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Lipid and phospholipid composition of the inner mitochondrial membrane of hepatocytes under conditions of glutamate-induced steatohepatosis and correction with nanocrystalline cerium dioxide

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The problem of studying the mechanisms of steatohepatosis development and its correction remains relevant, despite the significant number of scientific studies on the pathogenesis of metabolic-associated fatty liver disease. This is confirmed by the increase in morbidity, which may be associated with a wide range of factors that cause liver steatosis and the lack of effective therapeutic and preventive agents. The study aims to establish the lipid and phospholipid composition of the inner mitochondrial membrane of rat hepatocytes under conditions of diet- and glutamate-induced steatohepatosis and to evaluate the corrective effect of nanocrystalline cerium dioxide on the formation of steatohepatosis induced by neonatal administration of monosodium glutamate. The direction of the experiments included the study of the mechanisms of development of diet- and glutamate-induced visceral obesity in 4-month-old rats and the determination of the lipid and phospholipid composition of the inner membrane of hepatocyte mitochondria in rats under conditions of high-calorie diet and after neonatal administration of monosodium glutamate against the background of periodic administration of nanocrystalline cerium dioxide. It was established that rats that were on a high-calorie diet with a high content of fats, carbohydrates, and a reduced content of proteins for 4 months, and rats that were administered monosodium glutamate in the neonatal period, developed visceral obesity without the manifestation of hyperphagia, characterized by dyslipidemia and the development of steatohepatosis. A feature of the development of diet- and glutamate-induced steatohepatosis is mitochondrial dysfunction, which is characterized by changes in the lipid and phospholipid composition of the inner membrane of hepatocyte mitochondria. Not only structural changes occur in the membrane, but also dysfunctional changes in the mitochondria as a whole, manifesting themselves in the fact that ROS are generated in the respiratory chain instead of ATP, and this causes the development of oxidative stress in both the mitochondria and the entire hepatocyte. Periodic administration of nanocrystalline cerium dioxide to rats with glutamate-induced steatohepatosis significantly restored the lipid and phospholipid composition of the inner membrane of hepatocyte mitochondria, reduced manifestations of oxidative stress, and reduced the content of oxidized forms of phosphatidylcholine and phosphatidylethanolamine in the inner membrane of hepatocyte mitochondria against the background of normalization of cardiolipin content, which indicates the antioxidant effect of this drug and the possibility of its use for the prevention of steatohepatosis.

Keywords: sodium glutamate; obesity; hepatic steatosis; nanocrystalline cerium dioxide.

Introduction

Mitochondria play an important role in hepatocyte metabolism, being the main site of fatty acid oxidation and the process of oxidative phosphorylation. Most studies indicate the development of structural and functional damage to hepatocyte mitochondria, which is associated with both an increase and a decrease in β-oxidation activity. It has been established that with an increase in the activity of β-oxidation enzymes, an increase in ROS formation is observed, and with a decrease in activity, diacylglycerol accumulation occurs and simultaneous activation of the protein kinase C pathway with inhibition of the insulin signal. Some studies show that an increase in β-oxidation activity can serve as an adaptive mechanism to limit the lipotoxicity of free fatty acids. It has been established that as a result of such activation of lipid oxidation enzymes, a large number of reduced NADH equivalents are formed, regardless of the energy needs of the cell. Whether this is followed by further inhibition of the lipid oxidation cycle is unknown, but other studies of the pathogenesis of steatohepatosis have shown, on the contrary, inhibition of β-oxidation, in which the development of oxidative stress with a large number of ROS was noted (Pessayre, 2007; Wei et al., 2008; Vial et al., 2011).

The development of oxidative stress leads to impaired mitochondrial functioning (Gutyj et al., 2022) and the development of other complications, among which hyperglycemic complications and type 2 diabetes play a leading role (Verveha et al., 2023; Kondro et al., 2024). It is the increase in the production of reactive oxygen species (ROS) that is observed in hyperglycemia, as a result of which an imbalance in redox reactions occurs. Since mitochondria are one of the sources of cellular ROS, they become both the main target of oxidative damage, which can be caused by a decrease in the level of antioxidants, such as glutathione, and a disruption of the antioxidant systems. ROS-mediated damage to mitochondria, which is observed in various pathological conditions, causes a change in the shape of the organelle and a decrease in their functional activity. Increased ROS production in the mitochondrial electron transport chain (ETC) leads to an increase in the formation of the toxic radical peroxynitrite in the presence of nitric oxide. Peroxynitrite is able to covalently modify various proteins by nitration of tyrosine residues and S-nitrosylation of cysteine residues of amino acids. An increase in the ROS/AFN (active forms of nitrogen) ratio in pathological conditions inhibits the activity of various antioxidant enzymes, including glutathione peroxidase, glutathione reductase, superoxide dismutase, and catalase

(Bonomini et al., 2015). In addition to the functional component of mitochondria, namely its proteins, the development of oxidative stress also negatively affects its structural state, namely the lipid composition of the membrane. Lipids in the composition of mitochondrial membranes are involved in various processes, such as protein biogenesis, participation in energy production, membrane fusion, and apoptosis. Regarding energy production, membrane lipids can modulate mitochondrial respiration. Under physiological conditions, the phospholipid composition of mitochondria is formed due to the expression and activity of proteins involved in lipid synthesis and their interconversion. Phosphatidylcholine, phosphatidylethanolamine are the main phospholipids of both mitochondrial membranes, which are present in all cell types. It is phosphatidylethanolamine and cardiolipin that are mainly enriched in the inner membrane of mitochondria of all mammalian and plant cells. Phosphatidylinositol is present mainly in the outer mitochondrial membrane, and the amount of sterols varies depending on the specialization of the cells (Osman et al., 2011; Horvath & Daum, 2013).

ROS interact with the phospholipid components of the membrane, especially concerning long-chain polyunsaturated fatty acids, which are essential components of mitochondrial phospholipids. As a result of these reactions, various peroxides are formed, which change the spatial organization of phospholipids in the membrane. The appearance of these peroxides changes the organization of the bilayer in such a way that the fluidity and permeability of the membrane change, and there is also an inactivation of ETL components, including cytochrome c oxidase, by forming adducts with this enzyme. Collectively, these changes are capable of inactivating mitochondrial ETL complexes and oxidative phosphorylation processes (Pessayre, 2007; Lidofsky, 2008; Roberts et al., 2013).

Certain pathological conditions can contribute to changes in the lipid composition of mitochondria, leading to changes in their functioning. The main lipids affected by pathological factors are cardiolipins and cholesterol. Cardiolipins are unique phospholipids that are localized exclusively in the inner mitochondrial membrane and constitute approximately 15–20% of the total pool of phospholipids of the inner membranes. They play a central role in mitochondrial metabolism, in a number of bioenergetic processes, and in the stability and dynamics of mitochondrial membranes. Therefore, significant changes in the content of cardiolipins can change the fluidity of membranes, the organization and functioning of respiratory chain complexes, which may be associated with increased production of ROS by mitochondria (Rolo et al., 2012).

The ability of nanocrystalline cerium dioxide (NCD) to perform enzyme functions, regenerative, antioxidant, and antiradical properties opens up prospects for the prevention and treatment of pathological processes associated primarily with oxidative stress and inflammation (Caputo et al., 2017; Moridi et al., 2018; Louro et al., 2019; Kobyliak et al., 2019). Therefore, the use of nanocrystalline cerium dioxide as a prebiotic agent will allow it to be considered as a potential and medicinal drug for steatohepatitis.

The aim of the study was to determine the total content of lipids, cholesterol, and its esters and the phospholipid composition of the inner mitochondrial membrane of rat hepatocytes under conditions of diet- and glutamate-induced steatohepatosis and to evaluate the corrective effect of nanocrystalline cerium dioxide on the formation of steatohepatosis induced by neonatal administration of monosodium glutamate.

Materials and methods

The experiments were conducted on 70 white non-linear male rats, which were kept in the vivariums of the Danylo Halytskyi Lviv National Medical University and the Taras Shevchenko National University of Kyiv in compliance with the rules of the Council of Europe Convention for the Protection of Vertebrate Animals Used for Research and Other Scientific Purposes (Strasbourg, 1986) and approved by the First National Congress on Bioethics of Ukraine (Kyiv, 2001). The Bioethics Commission of the Danylo Halytskyi Lviv National Medical University (protocol No. 5 dated 06/22/2020) and the Educational and Scientific Center "Institute of Biology and Medicine" of the Taras Shevchenko National University of Kyiv (protocol No. 1 dated 02/04/2019) did not identify any moral and ethical violations in conducting experimental research. The animals were maintained on standard food "Purina rodent chow" (fat – 20.6%, protein – 32.4%, carbohydrates – 47%) and water ad libitum.

The first model consisted of using a high-calorie diet (HCD) (diet C 11024, Research Diets, New Brunswick, NJ (West, 1992)), consisting of standard feed "Purina rodent chow" (47%) and sweetened condensed milk (44%), vegetable oil (8%), vegetable starch (1%) (fat – 29.6%, protein – 14.8%, carbohydrates – 55.6%). For these studies, 3-month-old rats with an initial weight of no more than 200 g were selected, and they were randomly divided into two groups. The first group continued to stay on standard feed and served as the control (control group), and the second was transferred to a high-calorie diet (experimental group). The experiment lasted 16 weeks. The general condition of the animals, food, and water consumption were monitored daily, and the weight was determined once a week. Every 3 weeks, 10 animals were selected from each group.

The second obesity model consisted of neonatal administration of 4 mg/g monosodium glutamate dissolved in 8 μl/g water for injecting rats (Savcheniuk et al., 2014). Monosodium glutamate was administered subcutaneously on days 2, 4, 6, 8, and 10 after birth. The total number of injections was 5. At 1 month of age, rats were randomly divided into two groups, with intact rats of the appropriate age serving as controls. These rats were administered 0.25 mL/100 g of water after neonatal administration of monosodium glutamate (this was a 2-week course of administration for 3 months after 1 month of age). The second group of rats, after neonatal administration of monosodium glutamate, was administered nanocrystalline cerium dioxide (NCD) (1 mg/kg) dissolved in water at a volume of 0.29 mL/100 g (2-week course administration for 3 months after 1 month of life). At the end of the experiment, at the age of 4 months, the rats were weighed and euthanized by cervical dislocation. Then, the visceral adipose tissue was dissected and weighed. According to this method, the administration of monosodium glutamate in large doses to rats in the early neonatal period led to the destruction of the arcuate nuclei, as a result of which obesity and insulin resistance developed in rats at the age of 16 weeks, and liver damage was also recorded (Nakanishi et al., 2008). In rats that were administered monosodium glutamate in the early neonatal period, body weight and food consumption were recorded once a month, starting 1 month after birth.

Studies devoted to developing methods for the prevention and treatment of obesity have been conducted only on the model of glutamate-induced obesity, since the correction of obesity caused by a high-calorie diet consists, first of all, in changing the nature of nutrition and lifestyle. Instead of foods high in fat and easily digestible carbohydrates, the diet should be limited in fat and enriched with dietary fiber. When it comes to humans, physical activity should increase (Balducci, 2020; Kong & So, 2021). In the case of glutamate-induced obesity, the situation is much more complicated, since monosodium glutamate E_{621} (sodium salt of the amino acid glutamine) is one of the most well-known and popular flavor enhancers, salt substitutes and, finally, substances that mask old and spoiled products, making them look and taste fresh (Hernández-Bautista et al., 2019).

In each animal, the presence of obesity was determined by the Lee Index, which is the ratio of the cube root of body weight (g) to the naso-anal length of the rat (cm). Rats with the Lee Index value greater than 0.300 $g^{1/3}$/cm were classified as obese rats, and rats with a Lee Index value close to or less than 0.300 $g^{1/3}$/cm were classified as normal rats (Bernardis & Patterson, 1968). However, hyperphagia did not develop, as daily food intake did not change. The data obtained allow us to conclude that glutamate-induced obesity is not associated with excessive calorie intake, but is the result of a metabolic disorder.

A modified method for non-enzymatic hepatocyte production was developed based on the analysis of methodological approaches to obtain functionally intact cells (Hwang et al., 2001). The principle of obtaining submitochondrial fragments (particles) of hepatocytes consists in extracting the crushed tissue with a saline buffer solution, destroying it with abrasive materials, and fractionating it by stepwise

centrifugation in Tris-sucrose buffer (Ardail et al., 1990). Lipid extraction was performed using the Folch method. An extracting mixture of chloroform: methanol: water 1:2:0.8 was added to an aliquot of the membrane preparation at the rate of 3 mg of protein. (Folch et al., 1957; Aoun et al., 2012). The content of total lipids was determined by the SF-46 spectrophotometric method. The principle of the process is that the products of the decomposition of unsaturated lipids, after hydrolysis with sulfuric acid, interact with the phosphonovanillin reagent to form a colored compound with an absorption maximum at a wavelength of 530 nm. The content of total lipids was expressed in μg/mg of protein of the suspension of submitochondrial particles. Separating phospholipids was carried out by two-dimensional microthin-layer chromatography (Brockhuyse, 1968) using Sorbfil plates measuring 10*10 cm. Before use, they were activated at 110 °C for 30 min. Chromatography was carried out in glass chambers 18–20 cm high and 16–17 cm in diameter in two directions. After cooling the plates, the obtained lipid extracts were applied at a distance of 2.5 cm from the edge of the plate and dispersed in the prepared systems. In the first direction, the system chloroform: methanol: 28% ammonia (90:54:11) was used, and in the second, chloroform: methanol: glacial acetic acid: water (90:40:12:2). After passing in each direction, especially after the first, the plates were dried to completely remove the residues of the chromatographic systems and used for qualitative and quantitative analysis of phospholipid classes. The chromatography time in two directions was 60-65 min. The dried plates were stained in iodine vapor for 10 min to identify phospholipids. The identification was completed by comparing the results of qualitative reactions to phospholipids with the chromatographic behavior of phospholipid standards with a known Rf value. The phospholipid content was expressed in percentages, which was calculated using the ImageJ program. The determination of free and esterified cholesterol content was based on the reaction of a color reagent (0.1% ferric chloride) with cholesterol under different temperature conditions (Aoun et al., 2012). The method included two stages: first, free cholesterol was determined, then the total amount of free and bound cholesterol.

Statistical processing of the study results was carried out using generally accepted methods of variational statistics. The Shapiro-Wilk W test was used to determine the data distribution type. Since the data distribution was normal, the probability of the difference between the control and experimental measurements was estimated using Student's t-test. The difference between the comparative indicators was considered probable at $P < 0.05$. Calculations and graphing were performed on a computer using the "OriginLab Origin 8.0" application programs.

Results

After 16 weeks from the beginning of the experiment, the body weight of the rats in the control group had increased by 51.3% ($P < 0.01$) compared to the first day of the experiment. In rats on a high-calorie diet, the body weight increased by 57.0% ($P < 0.01$) over the same period. There was no statistically significant difference between the body weight of rats receiving standard food and rats on a high-calorie diet. At the same time, the increase in growth in rats on a high-calorie diet occurred more slowly. And the visceral fat mass at all observation periods was statistically significantly greater than the corresponding control. If in rats after 3 weeks of being on a high-calorie diet, the mass of visceral fat was 41.6% ($P < 0.05$) higher compared to the control, then after 16 weeks this difference was 180.0% ($P < 0.001$). Thus, in rats that were on high-calorie diet, the development of visceral obesity was noted.

Thus, in rats aged 16 weeks, which were administered monosodium glutamate in the early neonatal period, the body weight and nasoanal length were respectively 9.4% ($P < 0.001$) and 23.7% ($P < 0.001$) lower than the similar indicators in rats of the control group. The decrease in body weight after neonatal administration of monosodium glutamate was slight, although statistically significant. The introduction of monosodium glutamate in the neonatal period led to a pronounced visceral obesity, since the mass of visceral fat in these rats was 107.2% ($P < 0.001$) greater than in rats of the control group. In the experiments, the Lee index in rats in the control group was 0.29 ± 0.02, and in rats after neonatal administration of monosodium glutamate, it was 0.36 ± 0.03 ($P < 0.001$). Therefore, the introduction of monosodium glutamate to rats in the neonatal period led to visceral obesity at 4 months of age.

Considering the study of the preventive effect of nanocrystalline cerium dioxide on the development of steatohepatitis in rats, we had to conduct several studies that allowed us to compare two models of visceral obesity. This applies to the lipid composition of the inner mitochondrial membrane and the activity of ETL enzymes. In the control group of rats fed a standard diet, the total lipid content in the inner mitochondrial membrane of hepatocytes did not undergo statistically significant changes throughout the entire experiment, which lasted 16 weeks. In the experimental group of rats fed a high-calorie diet, statistically significant changes in the studied indicator were recorded from the 9th week of the experiment. Thus, during the 9th, 12th, and 15th weeks of the experiment, the total lipid content in the inner mitochondrial membrane of hepatocytes increased by 28.5% ($P < 0.01$), 29.1% ($P < 0.01$), and 72.1% ($P < 0.01$), respectively, compared to the control (Table 1).

Table 1

Total lipid and phospholipid content in the inner membrane of rat hepatocytes during the dynamics of being on a high-calorie diet C 11024 (μg/mg protein, $n = 10$, $x \pm SD$)

Indicator	Group	3 weeks	6 weeks	9 weeks	12 weeks	15 weeks
Total lipids	control	232.1 ± 15.1^{a}	244.2 ± 16.3^{ab}	238.6 ± 18.2^{a}	240.6 ± 15.7^{ab}	260.8 ± 21.7^{b}
	high-calorie diet	265.8 ± 20.4^{bd}	273.5 ± 18.6^{b}	306.7 ± 24.1^{c}	310.5 ± 22.3^{c}	356.0 ± 18.2^{d}
Total phospholipids	control	220.2 ± 11.0^{a}	234.0 ± 16.8^{ab}	238.6 ± 16.2^{ab}	230.6 ± 14.7^{ab}	240.8 ± 22.1^{ab}
	high-calorie diet	225.8 ± 20.2^{a}	253.5 ± 18.6^{b}	313.1 ± 28.6^{c}	320.4 ± 26.3^{c}	346.0 ± 28.2^{d}

Note: letters indicate significant differences between the groups within one line ($P < 0.05$) according to the Tukey test.

A statistically significant difference between the lipid content in the inner membrane of hepatocytes of rats with different durations of maintenance on the used model of high-calorie diet was observed between the 3rd and 9th, 3rd and 12th, and 3rd and 15th weeks. After 6 weeks of maintenance on the high-calorie diet, the lipid content in the inner membrane of hepatocytes was the same as after 3 weeks. The total phospholipid content in the inner membrane of hepatocyte mitochondria did not undergo statistically significant changes after 3 and 6 weeks of maintenance on the high-calorie diet compared with the corresponding control (Table 1). After 9, 12, and 15 weeks of keeping rats on a high-calorie diet, the total phospholipid content increased by 31.2% ($P < 0.05$), 38.9% ($P < 0.05$), and 43.7% ($P < 0.05$), respectively, relative to the corresponding control. As in the case of total lipids, the content of phospholipids in the inner membrane of hepatocyte mitochondria did not increase immediately after changing the nature of the feed. Statistically significant changes were observed after 9 weeks of being on the high-calorie diet.

Further studies showed that the most significant changes were observed in the rats' cholesterol content in the inner membrane of hepatocyte mitochondria on a high-calorie diet. Unlike the total lipid and phospholipid content, the cholesterol content in the inner mitochondrial membrane of rat hepatocytes increased after 3 weeks of maintenance stay on a high-calorie diet (Table 2).

After 3, 6, 9, 12, and 15 weeks of exposure to a high-calorie diet, the cholesterol content in the inner mitochondrial membrane of rat hepatocytes increased by 66.0% ($P < 0.05$), 75.2% ($P < 0.05$), 82.6% ($P < 0.01$), 114.7% ($P < 0.05$), and 138.1% ($P < 0.05$), respectively, compared with the corresponding control.

Table 2
Cholesterol content in the inner membrane of hepatocytes of rats on a high-calorie diet C 11024 (μg/mg protein, n = 10, x ± SD)

Groups	3 weeks	6 weeks	9 weeks	12 weeks	15 weeks
Control	7.78 ± 0.67^{a}	7.56 ± 0.68^{a}	8.04 ± 0.76^{ab}	7.98 ± 0.89^{ab}	8.26 ± 0.75^{b}
High-calorie diet	12.91 ± 1.02^{c}	13.25 ± 1.16^{cd}	14.68 ± 1.86^{d}	17.13 ± 1.63^{e}	19.67 ± 1.82^{f}

Note: see Table 1.

Compared with 3 weeks of exposure to a high-calorie diet, statistically significant changes in the cholesterol content in the inner mitochondrial membrane of hepatocytes occurred after 12 weeks of exposure to a high-calorie diet. Therefore, the changes did not happen immediately. Our results are consistent with the literature, according to which in rats fed a high-calorie diet, the lipid composition of the inner membrane of hepatocyte mitochondria changes, leading to changes in ETL functioning and the oxidation of fatty acids.

The content of cholesterol esters in the fraction of the inner membrane of hepatocyte mitochondria in rats in the dynamics of being on a high-calorie diet underwent less pronounced changes, the nature of which was opposite (Table 3).

Table 3
The content of cholesterol esters in the inner membrane of hepatocytes of rats on a high-calorie diet C 11024 (μg/mg protein, n = 10, x ± SD)

Groups	3 weeks	6 weeks	9 weeks	12 weeks	15 weeks
Control	17.04 ± 1.62^{c}	16.98 ± 1.95^{c}	17.08 ± 1.84^{c}	16.95 ± 1.22^{c}	17.14 ± 1.03^{c}
High-calorie diet	17.81 ± 1.00^{d}	17.25 ± 1.26^{cd}	17.68 ± 1.68^{d}	13.54 ± 1.33^{b}	11.98 ± 1.02^{a}

Note: see Table 1.

The maintenance of rats on the used model of high-calorie diet for 3, 6, and 9 weeks did not affect the content of cholesterol esters in the inner mitochondrial membrane. In addition, after 12 and 15 weeks, the content of cholesterol esters decreased by 20.1% ($P < 0.05$) and 30.8% ($P < 0.05$), respectively, compared with the control group of rats. Changes in the content of cholesterol esters that occurred after 12 and 15 weeks of being on a high-calorie diet were statistically significant concerning rats that were on the same diet for 3 weeks.

The results show that the registered significant changes in the lipid composition of the inner mitochondrial membrane of hepatocytes are mainly due to cholesterol. Structural changes undoubtedly affect the functional state of the membrane: the activity of the electron transport chain (ETC) enzymes changes, which produces ROS, not ATP. As a result, oxidative stress develops in mitochondria and hepatocytes.

It is no less important to characterize the following indicators of the inner membrane, which determine its functional state. First of all, these include phospholipids: cardiolipin, phosphatidylcholine, phosphatidylethanolamine, and their oxidized forms. The relative content of cardiolipin in the inner membrane of mitochondria of hepatocytes of rats of the control group did not undergo statistically significant changes during all observation periods. As for rats that were on high-calorie diet, the relative content of cardiolipin in the inner mitochondrial membrane of hepatocytes increased after 3, 12, and 15 weeks by 25.0% ($P < 0.05$), 23.7% ($P < 0.05$), and 41.5% ($P < 0.05$), respectively (Table 4).

Table 4
Relative content of cardiolipin, phosphatidylcholine, phosphatidylethanolamine, and their oxidized forms in the inner mitochondrial membrane of rat hepatocytes under conditions of diet-induced steatohepatosis modeling (n = 10, x ± SD)

Indicator	Group	3 weeks	9 weeks	12 weeks	15 weeks
Cardiolipin	control	16.8 ± 1.4^{a}	16.9 ± 1.3^{a}	19.0 ± 1.4^{b}	19.5 ± 1.2^{b}
	high-calorie diet	21.0 ± 1.5^{bc}	17.8 ± 1.8^{ab}	23.5 ± 1.3^{c}	27.6 ± 1.2^{d}
Phosphatidylcholine	control	32.5 ± 2.5^{ab}	32.8 ± 3.2^{ab}	33.0 ± 1.8^{b}	33.8 ± 1.4^{b}
	high-calorie diet	27.9 ± 1.1^{a}	34.6 ± 3.6^{b}	32.8 ± 2.2^{ab}	33.0 ± 1.2^{ab}
Phosphatidylethanolamine	control	32.4 ± 1.5^{c}	32.9 ± 3.1^{c}	27.3 ± 2.1^{ab}	32.3 ± 1.3^{c}
	high-calorie diet	25.8 ± 1.4^{a}	29.8 ± 2.6^{b}	26.6 ± 1.8^{ab}	29.0 ± 2.3^{b}
Lysophosphatidylcholine	control	1.402 ± 0.023^{b}	1.379 ± 0.021^{b}	1.087 ± 0.058^{a}	1.184 ± 0.011^{ab}
	high-calorie diet	2.609 ± 0.057^{d}	2.424 ± 0.034^{d}	1.876 ± 0.241^{c}	1.962 ± 0.039^{c}
Lysophosphatidylethanolamine	control	0.443 ± 0.033^{a}	0.439 ± 0.041^{a}	0.558 ± 0.024^{ab}	0.570 ± 0.042^{b}
	high-calorie diet	0.559 ± 0.021^{ab}	0.461 ± 0.075^{a}	0.817 ± 0.061^{c}	0.784 ± 0.034^{bc}

Note: see Table 1.

The relative content of phosphatidylcholine in the inner membrane of mitochondria of hepatocytes of rats of the control group did not change until the end of the experiment (Table 4). Unlike cardiolipin, the relative content of phosphatidylcholine in the inner membrane of mitochondria of hepatocytes of rats that were on a high-calorie diet changed only after 3 weeks of being on the high-calorie diet: it decreased by 14.2% ($P < 0.05$). Interestingly, the content of the oxidized form of phosphatidylcholine – lysophosphatidylcholine – significantly increased at all observation periods. It should be noted that its content is typically very low. Still, after 3, 9, 12 and 15 weeks of high-calorie diet, the relative content of lysophosphatidylcholine increased by 85.7% ($P < 0.001$), 75.4% ($P < 0.01$), 72.5% ($P < 0.001$) and 66.1% ($P < 0.01$).

A similar trend was observed when determining the content of phosphatidylethanolamine and its oxidized form – lysophosphatidylethanolamine – in the inner membrane of hepatocyte mitochondria (Table 4). The relative content of phosphatidylethanolamine in the inner membrane of hepatocyte mitochondria in rats after 3 weeks of high-calorie diet decreased by 20.4% ($P < 0.05$). At all other observation points, the decrease in this indicator was statistically insignificant. And the relative content of the oxidized form of phosphatidylethanolamine-lysophosphatidylethanolamine in the inner membrane of hepatocyte mitochondria of rats after 3, 12, and 15 weeks increased by 27.3% ($P < 0.05$), 46.4% ($P < 0.01$), and 36.8% ($P < 0.01$), respectively.

As a result of the conducted studies, it was found that under a high-calorie diet, the content of minor lipids in the inner membrane of hepatocyte mitochondria changes. The relative content of phosphatidylinositol and phosphatidylserine in the inner mitochondrial membrane of rat hepatocytes after 3 weeks of high-calorie diet increased by 34.7% ($P < 0.05$); after 9 weeks, it decreased by 26.3% ($P < 0.05$). After 12 weeks, it recovered to the control level. After 15 weeks, it again reduced by 24.1% ($P < 0.05$) (Table 5). As for the content of sphingomyelin in the inner mitochondrial membrane of rat hepatocytes, their content also depended on the duration of exposure to a high-calorie diet (Table 5). After 3 weeks, it increased by 78.8% ($P < 0.01$), and after 9, 12, and 15 weeks it decreased by 36.4% ($P < 0.05$), 37.3% ($P < 0.05$), and 50.0% ($P < 0.001$).

Table 5
Relative content of phosphotidylinositol, phosphotidylserine, and sphingomyelin in the inner mitochondrial membrane of rat hepatocytes under conditions of diet-induced steatohepatosis modeling (n = 10, x ± SD)

Indicators	Groups	3 weeks	9 weeks	12 weeks	15 weeks
Phosphatidylinositol and phosphatedylserine	control	11.80 ± 1.23^{b}	13.29 ± 0.86^{c}	12.93 ± 1.22^{bc}	11.57 ± 0.64^{b}
	high-calorie diet	15.86 ± 1.14^{d}	9.77 ± 0.76^{ab}	13.28 ± 1.28^{c}	8.84 ± 0.42^{a}
Sphingomyelin	control	3.34 ± 0.03^{c}	4.39 ± 0.03^{d}	5.08 ± 0.35^{de}	2.55 ± 0.49^{b}
	high-calorie diet	5.85 ± 0.58^{e}	2.82 ± 0.23^{b}	3.24 ± 0.26^{c}	1.31 ± 0.34^{a}

Note: see Table 1.

Studies of the effect of glutamate-induced obesity on hepatocyte mitochondria in rats showed significant changes in the lipid composition of the inner mitochondrial membrane. Thus, the total lipid content increased by 53.4% ($P < 0.01$) compared with intact controls. Under the influence of periodic administration of nanocrystalline cerium dioxide to rats with glutamate-induced obesity, the total lipid content in the inner mitochondrial membrane decreased by 14.4% ($P < 0.05$) compared with rats with glutamate-induced obesity. Still, it remained higher by 31.3% ($P < 0.05$) compared with intact controls (Table 6). The total lipid content is known to be a mixture of phospholipids, cholesterol, cholesterol esters, and minor lipid forms. Therefore, the question arises: due to which forms does the total lipid content in the intramitochondrial membrane of hepatocytes increase?

Table 6
Lipid composition of the inner mitochondrial membrane of hepatocytes of rats with glutamate-induced steatohepatosis and its correction with nanocrystalline cerium dioxide (μg/mg protein, n = 10, x ± SD)

Indicators	Control	Glutamate-induced steatohepatosis	Glutamate-induced steatohepatosis + cerium dioxide
Total lipids	232 ± 11^{a}	356 ± 18^{b}	305 ± 15^{ab}
Total phospholipids	220 ± 11^{a}	335 ± 16^{b}	305 ± 15^{ab}
Cholesterol	3.88 ± 0.23^{a}	14.12 ± 0.65^{c}	6.79 ± 0.27^{b}
Cholesterol esters	9.03 ± 0.51^{b}	7.26 ± 0.42^{a}	14.72 ± 0.66^{c}

Note: see Table 1.

As a result of the experiments, it was found that, compared with the intact control, in rats with glutamate-induced obesity, the total content of phospholipids in the intramitochondrial membrane of hepatocytes increased by 52.3% ($P < 0.01$, Table 6). Under the conditions of periodic administration of nanocrystalline cerium dioxide to rats with glutamate-induced obesity, the total content of phospholipids in the intramitochondrial membrane of hepatocytes was 19.1% ($P < 0.05$) lower compared with the group of rats with glutamate-induced obesity without correction. At the same time, the studied indicator did not return to the level of the intact control and remained higher than it by 23.1% ($P < 0.05$).

The most pronounced changes were detected when determining the cholesterol content in the intramitochondrial membrane of hepatocytes, which increased by 261.5% ($P < 0.001$) against the background of a decrease in the content of cholesterol esters by 18.9% ($P < 0.05$, Table 6). According to the literature, such changes in the cholesterol content in the inner mitochondrial membrane lead to an increase in the electrochemical potential of the electronic component. In this state, the mitochondrial respiratory chain accumulates intermediate electron-transporting compounds, the property of which is the transfer of electrons to synthesizing reactive oxygen species.

Nanocrystalline cerium dioxide, when periodically administered to rats with glutamate-induced obesity, significantly affected the content of cholesterol and its esters in the inner mitochondrial membrane of hepatocytes. A 51.8% decrease in cholesterol content ($P < 0.001$) was observed, with a simultaneous increase in cholesterol ester content by 101.4% ($P < 0.001$). At the same time, cholesterol content did not reach intact control. It remained higher than that by 74.4% ($P < 0.01$), and cholesterol ester content even exceeded the similar indicator in intact rats by 63.3% ($P < 0.01$).

The established changes in the lipid composition of the inner mitochondrial membrane under glutamate-induced steatohepatosis are similar to the data obtained both in diet-induced steatohepatosis and under maintenance conditions on different high-calorie diets. Despite the similarity in the results, the possible mechanism of development of the simulated steatohepatosis may differ, since the main factors of pathogenesis have different natures and degrees of influence. The obtained data also suggest that structural changes in the membrane and specific dysfunctional changes in the mitochondria are observed. Among them, there is a possible effect on the functioning of the respiratory chain, in which not ATP, but ROS is generated, which in turn causes the development of oxidative stress both in the mitochondria and the entire hepatocyte. The positive effect of cerium dioxide is associated with its antioxidant properties.

Thus, glutamate-induced steatohepatosis in rats is accompanied by changes in the lipid composition of the inner membrane of hepatocytes: total lipid content, total phospholipid content, and cholesterol content increased against a background of a decrease in the content of cholesterol esters. The use of cerium dioxide nanoparticles positively affects the lipid composition of the inner membrane of hepatocytes in rats with glutamate-induced steatohepatosis. However, it does not return the studied parameters to normal values.

When studying the main phospholipids of the inner mitochondrial membrane of hepatocytes, it was shown that cardiolipin content in rats with glutamate-induced steatohepatosis increased by 51.3% ($P < 0.01$) compared to intact controls. In rats after neonatal administration of monosodium glutamate against the background of periodic administration of nanocrystalline cerium dioxide, cardiolipin content decreased to the level of intact controls (Table 7).

Table 7
The content of basic phospholipids in the inner mitochondrial membrane of rat hepatocytes under conditions of glutamate-induced steatohepatosis and its correction with nanocrystalline cerium dioxide (n = 10, x ± SD)

Indicators	Control	Glutamate-induced steatohepatosis	Glutamate-induced steatohepatosis + cerium dioxide
Cardiolipin	18.9 ± 0.9^{a}	28.6 ± 1.4^{c}	22.1 ± 1.1^{b}
Phosphatidylcholine	33.5 ± 2.8^{c}	19.6 ± 0.9^{a}	26.3 ± 2.0^{b}
Lysophosphatidylcholine	1.109 ± 0.116^{a}	10.396 ± 0.925^{c}	5.124 ± 0.429^{b}
Phosphatidylethanolamine	30.6 ± 2.1^{b}	22.2 ± 0.9^{a}	23.6 ± 1.7^{a}
Lysophosphatidylethanolamine	0.418 ± 0.033^{a}	2.041 ± 0.238^{c}	0.768 ± 0.145^{b}

Note: see Table 1.

The percentage of phosphatidylcholine in the inner mitochondrial membrane of hepatocytes from rats with glutamate-induced steatohepatosis was reduced by 41.5% ($P < 0.01$) compared with intact controls. Periodic administration of nanocrystalline cerium dioxide to rats after neonatal administration of monosodium glutamate increased the content of phosphatidylcholine in the inner mitochondrial membrane of hepatocytes by 34.2% ($P < 0.05$). Still, it did not reach the level of intact controls.

The decrease in the percentage of phosphatidylcholine in the inner mitochondrial membrane of hepatocytes of rats with glutamate-induced steatohepatosis was accompanied by a very significant increase in the rate of its oxidized form – lysophosphatidylcholine (by 845.5%, $P < 0.001$), which in healthy rats is contained in very low quantities in the mitochondrial membrane of hepatocytes (Table 7). In rats after neonatal administration of monosodium glutamate against the background of periodic administration of nanocrystalline cerium dioxide, the percentage of lysophosphatidylcholine decreased by 51% ($P < 0.001$). It remained significantly higher than this value in intact rats (control group).

A similar pattern was observed when determining the percentage of phosphatidylethanolamine and its oxidized form – lysophosphatidylethanolamine in the inner membrane of hepatocyte mitochondria in rats with glutamate-induced steatohepatosis: the percentage of phosphatidylethanolamine decreased compared to the intact control by 27.5% ($P < 0.05$) with a simultaneous increase in the rate of lysophosphatidylethanolamine by 400% ($P < 0.001$, Table 7).

In rats after neonatal administration of monosodium glutamate against the background of periodic administration of nanocrystalline cerium dioxide, the percentage of phosphatidylethanolamine in the inner membrane of hepatocyte mitochondria did not change, and the rate of lysophosphatidylethanolamine decreased by 60.0% ($P < 0.001$).

A significant increase in the content of the oxidized form of phosphatidylcholine and phosphatidylethanolamine in the inner mitochondrial membrane, which is usually observed in small amounts, against the background of a decrease in the content of cardiolipin in the inner mitochondrial membrane confirms the development of oxidative stress. The fact that nanocrystalline cerium dioxide significantly reduced the content of the oxidized forms of phosphatidylcholine and phosphatidylethanolamine in the inner mitochondrial membrane of hepatocytes against the background of normalization of the content of cardiolipin in the inner mitochondrial membrane indicates the antioxidant effect of the prebiotic and the possibility of its use in the prevention and treatment of steatohepatosis. As for the content of minor components of the inner mitochondrial membrane of hepatocytes in rats with glutamate-induced steatohepatosis, the percentage content of the mixture of phosphatidylinositol and phosphatidylserine did not undergo statistically significant changes. At the same time, the content of sphingomyelin increased by 81.3% ($P < 0.001$, Table 8).

Table 8

The content of minor phospholipids in the inner mitochondrial membrane of rat hepatocytes under conditions of glutamate-induced steatohepatosis and its correction with nanocrystalline cerium dioxide ($n = 10$, $x \pm SD$)

Indicators	Control	lutamate-induced steatohepatosis	Glutamate-induced steatohepatosis + cerium dioxide
Phosphatidylinositol and phosphatidylserine	12.4 ± 1.0^b	11.4 ± 0.8^a	17.7 ± 1.4^c
Sphingomyelin	3.24 ± 0.48^a	5.75 ± 1.13^c	4.53 ± 0.75^b

Note: see Table 1.

We assume that the established changes in the percentage of minor phospholipids arose due to a violation of the ratio of the total content of phospholipids. An additional effect of fluctuations in the phospholipid composition is changes in the fluidity of the inner mitochondrial membrane, which has an additional destabilizing impact on the regular functional activity of not only the membrane, but also the entire mitochondria as a whole. Therefore, the positive effect of nanocrystalline cerium dioxide on the lipid and phospholipid composition of the inner mitochondrial membrane of hepatocytes is apparent, although complete recovery did not occur.

Discussion

In addition to the ambiguity of data on the mechanisms of steatohepatosis development, the polyetiological nature of the development of this pathology is quite interesting. The first stages of the disease must be asymptomatic in most cases, which complicates not only the establishment of mechanisms but also does not allow timely and effective treatment. Thus, most scientists emphasize that the development of steatohepatosis is accompanied by obesity, insulin resistance, and problems with the cardiovascular system. However, there is evidence that some patients may not be overweight and insulin resistant, but are diagnosed with a rather advanced form of nonalcoholic fatty liver disease, accompanied by fibrosis and partial or complete loss of functional activity of the organ (Angulo et al., 2012; Goh et al., 2014). These data actualize the problem of both the characteristic features of the pathogenesis of steatohepatosis and the comparison of different types of nonalcoholic fatty liver disease with each other by etiology. Therefore, the studies were aimed at comparing the structural and functional changes in the mitochondrial membrane of rat hepatocytes under the conditions of steatohepatosis development caused by various etiological factors.

The results obtained showed that their direction is consistent with the literature data, according to which changes in the lipid composition of the inner membrane of hepatocyte mitochondria in rats do not occur immediately after changing their standard diet to high-calorie diet (Aoun et al., 2012; Monteiro et al., 2013).

Keeping experimental animals on a high-calorie diet with a high content of fats and carbohydrates for 16 weeks showed the development of a slight degree of obesity, in contrast to neonatal administration of monosodium glutamate. In both cases, there was a significant increase in visceral fat mass, especially with regard to the glutamate-induced model of steatohepatosis. The selected models allowed us to observe the development of steatohepatosis, which was caused by different etiological factors, although with the same diagnostic characteristics and terms of development (Kondro et al., 2013; Voieikova et al., 2016). This made it possible to compare the structural and functional changes of the liver that occurred in the mitochondrial membrane of hepatocytes and to compare the obtained data.

We draw attention, firstly, to the increase in the content of cardiolipin in the inner membrane of hepatocyte mitochondria, which regulates the functional activity of ETL complexes. We also concluded that changes in its content in the membrane correlate with the level of mitochondrial proton leak and affect the normal functioning of all ETL complexes (Voieikova et al., 2016). Secondly, we draw attention to the increase in oxidized forms of phosphatidylcholine and phosphatidylethanolamine, the content of which is normally insignificant, which indicates the development of oxidative stress (Rolo et al., 2012; Monteiro et al., 2013; Paradies et al., 2014).

It was found that under the conditions of neonatal administration of monosodium glutamate, lipid content indicators, namely the total content of lipids, phospholipids, and cholesterol, in the mitochondrial membrane of hepatocytes increased more significantly than under the conditions of maintenance on high-calorie diet for 16 weeks. This shows that the development of obesity due to neuroendocrine disorders, and therefore a more profound effect on the basic metabolism, changes the structure of the membrane in a more significant way. This is especially true for the content of cholesterol, which is one of the primary regulators of the electrochemical gradient and membrane permeability. Under the conditions of glutamate-induced steatohepatosis, we can assume a greater degree of development of oxidative stress than under the conditions of a changed diet. The only common feature in lipid content changes is the cholesterol esters' content, which de-

creased in both cases similarly (Aoun et al., 2012; Akanya et al., 2015).

An increase in cholesterol content in the inner mitochondrial membrane can induce the development of oxidative stress in hepatocytes and violate the antioxidant defense mechanism (Breslow et al., 2010; Bieganski et al., 2011). Against the background of an increase in cholesterol content, a simultaneous decrease in the content of cholesterol esters was observed. The latter are transport molecules and a source of cholesterol and fatty acids. Therefore, a reduction in the content of cholesterol esters may result from an excess of cholesterol in the inner mitochondrial membrane, eliminating the need for its transport. Thus, an increase in cholesterol levels is accompanied by a parallel decrease in cholesterol esters (Breslow & Weissman, 2010; Aoun et al., 2012).

Further study of the parameters of the structural and functional state of the inner mitochondrial membrane of hepatocytes confirmed the described accumulation of oxidized products due to changes in the enzymatic activity of the respiratory chain complexes. Thus, under maintenance conditions of a high-calorie diet, a slight decrease in the content of phosphatidylcholine and phosphatidylethanolamine is observed along with an increase in the content of their oxidized forms. Changes in the enzymatic activity of the electron transport chain complexes indicate that the respiratory chain takes an active part in the accumulation of oxidized products in the membrane. Thus, an increase in the activity of the enzymatic component of complex I against the background of a decrease in the activity of complex II is the cause of the accumulation of intermediate electron-transporting compounds, which in turn are restored to form ROS. A similar change in enzymatic activity in the case of complexes III and IV leads to similar consequences. At the same time, a significant decrease in H+ - ATPase activity is observed, which allows us to assume not only the development of oxidative stress, but also shows that the electron transport chain can take an active part in increasing the content of lysoforms in the membrane.

At the same time, under conditions of glutamate-induced obesity and steatohepatosis, a different picture of structural and functional rearrangements of the hepatocyte membrane was observed. As in the case of maintenance on high-calorie diet, a decrease in the content of phosphatidylcholine and phosphatidylethanolamine was observed, although more significantly. In this modeling method, the content of oxidized forms of phospholipids significantly increased, which may indicate a significant degree of development of oxidative stress. A substantial increase in the content of lysoforms in the membrane was accompanied by a decrease in the enzymatic activity of all complexes of the respiratory chain. Thus, a significant disruption of the basic metabolism due to the introduction of substantial doses of monosodium glutamate, resulting in steatohepatosis, caused profound structural and functional rearrangements of the inner mitochondrial membrane of hepatocytes. The only common feature inherent in both models is a decrease in H+ - ATPase activity, which in both cases indicates a reduction in the content of ATP in the cell in general (Kondro et al., 2024). Thus, glutamate-induced obesity causes the development of steatohepatosis, which is characterized by a greater degree of development of pathological transformations of the mitochondrial membrane of hepatocytes. Analysis of the results showed that the use of correction is more appropriate in the case of glutamate-induced steatohepatosis, as it will allow the analysis of the possibility of correcting a deeper degree of development of steatohepatosis.

Having analyzed the literature data and the results obtained, we assume that the established changes in the percentage content of minor phospholipids arose due to a violation of the ratio of the total phospholipids and do not have functional consequences for the inner mitochondrial membrane. The only additional effect in combination with changes in other phospholipids is a decrease in the fluidity of the inner mitochondrial membrane, which has an additional destabilizing impact on the regular functional activity of not only the membrane, but also the entire mitochondria as a whole (Okediran et al., 2014).

Assessment of the structural and functional state of the inner mitochondrial membrane of hepatocytes also showed a significant improvement in the lipid composition, although without complete restoration of the enzymatic activity of the respiratory chain complexes under the conditions of correction of steatohepatosis with nanocrystalline cerium dioxide. The phospholipid composition was close to control values, with a slight increase in oxidized forms. Although the activity of the electron transport chain complexes did not return to control values, compared to animals that did not receive nanoceria, the indicators were slightly higher (Kondro et al., 2019). This indicates a minor effect on the state of mitochondria and suggests that restoration of the inner mitochondrial membrane's normal functioning is possible with more extended use of the drug.

Conclusion

Rats fed a high-calorie diet with a high content of fats, carbohydrates, and a reduced content of proteins for 16 weeks, and 4-month-old rats given monosodium glutamate in the neonatal period, developed visceral obesity without hyperphagia, characterized by dyslipidemia, insulin resistance, and steatohepatitis. Periodic administration of nanocrystalline cerium dioxide to rats with glutamate-induced steatohepatitis significantly restored the morphofunctional state of the liver and prevents the development of steatohepatitis. A feature of the development of diet- and glutamate-induced steagepatosis is mitochondrial dysfunction, characterized by changes in the lipid composition of the inner membrane of hepatocyte mitochondria, an increase in oxidized products, and changes in the enzymatic activity of all components of the electron transport chain. Periodic application of nanocrystalline cerium dioxide significantly restored the lipid composition of the inner mitochondrial membrane of hepatocytes and the enzymatic activity of all components of the electron transport chain in rats that were administered monosodium glutamate in the neonatal period. In rats with steatoheatosis caused by a high-calorie diet or the administration of monosodium glutamate, the phospholipid composition of the inner mitochondrial membrane changed and not only structural changes occurred in the membrane, but also dysfunctional changes in the mitochondria as a whole, which manifested themselves in the fact that ROS were generated in the respiratory chain instead of ATP, and this caused the development of oxidative stress in both the mitochondria and the entire hepatocyte. The use of nanocrystalline cerium dioxide significantly reduced the manifestations of oxidative stress, reduced the content of oxidized forms of phosphatidylcholine and phosphatidylethanolamine in the inner membrane of hepatocyte mitochondria against the background of normalization of cardiolipin content, which indicates the antioxidant effect of this drug and the possibility of its use for the prevention and treatment of steatohepatosis.

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