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EFFECT OF LONG-TERM ETHANOL CONSUMPTION AND A HIGH-FAT DIET ON MITOCHONDRIAL RESPIRATION IN RAT PANCREATIC ACINAR CELLS AND HEPATOCYTES

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Chronic alcohol consumption may cause pancreatitis and alcohol-related liver diseases. Both adaptation and damage of liver mitochondria in animals on chronic ethanol and high-fat diets were demonstrated. It is currently not clear if ethanol or its metabolites such as fatty acid ethyl esters can cause mitochondrial damage to the pancreas. The present study aimed to evaluate the effect of chronic ethanol administration in combination with a high-fat diet on mitochondrial respiration in both pancreatic acinar cells and hepatocytes of rats. Wistar male rats on a high-fat diet (35% calories) were administered ethanol (6 g/kg body weight) by oral gavage for 14 days. Pancreatic acini cells and hepatocytes were isolated with collagenase digestion. The respiration of isolated cells was studied with a Clark electrode. Ethanol administration to rats kept on a high-fat diet was followed by a rapid loss of animal weight during the first 5 days of the experiment and diminished secretory response of pancreatic acini to acetylcholine, however, no changes in acinar cells ultrastructure, basal, oligomycin-insensitive or FCCP-uncoupled respiration were found. Meanwhile ethanol caused a significant (~40%) increase in basal and maximal FCCP-uncoupled respiration rate of isolated hepatocytes. In conclusion, chronic ethanol administration to rats on a high-fat diet does not cause mitochondrial damage in the pancreas, while mitochondria of the liver adapt to ethanol by increasing respiration rate.

K e y w o r d s: ethanol, pancreatic acinar cells, hepatocytes, mitochondrial respiration, high-fat diet.

Chronic alcohol consumption is the main cause of pancreatitis and alcohol-related liver disease in developed countries [1, 2]. Only a few people (less than 5%) with alcohol use disorder develop pancreatitis [3]. In animal models, a classic Lieber-DeCarli ethanol diet causes liver disease [4] but not pancreatitis [5-7]. It is thus believed that other factors in combination with alcohol ultimately trigger acute pancreatitis. Chronic ethanol feeding makes the pancreas more susceptible to cerulein-induced pancreatitis [6, 7]. Similarly, acute exposure to ethanol in combination with cholecystokinin *in vitro* or *in vivo* may cause pancreatic acinar cell death or blebbing [8]. Despite the low clinical relevance of hyperstimulation with cholecystokinin or its analogues, these experiments prove that ethanol exposure causes the accumulation of defects of some kind in pancreatic acinar cells.

Fatty acids seem to be another factor contributing to acute pancreatitis development. In patients with autosomal recessive lipoprotein lipase deficiency, recurring pancreatitis is observed [9]. Products of non-oxidative ethanol metabolism, fatty acid ethyl esters (FAEEs) increase in pancreatic tissue compared to other organs (such as a heart or lungs) of people who died after acute alcohol intoxication [10]. Studies on isolated pancreatic acinar cells and in animal models confirmed the capacity of FAEEs to induce acute pancreatitis [11-14]. The proposed mechanism of *in vitro* FAEEs toxicity involves sustained cytosolic Ca^{2+} elevation, mitochondrial Ca^{2+}-overload, depolarization and decrease in the NADH

level and ATP synthesis leading to cell death [13-15]. Mitochondrial damage is also observed in pancreatic tissues of patients with pancreatitis [16]. However, it is still not clear, if ethanol via naturally occurring FAEEs synthesis or by any other mechanism causes mitochondrial damage to the pancreas *in vivo.*

In the liver, mitochondrial respiration plays an important role in ethanol metabolism by regenerating the NAD^+ cofactor for alcohol dehydrogenase and aldehyde dehydrogenase. In mice, mitochondria of the liver adapt to chronic ethanol feeding by increasing the expression of mitochondrial respiratory chain proteins and respiration rate [17]. It is not currently known, if the mitochondria of pancreatic acinar cells adapt to chronic alcohol consumption. Thus, the present study aimed to evaluate the effects of chronic ethanol administration in combination with a high-fat diet on mitochondrial respiration of pancreatic acinar cells and hepatocytes of rats.

Materials and Methods

Reagents used in these experiments were purchased from Sigma-Aldrich (sodium chloride, glucose, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (HEPES) H3375, soybean trypsin inhibitor, bovine serum albumin (BSA), sodium pyruvate, glutamine, monomethyl-succinate, D-methyl-2-oxoglutarate, carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP), oligomycin A, collagenase type IV) or Merck Chemicals (Calcium chloride dihydrate). All other reagents were of the purest available grade.

The experiments followed the recommendations of the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 1986), and the EU Directive 2010/63/EU for animal experiments and laws of Ukraine. Experimental protocols were approved by the Animal Care and Use Committee of Ivan Franko National University of Lviv.

Experiments were carried out on Wistar white male rats (250-300 g). All animals were kept under the standard conditions of a vivarium at room temperature (18-20°C) on a standard diet. During the 14-day experiment, all animals were fed a special high-fat diet with ~35% of the total calories obtained from animal fat (lard). Rats in the “alcohol” group were also administered ~4 ml of water (control) or 40% ethanol solution (6 g/kg of body weight) with an oral gavage every evening (6-7 p.m.) for 14 days. Animals were sacrificed on day 15 in the morning after 12 h of fasting.

A suspension of isolated pancreatic acini was obtained following collagenase digestion (Sigma, type IV, 0.2 mg/ml) according to the previously reported method [18]. Acini were obtained and stored in extracellular solution containing (mM): 140.0 NaCl, 4.7 KCl, 1.3 $CaCl_2$, 1.0 $MgCl_2$, 10.0 HEPES, 10.0 glucose, 2.0 glutamine, 2.0 sodium pyruvate, 0.01% (wt/vol) soybean trypsin inhibitor, 0.25% (wt/vol) BSA and MEM amino acid supplement; pH 7.4 (NaOH). Hepatocytes were isolated with collagenase digestion (Sigma, type IV, 108 units/ml) using an *in vitro* liver perfusion method, as previously reported [19]. Hepatocytes were stored in extracellular solution containing (mM) at 20 °C: NaCl – 140.0, KCl – 4.7, $CaCl_2$ – 1.3, $MgCl_2$ – 1.0, glucose – 5.0, HEPES – 10.0; pH 7.4 (NaOH). Cell viability was evaluated with the trypan blue test (0.1%). Cell counting was performed using a hemocytometer.

Oxygen consumption was measured with a Clark oxygen electrode (Biological oxygen monitor YSI 5300, Yellow Springs Instrument, USA) in the closed 1.6-ml glass respiration chamber at 37°C. Before respiration measurement, suspension of pancreatic acini or hepatocytes was pre-incubated (15 min, 37°C) in respective solutions with glucose (10 mM) or with oxidative substrates (2 mM pyruvate, 2 mM glutamine or 2 mM monomethyl-succinate). Maximal uncoupled respiration was studied with protonophore FCCP added step-wise to reach the final concentrations 0.5, 1, 1.5, and 2 μM as previously described [20]. Oligomycin (5 μM) was added to inhibit ATP-synthase.

Acetylcholine (ACh)-stimulated amylase release from pancreatic acini was studied *in vitro.* Suspension of pancreatic acini was pre-incubated (30 or 60 min, 37°C). Pancreatic acini were stimulated by 0.1, 1.0 or 10 μM ACh for 30 min at 37°C. Acini were sedimented with centrifugation and supernatant was collected for amylase assay. A separate aliquot of acini in suspension was homogenized in lysis solution, containing 0.1% Triton X-100 in addition to other components described earlier. Total amylase activity was determined in the lysate. Amylase activity was studied with turbidimetric and kinetic assay using a DENOVIX spectrophotometer DS-11 FX+ in the 1 cm cuvette at a wavelength of 300 nm at 37°C.

The ultrastructure of pancreatic acinar cells was studied by electron microscopy. Suspension of isolated pancreatic acini was processed by a 1.5% solution of OsO_4 in a 0.2 M solution of sodium cacodylate (pH 7.2) for 2-2.5 h. The samples were gradually placed for 30 min at room temperature in

ethanol solution (50, 70, 90°, and absolute alcohol), and then coated with an epoxy resin. The slices were contrasted in a 1.5% solution of uranyl acetate and by Reynolds and studied with transmission electron microscope PEM-100.

Statistical analysis of the data was performed using the software package Microsoft Excel and Origin Pro 2018. The statistical significance of the difference between groups was determined with ANOVA followed by Holm-Bonferroni-corrected post-hoc t-tests.

Results and Discussion

30 animals were split equally and randomly between two experimental groups. Three rats from the ethanol group died during the experiment: one animal on the 4th day of the study, and two others – on the 7th day. Additional animals had to be studied to compensate for the premature death due to ethanol intoxication. The condition and weight of the animals were monitored daily during 14 days of the experiment. It was found that ethanol administration caused rapid loss of animal weight during the first 5 days of the experiment (Fig. 1, *A*). This is consistent with the results of earlier study [17]. Control animals (on a high-fat diet only) slowly gained more weight during the first 7 days; afterwards, the significant weight difference between the two groups was more or less stable (Fig. 1, *A*). Overall, during the experiment (14 days), the weight of control animals increased by 7.28%, while in alcohol-fed animals it decreased by 9.15 %. Despite these evidences of

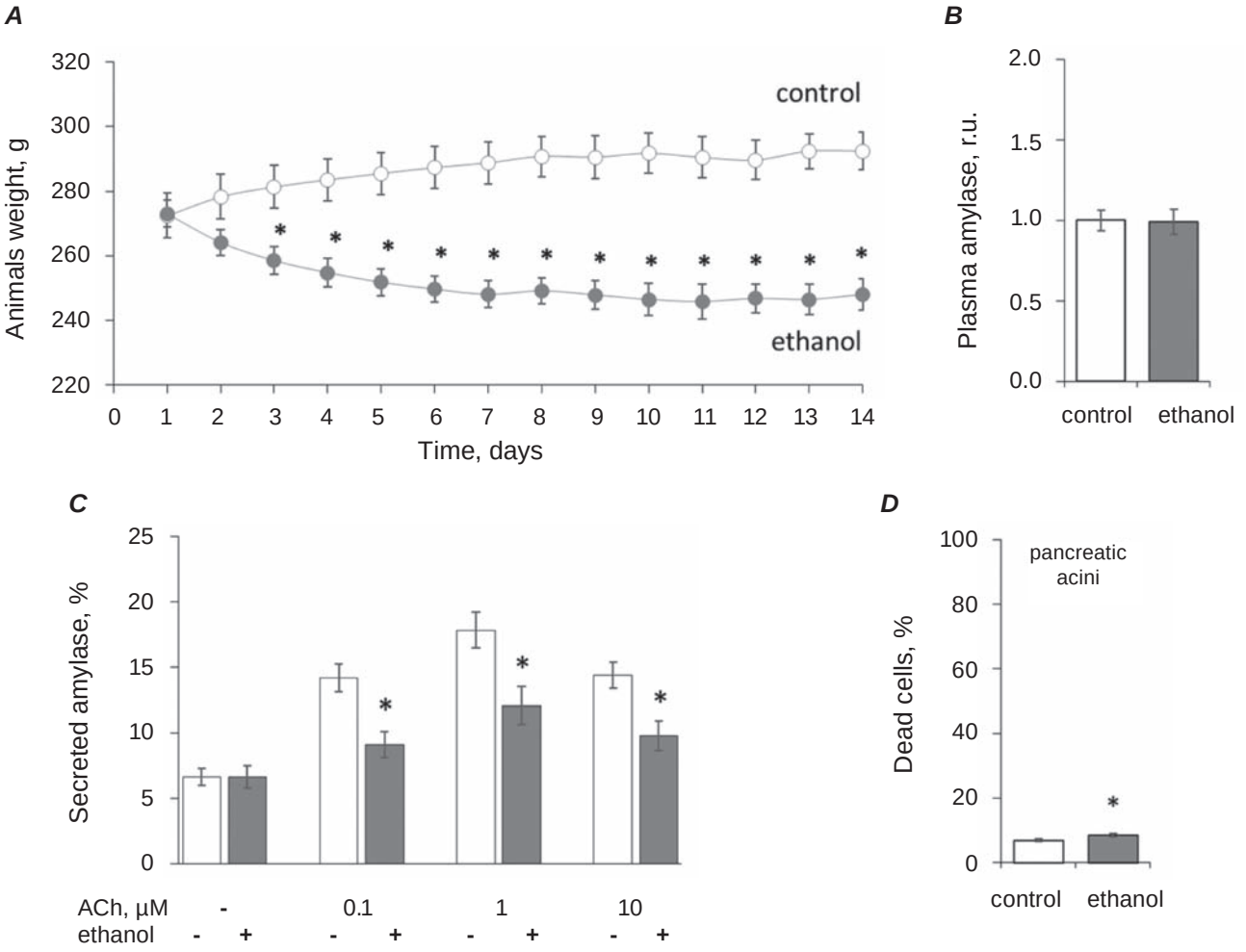

*Fig. 1. Effects of 14-day ethanol administration to rats on body weight (**A**), plasma amylase activity on day 15 (**B**), amylase secretion by isolated pancreatic acini (**C**) and viability of isolated pancreatic acini (D) and liver cells (**E**); ACh – acetylcholine, *statistically significant difference compared to control with $P < 0.05$, mean ± SEM, n = 12–15 or 10–12 for isolated pancreatic acini and liver cells, respectively*

ethanol toxicity, we did not observe any changes in plasma amylase activity on day 15 of the experiment, indicating the lack of pancreatic damage (Fig. 1, *B*).

The viability of pancreatic acini and liver cells, isolated on the 15th day of the experiment, was evaluated with the trypan blue test. A small but significant increase in the number of trypan-positive dead pancreatic acinar cells was found in the ethanol-fed animal group (8.6 ± 0.6 %) compared to control (7.1 ± 0.4 %, Fig. 1, *D*). Ethanol did not affect liver cell viability (Fig. 1, *E*).

The functional activity of isolated pancreatic acini was assessed by amylase release assay. Secretion was stimulated by ACh (0.1, 1 and 10 μM, Fig. 1, *C*) for 30 min. Basal unstimulated amylase release was low (~6 % of total amylase content) and did not change in the ethanol animal group (Fig. 1, *C*). In control animals, acini displayed a typical secretory response to ACh with peak amylase release at 1 μM ACh (17.80 ± 1.38% of total amylase). In acini isolated from ethanol-fed rats, the secretory response to ACh stimulation was significantly lower for each ACh concentration, with a peak of 12.10 ± 1.45% at 1 μM ACh (Fig. 1, *C*). Similar results were obtained by Bragado et al. [21] in a longer 6-month study.

In addition, we have assessed the morphological state of isolated pancreatic acini by electron microscopy. We did not observe any changes in mitochondrial or endoplasmic reticulum ultrastructure in pancreatic acinar cells in the alcohol group (Fig. 2).

We studied mitochondrial functional capacity upon various substrates availability: 1) isolated pancreatic acini or hepatocytes were pre-incubated in solution with glucose to support glycolytic oxidative pathways; 2) NAD-dependent mitochondrial oxidative substrates were present (for pancreatic acini cells, we used a combination of glucose with glutamine and pyruvate, and for liver cells – pyruvate, which was shown earlier to enhance the maximal oxidative capacity of these cells [20, 22]); 3) monomethyl-succinate, a membrane-permeable methyl ester of FAD-dependent succinate was in the medium. As expected, the presence of NAD-dependent substrates caused a substantial increase in maximal uncoupled respiration of liver and pancreatic cells (Fig. 3).

The response of respiration of pancreatic acinar cells and hepatocytes to protonophore FCCP was typical for these cells. Chronic administration of alcohol did not affect basal or uncoupled respiration of isolated pancreatic acini, irrespectively of the oxidative substrate (Fig. 3, *A*). In addition, oligomycin-insensitive respiration of pancreatic acini was unaffected by ethanol feeding, indicating normal coupling between respiration and oxidative phosphorylation.

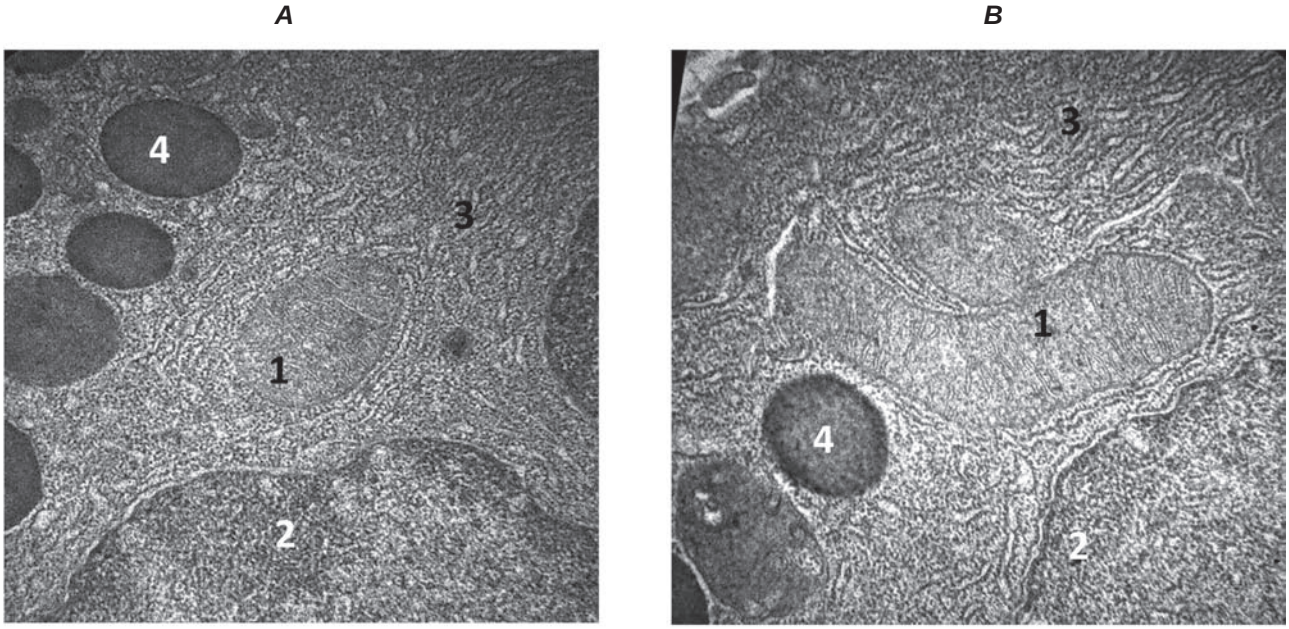

*Fig. 2. Electron micrographs of pancreatic acinar cells in control (**A**) and alcohol group (**B**) display normal ultrastructure of mitochondria (1), nucleus (2), endoplasmic reticulum (3) and secretory vesicles (4); magnification ×10 000*

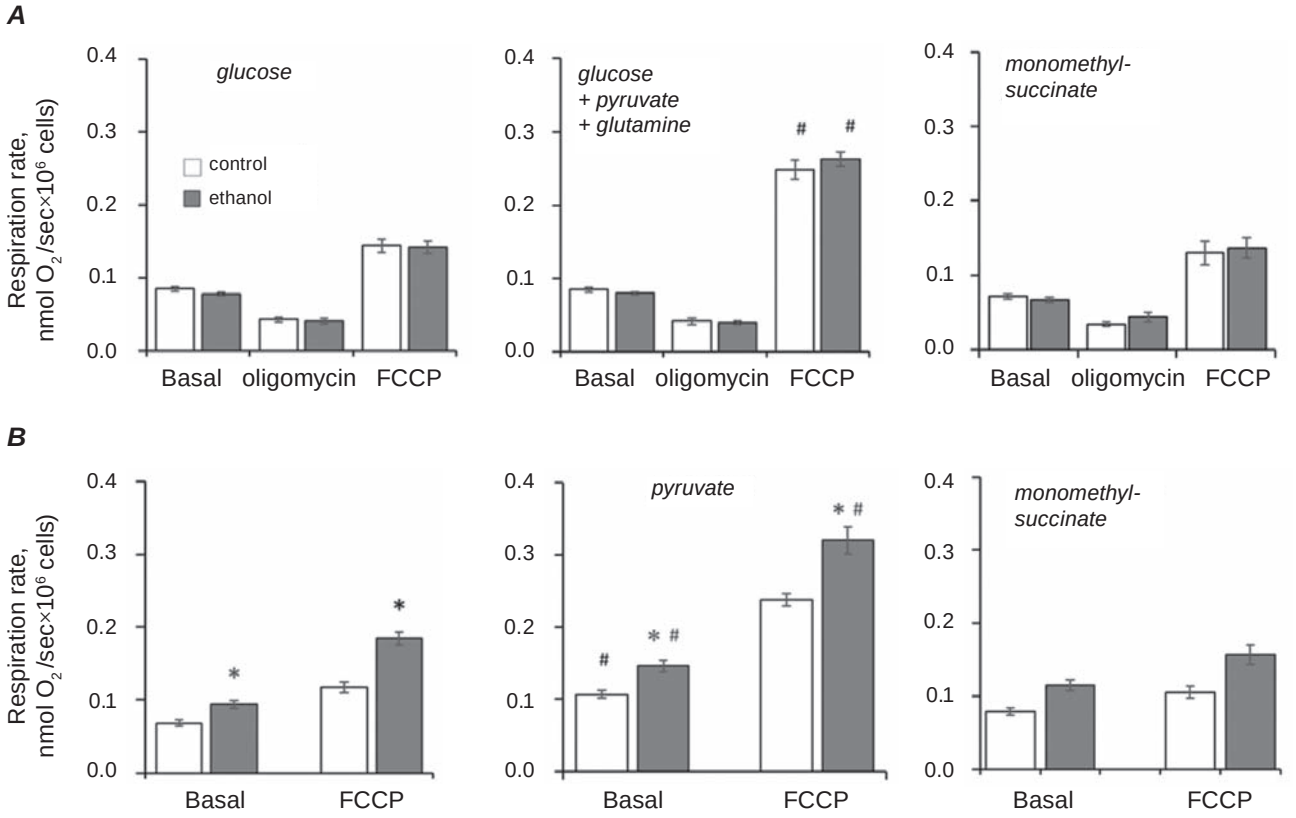

*Fig. 3. The effects of in vivo ethanol administration (14 days) to rats on respiration of pancreatic acini (**A**) and liver cells (**B**): [glucose] = 10 mM, [pyruvate] = 2 mM, [glutamine] = 2 mM, [monomethyl-succinate] = 2 mM, [FCCP] = 0.5–2 μM, [oligomycin] = 5 μM; *statistically significant difference compared to control with $P < 0.05$, #difference significant compared to glucose, $P < 0.05$; mean ± SEM, $n = 13–14$ and $n = 10–11$ for isolated pancreatic acini and liver cells, respectively*

In contrast, alcohol feeding caused a significant increase in the respiration of liver cells (Fig. 3, *B*). Notably, basal and maximal uncoupled respiration rates increased proportionally irrespectively of oxidative substrates: by 37 and 57% in case of glucose oxidation; 37 and 35% when pyruvate was present or 45 and 48% in case of monomethyl-succinate oxidation.

A similar adaptation of liver mitochondria to 2–4 weeks of ethanol feeding was demonstrated in mice [17]. However, a 6-week study of ethanol feeding to rats found a decrease in State 3 of isolated liver mitochondria respiration, and no increase of respiratory chain proteins [23]. The different results of our study may be explained by its shorter duration and/or higher ethanol dose. In addition, the number of mitochondria per cell, which increased in mice [17], was not studied in rats [23]. Thus, considering the uniform increase of hepatocyte respiration in our study, we assume that 2-week ethanol feeding to rats causes an increase of a number of mitochondria in the liver. While the damage to mitochondria was found in patients with a history of long-term alcohol abuse [24], evidence for short-term mitochondrial respiration enhancement in humans is still lacking.

To our knowledge, this is the first study of chronic (two-week) ethanol administration to rats on pancreatic mitochondrial respiration. We have demonstrated that ethanol does not cause mitochondrial damage in the pancreas. The important aspect of this study was that rats were on a high-fat diet to model the natural synthesis of FAEEs, which are detrimental to mitochondria *in vitro* [14]. We have recently found that a 7-week high-fat diet (without ethanol) causes only a minor decrease in basal but not maximal respiration of pancreatic acinar cells [25]. While we did not evaluate lipid metabolism in rats on a high-fat diet in present study, a 7-week high-fat diet did not cause significant changes in lipid plasma profile [25]. Therefore, further studies

are required to evaluate if FAEEs are indeed accumulated in the pancreas under such experimental conditions. The dose of ethanol in the present study was high enough to cause ~20% lethality in addition to a substantial decrease in body weight. While no evidence of pancreatitis was detected, a decreased secretory function of the pancreas was found.

Conclusion. The data of this study do not support the hypotheses that chronic ethanol consumption causes accumulation of mitochondrial damage in the pancreas or that naturally occurring ethanol metabolites, such as FAEEs, negatively affect mitochondrial respiration in pancreatic acinar cells *in vivo.*

Conflict of interest. The authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

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ВПЛИВ ДОВГОТРИВАЛОГО СПОЖИВАННЯ ЕТАНОЛУ ТА ДІЄТИ З ВИСОКИМ ВМІСТОМ ЖИРІВ НА МІТОХОНДРІАЛЬНЕ ДИХАННЯ В АЦИНАРНИХ КЛІТИНАХ ПІДШЛУНКОВОЇ ЗАЛОЗИ І ГЕПАТОЦИТАХ ЩУРІВ

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Хронічне вживання алкоголю може спричинити панкреатит і захворювання печінки. У тварин, що перебували на високожировій дієті у поєднанні з алкоголем, було показано як адаптацію так і пошкодження мітохондрій печінки. На сьогодні не відомо, чи здатний алкоголь або його метаболіти викликати порушення функцій мітохондрій у підшлунковій залозі *in vivo.* Це дослідження мало на меті оцінити вплив тривалого введення алкоголю в поєднанні з високожировою дієтою на мітохондріальне дихання ацинарних клітин підшлункової залози та гепатоцитів щурів. Щурі лінії Wistar перебували на високожировій дієті (35% калорій), яким перорально вводили алкоголь (6 г/кг маси тварини) протягом 14 днів. Дихання панкреатичних ацинусів та ізольованих гепатоцитів досліджували з використанням електроду Кларка. Введення алкоголю щурам спричиняло швидку втрату ваги протягом перших 5 днів експерименту. Панкреатичні ацинуси цих тварин мали знижену здатність до секреції амілази за стимуляції ацетилхоліном. Однак у групі тварин, які отримували алкоголь, не було виявлено змін ультраструктури ацинарних клітин підшлункової залози. Рівень амілази у плазмі крові не відрізнявся між групами тварин. Алкоголь спричинив значне (~40%) збільшення базальної та максимальної FCCP-стимульованої швидкості дихання ізольованих гепатоцитів. Введення алкоголю не вплинуло на базальне, олігоміцин-нечутливе або FCCP-стимульоване дихання у ізольованих панкреатичних ацинусах. Отже, хронічне введення алкоголю щурам, що перебували на високожировій дієті, не спричинило пошкодження мітохондрій підшлункової залози щурів, тоді як мітохондрії печінки адаптуються до алкоголю шляхом підвищення швидкості дихання.

Ключові слова: етанол, ацинарні клітини підшлункової залози, гепатоцити, мітохондріальне дихання, високожирова дієта.

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