.001$ ). РОТ также увеличивал на 25.6 % ( $p < 0.001$ ) долю кислого пути в биосинтезе ЖК. Применение 2,3-ДП приводило к полному восстановлению ТДП, или частичному — оттока желчи, концентраций ЖК и их соотношения в желчи крыс с БП. **Заключение.** 2,3-ДП существенно влияет на функцию паренхимы печени у крыс с РОТ моделью БП. Этот препарат восстановил интенсивность ТДП, оттока желчи и заметно улучшил ее химический состав, нарушенный применением РОТ.

**Ключевые слова:** болезнь Паркинсона, 2,3-диазепин, тканевое дыхание печени, отток желчи, желчные кислоты.

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