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The Phospholipid Composition Of Internal Membrane Of Hepatocytes In Rats With Glutamat-Induced Steatohepatitis And Its Correction By Cerium Dioxide Nanoparticles.

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ABSTRACT

Lipids are main mitochondrial fuels however these molecules can also behave as uncouplers and inhibitors of oxidative phosphorylation. The development of oxidative stress entails not only a malfunction of mitochondria but also the development of the complications among which the main role play hyperglycemic complications and type 2 diabetes. The aim of our work was to investigate the effect of cerium dioxide nanocrystals on the phospholipid content of the internal mitochondrial membrane of hepatocytes under conditions of glutamate-induced steatohepatitis. As a result of our work it was found that in rats with glutamate-induced steatohepatitis, the percentage content of cardiolipin, lysophosphatidylcholine and lysophosphatidyle than olamine were higher compared with intact control. Under the influence of periodic administration of cerium dioxide nanocrystals in rats with monosodium glutamate induced steatohepatitis, the percentage of cardiolipin in the internal mitochondrial membrane was restored to the level of intact control. In rats with glutamate-induced steatohepatitis, periodic administration of cerium dioxide nanoparticles increased the percentage content of the mixture of phosphatidylinositol and phosphatidylserine compared with intact control and to rats with glutamate-induced steatohepatitis without correction. Our data testify to the positive effect of cerium dioxide nanoparticles on the phospholipid content of the internal membrane of mitochondria, which undergoes significant changes in the development of glutamate-induced steatohepatitis.

Keywords: monosodium glutamate induced steatohepatitis, Wistar rats, cerium dioxide nanoparticles, phosphatidylcholine

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INTRODUCTION

Mitochondria play an important role in the metabolism of hepatocytes, being the main site of oxidation of fatty acids and the process of oxidative phosphorylation. Most studies point to the development of structural and functional damage to hepatocyte mitochondria, which is called "mitochondrial dysfunction". The development of this disorder is associated with both an increase and a decrease in the activity of β-oxidation. It was established that with increasing activity of β-oxidation enzymes, an increase in the formation of active forms of oxygen is observed, and when activity decreases, the accumulation of diacylglycerol and the simultaneous activation of the pathway involving protein kinase C with inhibitory signal from insulin are observed. Some studies have shown that increased activity of β-oxidation can serve as an adaptive mechanism for limiting lipotoxicity of free fatty acids. They showed that as a result of such activation of the oxidation of lipids, a large number of recovered nicotinamide adenine dinucleotide hydrate (NADH) equivalents is formed, regardless of the energy needs of the cell. Whether subsequent suppression of activity of the cycle of lipid oxidation follows, it is not known, but other studies of the pathogenesis of steatohepatosis showed the inhibition of β-oxidation, which marked the development of oxidative stress with a large number of active forms of oxygen (AFO) [1, 2, 3, 4].

The development of oxidative stress entails not only a malfunction of mitochondria, but also the development of other complications among which the main role play hyperglycemic complications and type 2 diabetes. The increase in the production of AFO is observed with hyperglycemia, resulting in an imbalance in oxidative-reduction reactions. Since mitochondria are one of the sources of cellular AFO, they become both the main target of oxidative damage, which may be caused by a decrease in antioxidants such as glutathione and a violation of antioxidant systems. AFO-mediated mitochondrial damage, which is observed in various pathological conditions, causes organoleptic alteration and a decrease in their functional activity. Increasing of AFO-production in the electron transport chain of mitochondria leads to an increase in the formation of a very toxic peroxynitrite radical 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. Increasing of the ratio of AFO/active forms of nitrogen (AFN) in pathological conditions stops the activity of various antioxidant enzymes, including glutathione peroxidase, glutathione reductase, superoxide dismutase and catalase [5, 6].

In addition to the functional component of mitochondria, and in particular its proteins, the development of oxidative stress negatively affects its structural state - the lipid composition of the membrane. Lipids in the inner mitochondrial membrane are involved in a variety of processes, such as protein biogenesis, participation in energy generation, membrane fusion, and apoptosis. Regarding energy production, membrane lipids are able to modulate mitochondrial breathing. Under physiological conditions, the phospholipid composition of mitochondria is formed due to the expression and activity of the proteins involved in the synthesis of lipids, as well as their inter conversion. Phosphatidylcholine, phosphatidylethanolamine are the main phospholipids of both mitochondrial membranes that are present in all cell types. Phosphatidylethanolamine and cardiolipin are mainly enriched with an internal membrane of mitochondria in all mammalian and plant cells. Phosphatidylinositol is present mainly in the outer membrane of mitochondria, and the number of sterols varies depending on the specialization of cells [7, 8].

Previously, we have shown that in rats with steatohepatitis caused by a high calorie diet, and in rats with MSG-induced steatohepatitis, the phospholipid content of the internal membrane of mitochondria changes [9]. The evaluation of the phospholipid composition, as one of the main components of the internal mitochondrial membrane, allows us to assess not only structural alterations, but also the functional state under the conditions of steatohepatitis.

Taking into account the above, it is important to search for methods of influencing the phospholipid content of the membrane of mitochondria in conditions of MSG-induced steatohepatitis.

Perspective in this issue, in our opinion, is the use of nanocrystalline cerium dioxide ($nCeO_2$), which has the expressed antioxidant properties, and its periodic administration to rats from the 2^{nd} to the 4^{th} month prevented the development of steatohepatitis in 4-month rats, which in the early neonatal period injected with glutamate sodium [10].

Cerium dioxide is an inorganic material that is widely used in sensors and electrochromic anticorrosive coatings. It is part of the catalysts of selective oxidation and dehydrogenation. When transitioning to a nanocrystalline state, cerium dioxide significantly changes its physical and chemical properties, and the nature of these changes is rather unusual [11]. Namely, unlike a number of substances, the parameter of the elemental cell CeO_2 increases with decreasing particle sizes [12].At the same time, the oxygen nonstoichiometry changes as a result of an increase in the proportion of atoms on the surface of the particles. It is shown that the surface state of $nCeO_2$ plays a key role in the inactivation of free superoxide radicals [13]. Thus, nanocrystalline CeO_2 is characterized by superoxide dismutase activity, which determines the prospect of its use in the prophylaxis and treatment of steatohepatitis.

Thus, the purpose of the work was to investigate the effect of cerium dioxide nanocrystals on the phospholipid content of the internal mitochondrial membrane of hepatocytes under conditions of glutamate-induced steatohepatitis.

MATERIALS AND METHODS

Experiments have been carried out in compliance with the general principles of bioethics in accordance with the international recommendations of the European Convention for the Protection of Vertebrate Animals used for experimental and other scientific purposes (Strasbourg, 1986) and approved by the First National Congress on Bioethics of Ukraine (September 2001).

The studies were conducted on 30 white non-linear male rats born from several females with a difference of 1-3 days. Newborn rats were randomly divided into 3 groups of 10 animals in each. The first group served as control: at 2, 4, 6, 8 and 10 days after birth, the physiological solution was injected subcutaneously at a dose of 8 µl/g. Rats of the second and third groups at 2^{nd}, 4^{th}, 6^{th}, 8^{th}, and 10^{th} days after birth, subcutaneously were injected MSG at a dose of 4 mg/kg dissolved in a physiological solution [14].

The volume of the injected solution of MSG was 8 µl/g of weight of the rat. Starting from 1^{st} month from the date of birth and for the next three months, the rats from the first group (control) and second group (after the neonatal injection of MSG) received the oral water (2,9 ml/kg). Rats from the third group (after the neonatal injection of MSG), starting from 1 month of birth and over the next three months, were injected nanocrystalline cerium dioxide at a dose of 4 mg/g, dissolved in water for injection, volume of which was 2.9 ml/kg.

Morphologically and functionally intact liver cells were obtained using a modified non-enzymatic method for the allocation of hepatocyte fraction of liver cells [15]. Further subsequent ultracentrifugation allowed to receive the preparations of the internal mitochondrial membrane [16].

The separation of phospholipids was carried out by two-dimensional micro transparent chromatography on the plates “Sorbfil” of size of 10×10 cm [17], in which different classes of lipids are fractionated in various solvents. Before use, the plates were heated at 110 °C for 30 minutes. Chromatography was carried out in glass chambers with the height of 18-20 cm and a diameter of 16-17 cm in two directions. The plates were then cooled down, and the received lipid extracts were applied at a distance of 2,5 cm from the edge of the plate and dispersed them in prepared systems. In the first direction we used a system–chloroform: methanol: 28% ammoniain the correspondence 90:54:11, and in the other direction we used a system - chloroform: methanol: glacial acetic acid: water in the correspondence 90:40:12:2. After passing in each direction, especially after the first, the plate was well dried to the complete removal of the residues of the chromatographic systems and used for qualitative and quantitative analysis of the phospholipid classes.

The duration of chromatography in two directions was 60-65 minutes. For the identification of phospholipids, dried plates were stained in the iodine vapors during 10 min. The identification was completed by comparing the results of qualitative reactions on phospholipids with the chromatographic behavior of phospholipid standards with known values of Rf. The content of phospholipids was expressed as a percentage, calculated using the program ImageJ.

Statistical processing of the results of the research was carried out by generally accepted methods of variation statistics, according to which the data were verified on the normality of distribution for Shapiro-Wilka

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test. Since the results were normally distributed, Student's t-criterion for independent samples was used for statistical processing.

RESULTS AND DISCUSSION

As a result of the studies, it was found that in rats with glutamate-induced steatohepatitis, the percentage content of cardiolipin was on 51.3% ($p < 0.01$) higher compared with intact control (Figure 1).

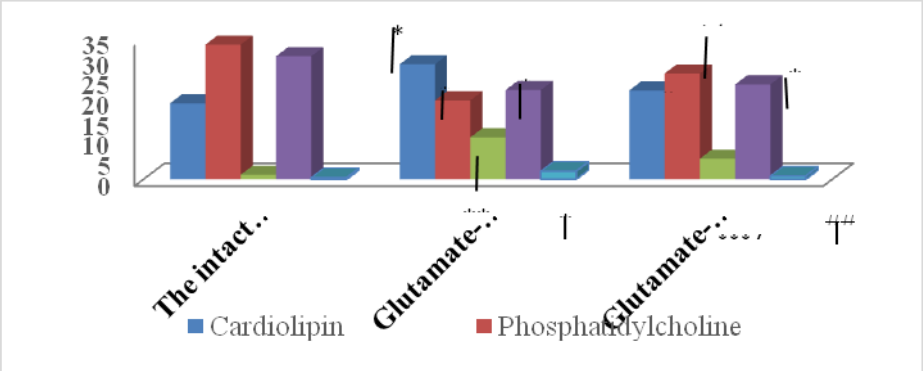

Figure 1: Content (%) of the main phospholipids of the internal mitochondrial membrane of rat hepatocytes under conditions of glutamate-induced steatohepatosis (n = 10, M ± m)* - p <0,05, ** - p <0,01, * - p <0,001 in comparison with the control group;# - p <0,05, ### - p <0,001 - in comparison with the group of rats with glutamate-induced steatohepatosis.**

Under the influence of periodic administration of $nCeO_2$in rats with glutamate-induced steatohepatitis, the percentage content of cardiolipin in the internal mitochondrial membrane was restored to the level of intact control (Figure 1).

Functionally, cardiolipin is able to regulate the permeability of mitochondrial pores, as well as maintain the functional activity of some electron transport layer (ETL) complexes. That is why the changes of its content in the membrane correlate with the level of mitochondrial leakage of protons and affects the normal functioning of all ETL complexes. In steatohepatosis, which we have demonstrated, despite the increased content of cardiolipin compared with the control, we assume that this is due to the appearance of oxidized forms of cardiolipin, which are functionally inactive due to the changed spatial organization.

The percentage content of phosphatidylcholine in the internal mitochondrial membrane of hepatocytes in rats with glutamate-induced steatohepatosis decreased by 41,5% ($p < 0.01$) compared with intact controls (Figure 1).

The decrease of the percentage phosphatidylcholine in the internal mitochondrial membrane of hepatocytes in rats with glutamate-induced steatohepatosis was accompanied by a very significant increase in the percentage content of its oxidized form - lysophosphatidylcholine (by 845,5%, $p < 0.001$), which is present in very low numbers in healthy rats in the mitochondrial membrane of hepatocytes (Figure 1).

A similar picture was observed during determining the percentage content of phosphatidylethanol amine and its oxidised form–lysophosphatidylethanol amine in the internal membrane of hepatocytemitochondria in rats with glutamate-induced steatohepatose: the percentage content of phosphatidylethanol amine decreased in comparison with intact control by 27,5% ($p < 0.05$) with a simultaneous increase of the percentage of lysophosphatidylethanol amine by 400,0% ($p < 0.001$) (Figure 1).

Significant increase of the content of oxidized forms of phosphatidylcholine and phosphatidylethanolamine in the internal membrane of mitochondria, which normally are observed in a small amount, with the increase of the content of cardiolipin in the internal membrane of mitochondria confirms the development of oxidative stress [18,19, 20, 21, 22].

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The percentage content of phosphatidylcholine under the influence ofnanocrystalline cerium dioxide cerium increased by 34,2% ($p<0.05$), and the content of its oxidised form of lysophosphatidylethanolamine decreased by 51,0% ($p<0.001$) compared to rats with glutamate-induced steatohepatosis without correction. At the same time, these indicators did not reach the level of control (Figure 1).

The percentage of phosphatidylethanol amine in the internal membrane of hepatocytes in rats with glutamate-induced steatohepatosison the background of the periodic administration of nanocry stalline cerium dioxidehad only a tendencytoincrease, while the content ofoxidisedform of lysophosphatidylethanol amine was decreasing on 60,0% ($p<0.001$), indicating the restoration of this indicator to the level of control (Figure 1).

Regarding the content of the minor components of the internal membrane of hepatocyte mitochondria in rats with glutamate-induced steatohepatosis, the percentage content of the mixture of phosphatildylinositol and phosphatildylserine did not undergo statistically significant changes (Figure 2). At the same time, the content of sphingomyelin increased by 81,3% ($p<0.01$).

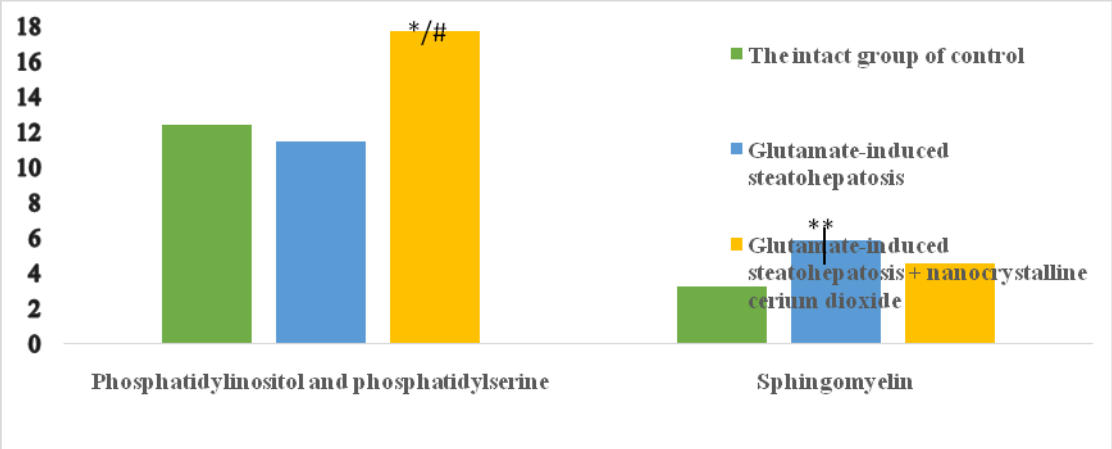

Figure 2: The content (%) of minor phospholipids of the internal mitochondrial membrane of rat hepatocytes under conditions of glutamate-induced steatohepatosis(n= 10, M ± m)* - p <0,05, ** - p <0,01 - in comparison with the control group;# - p <0,05 - in comparison with the group of rats with glutamate-induced steatohepatosis.

In rats with MSG-steatohepatitis, periodic administration of $nCeO_2$ increased the percentage content of the mixture of phosphatidylinositol and phosphatildylserineby 42,7% ($p<0.05$) compared with intact control and by 55.3% ($p<0.05$) compared to rats with glutamate-induced steatohepatosis without correction (Figure 2). On the background of the administation of nanocrystalline ceriumdioxide, the percentage of sphingomyelin was restored to the level of intact control.

Thus, the obtained data testify to the positive effect of $nCeO_2$ on the phospholipid content of the internal membrane of mitochondria, which undergoes significant changes in the development of glutamate-induced steatohepatitis. We associate this effect with the antioxidant properties of $nCeO_2$.

Studies of the interaction of $nCeO_2$ with hydrogen peroxide using X-ray photoelectron spectroscopy and UV-visible spectroscopy [13] showed that an increase in the Ce^{3+}/Ce^{4+} ratio in a nanoparticle directly correlates with its ability to perform functions of superoxide dismutase. These results convincingly confirm that the surface state of $nCeO_2$ plays a key role in the inactivation of free superoxide radicals, and the presence of cerium (III) in the surface layer is the most significant factor.

It has been shown that $nCeO_2$ (unlike superoxide dismutase (SOD)) can also inactivate the hydroxyl radical[22].

This fact was confirmed in the scientific work [21], where electron magnetic resonance method was used to confirm the ability of cerium dioxide nanoparticles in size 3–5 nm in concentrations of 1 mM and 10

μM to inactivate both superoxide and hydroxyl radicals. Using the example of a water-soluble nitroxyl radical, it was shown [6] that $nCeO_2$ can inactivate not only short-lived, but also stable radicals. Two types of CeO_2 nanoparticles 1–2 nm in size (stabilized with sodium citrate) and 3–5 nm (stabilized with sodium adenosine triphosphate) were investigated.

Interestingly, that the speed of inactivation of the studied free radicals directly depends on the size of the particles and increases proportionally with their decrease. As far as the size of $nCeO_2$ decreases the portionof Ce (III) on the surface increases, obviously that the crucial role in the inactivation of radicals play the ions of trivalent cerium.

The ability of $nCeO_2$ to regeneration is a specific and very important feature of this material. Traditional antioxidants (ascorbic acid, tocopherol, methionine, etc.) are able to participate in only one redox cycle, and then go into an oxidized inactive state or can be destroyed. Probably, cerium dioxide nanoparticles in this aspect has an advantage over existing antioxidants and in some cases surpasses them in it.

CONCLUSIONS

1. As a result of the research, it was found that in rats with glutamate-induced steatohepatitis, the percentage content of cardiolipin, lysophosphatidylcholine and lysophosphatidylethanolamine were higheron 51,3% ($p<0,01$) compared with intact control. Under the influence of periodic administration of $nCeO_2$ in rats with MSG-steatohepatitis, the percentage of cardiolipin in the internal mitochondrial membrane was restored to the level of intact control.
2. The percentage of phosphatidylcholine and phosphatidylethanolamine in the internal mitochondrial membrane of hepatocytes in rats with glutamate-induced steatohepatitis decreased by 41.5% ($p <0.01$) and 27.5% ($p <0.05$), respectively, in comparison with intact control with simultaneous increase of the percentage of their oxidized forms - lysophosphatidylcholine (by 845,5%, $p <0.001$) and lysophosphatidylethanolamine (by 400.0%, $p <0.001$).The percentage content of phosphatidylcholine under the influence of nanocrystalline cerium dioxide cerium increased by 34,2% ($p<0.05$), and the content of its oxidised form of lysophosphatidylethanolamine decreased by 51,0% ($p<0.001$) compared to rats with MSG-steatohepatitis without correction. At the same time, these data did not reach the level of control. The percentage of phosphatidylethanolamine in the internal hepatocyte membrane in rats with MSG-steatohepatitis on the background of the periodic administration of cerium dioxide had only a tendency to increase with the fall of the content of its oxidised form of lysophosphatidylethanolamine to the control level.
3. 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% ($p<0.01$). In rats with glutamate-induced steatohepatitis, periodic administration of cerium dioxide nanoparticles increased the percentage content of the mixture of phosphatidylinositol and phosphatidylserine by 42.7% ($p <0.05$) compared with intact control and by 55.3% ($p <0.05$) in compared to rats with glutamate-induced steatohepatitis without correction. On the background of the introduction of cerium dioxide, the percentage of sphingomyelin was restored to the level of intact control.

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