ISSN 0206-5657. Вісник Львівського університету. Серія біологічна. 2025. Випуск 94. С. 95–103
Visnyk of the Lviv University. Series Biology. 2025. Issue 94. P. 95–103
UDC 612.35 : 612.26 : 577.23 HTTPS://DOI.ORG/10.30970/VLUBS.2025.94.10

ETHANOL *IN VITRO* DOES NOT AFFECT THE OXIDATIVE PROCESSES OF HEPATOCYTE MITOCHONDRIA UPON GLUCOSE, PYRUVATE, OR MONOMETHYL SUCCINATE OXIDATION

H. Mazur, O. Ruda, B. Manko, V. Manko

Ivan Franko National University of Lviv
4, Hrushevskyi St., Lviv 79005, Ukraine
e-mail: halyna.mazur@lnu.edu.ua

Most of the alcohol that enters the body is metabolized in the liver. In the case when the rate of ethanol oxidation is lower than the rate of its intake, the accumulation of ethanol leads to liver cell damage. Since reduced forms of NADH are formed in ethanol oxidation, mitochondrial respiration plays an important role in alcohol metabolism by regenerating NAD^+, which regulates the level of both ethanol oxidation enzymes and is necessary for ethanol-acetaldehyde metabolism. The aim of the study was to investigate the effect of glucose, pyruvate and monomethyl succinate on the oxidative processes of hepatocyte mitochondria under the influence of ethanol *in vitro*.

Experiments were performed on male Wistar rats weighing 220–250 g. Isolation of hepatocytes was carried out by the two-stage Seglen method. The oxygen consumption rate was determined with a Clark electrode. Hepatocytes were incubated for 60 min with ethanol (50 mM) in a medium with glucose (10 mmol/L), pyruvate, or monomethyl succinate (2 mmol/L each. An Olympus IX73 fluorescent microscope with a DP-74 digital camera was used to study mitochondrial membrane potential and NADH autofluorescence.

Ethanol did not affect the basal and maximal FCCP-stimulated respiration of hepatocytes, membrane potential, and NADH autofluorescence upon glucose, pyruvate, or monomethyl succinate oxidation. Monomethyl succinate increased basal and FCCP-uncoupled respiration, both in the presence and absence of ethanol. The analysis confirmed the influence of the oxidative substrates on the NADH autofluorescence of hepatocytes. The presence of monomethyl succinate ameliorated the FCCP-induced reduction of NADH autofluorescence of hepatocytes. When mitochondrial complex I was inhibited with rotenone, pyruvate caused a decrease of NADH-autofluorescence of hepatocytes, compared to glucose or monomethyl succinate presence.

Ethanol *in vitro* does not cause hypermetabolic state in mitochondria of isolated hepatocytes irrespectively of pyruvate or monomethyl succinate presence.

Keywords: ethanol, uncoupled respiration, oxidation substrates, hepatocytes

The liver is the main organ that metabolizes alcohol. Due to its small size and hydroxyl group, the ethanol molecule is well soluble in aqueous and lipid environments, respectively, and it penetrates well into the cytoplasm of cells [13]. The main part of alcohol entering the body is metabolized in the liver in three main pathways: by the enzyme alcohol dehydrogenase, through the microsomal P450-ethanol-oxidizing and catalase systems. The process of ethanol oxidation is irreversible and unregulated, so the rate depends only on the concentration and activity of enzymes.

In hepatocytes, ethanol oxidation is catalyzed mainly by two enzymes – alcohol dehydrogenase (ADH) and aldehyde dehydrogenase (ALDH). ADH in the cytoplasm of hepatocytes catalyzes the first stage of ethanol oxidation, which requires NAD^+ to accept reducing equivalents from alcohol. As a result of this reaction, ethanol is oxidized to acetaldehyde, and the vitamin cofactor is reduced to NADH [5].

Next, in the mitochondria, acetaldehyde is oxidized to acetate with the participation of ALDH. In this irreversible reaction, NAD^+ is also reduced. Part of the acetate formed as a result of the acetaldehyde oxidation circulates to peripheral tissues, where it is activated to the key product – acetyl-CoA, which enters the Krebs cycle.

As a result of ethanol oxidation, an additional infrow of reducing equivalents occurs, which leads to a decrease in both cytoplasmic and intramitochondrial NAD^+ and an increase in the ATP / ADP ratio [17]. Since reduced forms of NADH are formed in the process of ethanol oxidation, mitochondrial respiration plays an important role in alcohol metabolism by regenerating NAD^+, which regulates the activity of both enzymes and is necessary for ethanol-acetaldehyde metabolism [4, 20].

Acetaldehyde is a key toxin in alcohol-induced liver injury and induces cellular damage, inflammation, extracellular matrix remodeling and fibrogenesis [7, 11]. In response to the influence of alcohol in the liver, there is an increase in ATP synthesis, an increase in the formation of reactive oxygen species, lipid peroxidation, and inhibition of fatty acid oxidation [1], which leads to the development of steatosis [12]. Adducts of alcohol metabolism can cause disturbances in the formation of the extracellular matrix, leading to the formation of scar tissue in the liver (hepatic fibrosis) [16]. In the case when the rate of ethanol oxidation is lower than the rate of its intake, the accumulation of ethanol leads to liver cell damage [3, 8], and subsequently to the development of alcoholic liver disease [6, 18]. Thus, increasing the rate of ethanol oxidation may ameliorate its toxic effects in liver.

A hypothesis has been suggested that ethanol oxidation rate in the liver is limited by the mitochondrial respiratory chain capacity [4, 20]. Since, alcohol dehydrogenase activity does not change in response to ethanol consumption, hepatocytes adapt metabolism by rapidly increasing the alcohol metabolism, mitochondrial respiration, and uncoupling of mitochondrial oxidative phosphorylation [2, 19].

Administration of pyruvate may accelerate ethanol oxidation by trapping reducing equivalents during conversion of pyruvate to lactate [14]. Recently, we have shown that pyruvate *in vitro* protects pancreatic acinar cells from toxic effects of ethanol and cholecystokinin [9]. Also we used monomethyl succinate as a membrane-permeable form of succinate. Monomethyl succinate easily enters intact hepatocytes, where it is hydrolyzed by intracellular esterases to yield succinate inside the mitochondria, allowing direct stimulation of mitochondrial complex II. Thus, the aim of the study was to investigate the effect of glucose, pyruvate and monomethyl succinate on the oxidative processes of hepatocyte mitochondria under the influence of ethanol *in vitro*.

Materails and methods

Materials. Reagents used in experiments were purchased from Sigma-Aldrich (sodium chloride S7653, glucose G8270, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) H3375, bovine serum albumin (BSA) A6003, sodium pyruvate P2256, mono-methyl-succinate M81101, Ethylene glycol-bis(2-aminoethylether)-N, N, N, N-tetraacetic acid (EGTA), carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP) C2920, collagenase type IV C5138 or Merck Chemicals (Calcium chloride dihydrate, 1725701000). All other reagents were of the purest available grade.

Experimental animals. All manipulations with animals were carried out according to the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes, and the Law of Ukraine “On protection of animals from cruelty”. Experiments were performed on male Wistar rats weighing 220–250 g. The animals were kept in stationary vivarium conditions at a constant temperature and the standard diet. Before the exper-

iment, the animals were starved for 18 hours with free access to water.

Isolation and viability assessment of hepatocytes. Hepatocytes were isolated with a collagenase perfusion method as previously described [10].

After the isolation, hepatocytes were stored at room temperature in the basic extracellular medium containing, mM: NaCl – 140.0, KCl – 4.7, $CaCl_2$ – 1.3, $MgCl_2$ – 1.0, glucose – 5.0; HEPES – 10.0; pH 7.4.

Hepatocytes were counted with haemocytometer. The plasma membrane integrity of hepatocytes was evaluated by staining the cells with a 0.1 % trypan blue solution. The number of intact cells was 85.7±0.92 %.

Oxygen consumption. The rate of oxygen consumption was measured with Clark oxygen electrode at 37 °C using SI929 6-channel Oxygen Meter (Strathkelvin). Rat hepatocytes were incubated for 60 min with ethanol (50 mmol/L) in a medium with glucose (10 mmol/L) and oxidation substrates (pyruvate or monomethyl succinate, 2 mmol/L) or without them. After that, to determine the respiration rate, hepatocytes were introduced into the polarographic chamber and protonophore was added in increasing concentrations – up to 0.25, 0.5 and 1 μM.

Fluorescent studies. An Olympus IX73 fluorescence microscope with a DP-74 digital camera was used to record the mitochondrial membrane potential and observe NADH autofluorescence. Mitochondrial membrane potential was recorded using rhodamine 123 dye (excitation filter 470–490 nm, beam splitter 505 nm, barrier filter 515 nm). NADH fluorescence signal (excitation filter 340–390 nm, beam splitter 410 nm, barrier filter 420 nm) was used to evaluate the effect of alcohol on the mitochondrial respiratory chain. For this purpose, hepatocytes were incubated for 60 min with ethanol (50 mmol/L) in a medium with appropriate oxidation substrates. Next, the hepatocyte suspension was incubated for 5 min at a temperature of 37 °C with FCCP (0.1 or 2 μM) or rotenone (0.5 μM), and then for 10 min with rhodamine 123 (0.1 μM).

Then 5 variants of cell images in the visible and fluorescent light spectrum were randomly selected and the cells were photographed on a fluorescent microscope. Fluorescence intensity was analyzed with ImageJ software, using the green channel to measure rhodamine 123 fluorescence and the blue channel to measure NADH autofluorescence.

Statistical analysis. Statistical analysis was performed using Origin Pro 2018 (Northampton, Mass) software. The significance of difference between the groups was determined with a two-way ANOVA followed by a Turkey corrected post-hoc t-tests in case of a significant difference according to ANOVA.

Results and disscussion

We have studied respiration of isolated hepatocytes as a parameter to assess the effect of ethanol *in vitro* on the oxidative processes of hepatocyte mitochondria. Namely, we evaluated changes in basal respiration and the maximal uncoupled respiration rate under ethanol exposure *in vitro, similarly to our previous work* [10]. To assess the potential role of pyruvate in eliminating NADH excess from ethanol oxidation process, hepatocytes were incubated in a basic extracellular solution supplemented with this substrate. A membrane-permeable ester form of FAD-dependent substrate succinate (monomethyl succinate) substrate was also used in parallel as a negative control

Ethanol did not affect the basal respiration of hepatocytes upon glucose, pyruvate, or monomethyl succinate oxidation. Therefore, it can be assumed that the mitochondrial oxidation of ethanol by hepatocytes under such conditions is insignificant. The lack of influence of ethanol on oxygen consumption rate was confirmed by ANOVA (fig. 1). Also, ethanol did not affect maximal FCCP-stimulated respiration in the presence of glucose, pyruvate, or monomethyl succinate in the medium (fig. 1). However, monomethyl succinate did stimulate both basal and maximal uncoupled respiration rate, as was shown in our previous study [10].

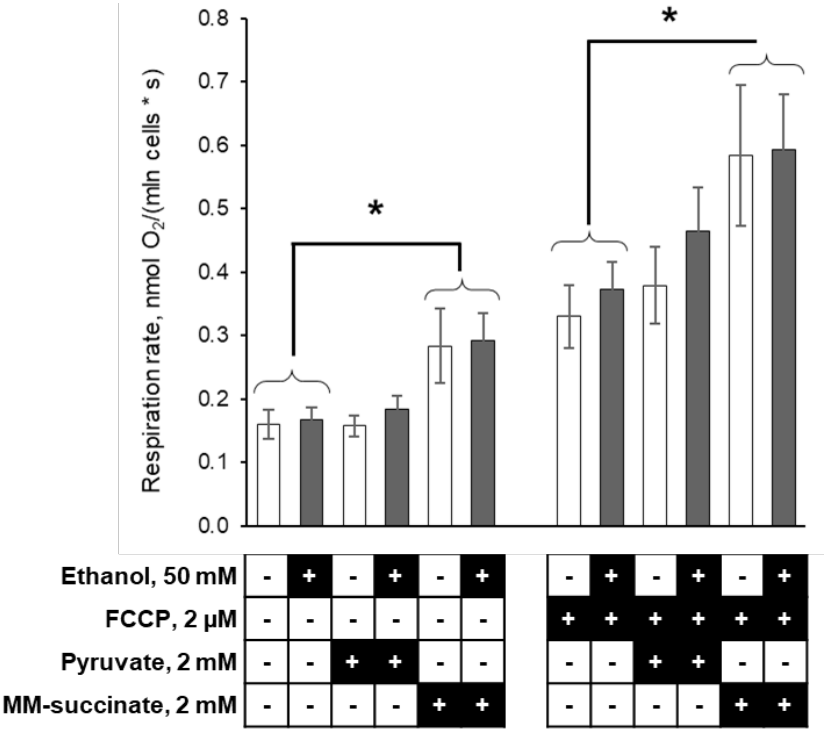

Fig. 1. The effect of ethanol *in vitro* on the hepatocyte respiration rate upon glucose, pyruvate, and monomethyl succinate oxidation: [glucose] = 10 mM; [pyruvate], [monomethyl succinate] = 2 mmol/L; * – statistically significant difference compared with glucose; n=5

Using fluorescence microscopy and rhodamine 123 dye, we investigated the effect of ethanol on the mitochondrial membrane potential of hepatocytes (fig. 2, 3).

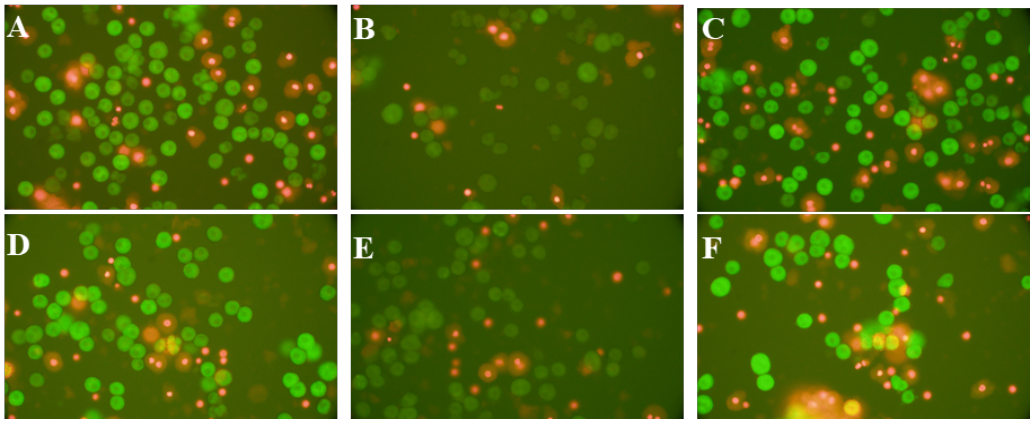

Fig. 2. Photomicrographs of fluorescence of isolated hepatocytes: green – fluorescence of Rhodamine123, orange – fluorescence of propidium iodide; A, D – control; B, E – 2 µM FCCP; C, F – rotenone; D, E, F – ethanol

It was established that ethanol did not affect the membrane potential of hepatocyte mitochondria upon glucose, pyruvate or monomethyl succinate oxidation. ANOVA analysis of vari-

ance showed no effect of ethanol or oxidative substrates on the membrane potential of hepatocyte mitochondria (fig. 3).

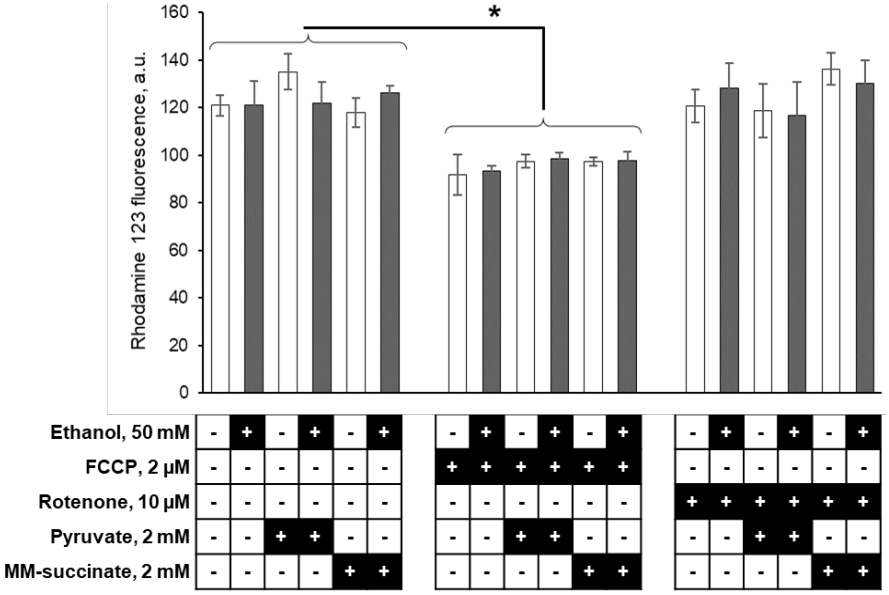

Fig. 3. Effect of ethanol *in vitro* on the membrane potential of hepatocyte mitochondria: for the oxidation of glucose, pyruvate or monomethyl succinate; [glucose] = 10 mM; [pyruvate] and [monomethyl succinate] = 2 mM; * – statistically significant difference compared to the control (without FCCP); n=5

As expected, the protonophore FCCP, but not mitochondrial complex I inhibitor rotenone, caused a decrease of rhodamine 123 fluorescence indicating depolarization of the inner mitochondrial membrane of hepatocytes irrespectively of the oxidative substrate (fig. 3).

In experiments *in vivo*, it was shown that ethanol may both increase and decrease the autofluorescence of NADH in hepatocytes, depending on the membrane potential of mitochondria [20]. We have found that 60 min of incubation with ethanol (50 mM) did not affect NADH level in hepatocytes upon the studied substrates presence (fig. 4, 5).

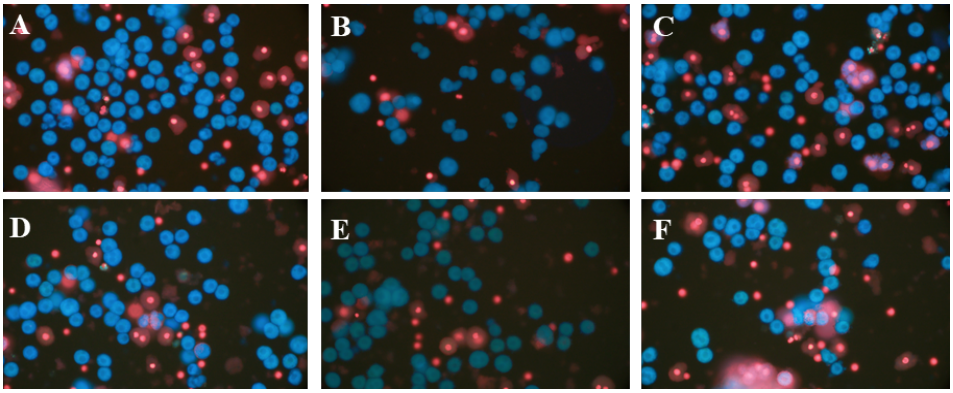

Fig. 4. Photomicrographs isolated hepatocytes with NADH (blue) and propidium iodide (red) fluorescence: A, B, C – control; D, E, F – incubated with ethanol (1h, 50 mM); A, D – without FCCP and rotenone; before measurement 2 μM FCCP (B, E) or 2 μM rotenone (C, F) were added

Surprisingly, in the presence of monomethyl succinate, FCCP (2 μM) caused a significantly smaller effect on NADH autofluorescence of hepatocytes compared to glucose control (fig. 5). Thus, succinate is an additional source of reducing equivalents ($FADH_2$) to compensate for mitochondrial depolarization with FCCP.

On the other hand, under the influence of rotenone, an inhibitor of I-complex of the mitochondrial respiratory chain, regardless of the presence or absence of ethanol in the environment, NADH autofluorescence of hepatocytes paradoxically decreased due to the oxidation of the NADH-dependent substrate pyruvate (fig. 5). This indicates the predominance of the reaction of lactate formation from pyruvate over its oxidation in the mitochondria of hepatocytes under conditions of inhibition of mitochondrial respiration by rotenone.

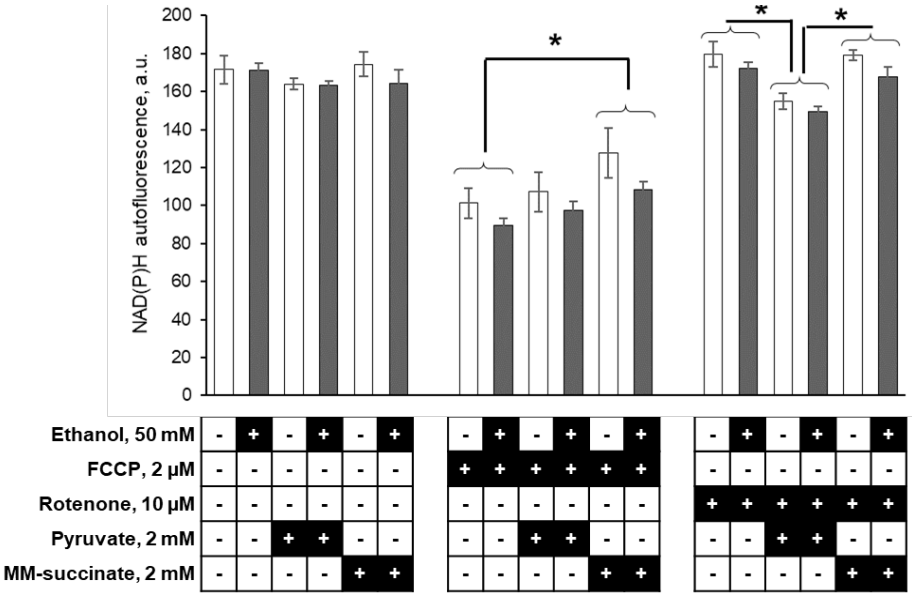

Fig. 5. Effect of ethanol *in vitro* on NADH autofluorescence of hepatocyte mitochondria: upon oxidation of glucose, pyruvate, or monomethyl succinate; [glucose] = 10 mM; [pyruvate] and [monomethyl succinate] = 2 mM; * – statistically significant difference compared to the control (without ethanol); n=5

According to literature sources, alcohol consumption leads to an adaptive increase in ethanol metabolism. This hypermetabolic state is characterized by a rapid increase in alcohol metabolism, an increase in mitochondrial respiration, and uncoupling of oxidative phosphorylation, [15]. However, the above-described effect of alcohol was not confirmed when it affected the already isolated liver [15]. The difference in the effects of ethanol when exposed *in vitro* and *in vivo* was shown by Zhong et al. Acute ethanol consumption caused reversible depolarization of mitochondria *in vivo* for a period of 1 to 24 hours, but ethanol-induced mitochondrial depolarization did not occur *in vitro* in isolated liver cells [20]. This may be explained by the difference in the experimental solution composition from blood (e.g., lack of oxidative substrates other than glucose) or some systemic effects (e.g., mixed substrate availability, Kupffer cell activation, cytokine release, hepatic blood flow) of alcohol *in vivo*. In our experiment, we added pyruvate and monomethyl succinate to solution, but still did not detect any effects of ethanol on respiration, mitochondrial membrane potential and NADH level in isolated hepatocytes. Therefore, the results of our study are not sufficient to confirm or reject the hypothesis that pyruvate may accelerate ethanol oxidation.

Conclusion

Ethanol *in vitro* does not cause hypermetabolic state in mitochondria of isolated hepatocytes irrespectively of pyruvate or monomethyl succinate presence. Namely, ethanol did not affect the basal and maximal FCCP-stimulated respiration of hepatocytes, membrane potential, and NADH autofluorescence upon the oxidation of the studied substrates. However, the oxidative substrates affected the NADH autofluorescence of hepatocytes. Monomethyl succinate increased basal and FCCP-uncoupled respiration in the presence and absence of ethanol.

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Стаття надійшла до редакції 08.04.25

доопрацьована 03.06.25

прийнята до друку 09.06.25

ЕТАНОЛ *IN VITRO* НЕ ВПЛИВАЄ НА ОКИСНІ ПРОЦЕСИ МІТОХОНДРІЙ ГЕПАТОЦИТІВ ЗА ОКИСНЕННЯ ГЛЮКОЗИ, ПІРУВАТУ ЧИ МОНОМЕТИЛ-СУКЦИНАТУ

Г. Мазур, О. Руда, Б. Манько, В. Манько

Львівський національний університет імені Івана Франка
вул. Грушевського, 4, Львів 79005, Україна
e-mail: halyna.mazur@lnu.edu.ua

Основна частина алкоголю, що потрапляє в організм, метаболізується у печінці. Якщо швидкість окиснення етанолу нижча за швидкість його надходження, то накопичення етанолу призводить до пошкодження клітин печінки. Оскільки у процесі окиснення етанолу утворюються відновлені форми НАДН, то мітохондріальне дихання відіграє важливу роль в метаболізмі алкоголю шляхом регенерації $НАД^+$, які регулюють рівень обох ферментів окиснення етанолу та є необхідними для етанол-ацетальдегід метаболізму. Метою нашого дослідження було вивчити вплив глюкози, пірувату і монометил-сукцинату на окисні процеси мітохондрій гепатоцитів за дії етанолу *in vitro*.

Дослідження проводили на щурах-самцях лінії Вістар масою 220–250 г. Ізолювання гепатоцитів здійснювали двостадійним методом Сеґлена. Швидкість споживання кисню визначали за допомогою 6-канального вимірювача кисню SI929 (Strathkelvin). Гепатоцити інкубували протягом 60 хв з етанолом (50 мМ) у середовищі з глюкозою (10 ммоль/л) та відповідним субстратом окиснення (піруватом чи монометил-сукцинатом (по 2 ммоль/л). Після внесення гепатоцитів у полярографічну комірку та реєстрації базального дихання додавали протонофор у наростаючих концентраціях, до 0,25, 0,5 та 1 мкмоль/л. Для реєстрації мембранного потенціалу мітохондрій і спостереження НАДН-автофлуоресценції використовували флуоресцентний мікроскоп Olympus IX73 із цифровою камерою DP-74. Вірогідність змін визначали, використовуючи ANOVA.

Етанол не впливав на базальне та максимальне FCCP-стимульоване дихання гепатоцитів, на мембранний потенціал і НАДН-автофлуоресценцію за окиснення глюкози, пірувату чи монометил-сукцинату. Монометил-сукцинат підвищував базальне та FCCP-роз'єднане дихання як за наявності, так і за відсутності етанолу. Аналіз підтвердив вплив субстрату окиснення на НАДН-автофлуоресценцію гепатоцитів. За окиснення монометил-сукцинату НАДН-автофлуоресценція гепатоцитів зростала за

впливу FCCP у концентрації 2 мкмоль/л порівняно до окиснення глюкози. За впливу ротенону і окиснення піруват НАДН-автофлуоресценція гепатоцитів знижувалася, порівняно із дослідом з окисненням глюкози чи монометил-сукцинату.

Етанол не впливав на швидкість дихання, мембранний потенціал і НАДН-автофлуоресценцію мітохондрій гепатоцитів. Монометил-сукцинат підвищував базальне та FCCP-роз'єднане дихання незалежно від наявності у середовищі етанолу.

Ключові слова: етанол, максимальна швидкість FCCP-роз'єднаного дихання, субстрати окиснення, гепатоцити, FCCP