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INFLUENCE OF FLAVOSAN ON CALCIUM TRANSPORT, ENERGY AND REACTIVE OXYGEN SPECIES FORMATION IN LIVER MITOCHONDRIA AT HEPATITIS

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ABSTRACT
At heliotrine hepatitis, flavosan slows down the entry of calcium into mitochondria of rat liver, reduces the formation of reactive oxygen species, and accelerates the efficiency of oxidative phosphorylation. Flavosan, improving internal membranes compactness and reducing the release of cytochrome c in the intermembrane space increases rotenone-sensitive NADPH oxidase activity and on the contrary lowers of rotenone insensitive NADPH oxidase activity.

KEYWORDS: heliotrine**,** hepatitis, rat, liver, mitochondria, flavosan.

1. INTRODUCTION

Liver diseases of different etiology are rather widespread pathology.[1] According to WHO, about 2 billion people exposed to liver disease. Hepatotropic toxic substances are divided into a group of endogenous and exogenous affecting compounds. Toxic products of endogenous substances metabolism include allergic, immunopathological substances and radiation, and exogenous substances include chemical compounds, toxic substances of plants and animals. Inflammation of the liver depends on the structure, dosage and duration of hepatotoxic substances effect. Pervasion of seneciphyline from cereals seed (wheat, barley, corn, millet) and heliotrine from seed of heliotrope hebecarpous (*Heliotropium lasiocarpum*) causes toxic hepatitis. Chronic poisoning of liver by products of abovementioned plants is widespread in Central Asian Republics.[2] "Real hepatoprotectors" are few among the medicines used in the complex treatment of liver diseases[3-6, 1] and their efficiency does not meet the requirements of clinicians.[7-14, 1]

Mitochondria is considered the cell power plant that provides the cell energy necessary for all energy-dependent functions. Biological oxidation of proteins, fats and carbohydrates occurs in mitochondria and energy released in this process is spent for ATP synthesis. ATP is consumed in biosynthesis of protein compounds, active transport of molecules, cell division and cell movement. In other words, normal life of every cell in orgasm depends on mitochondria. Therefore, many scientists believe that the violation of the structure and function of the cell (in general of whole organism) is a result of mitochondrial damage, i.e. energy crisis.[15, 16] Reduced intracellular ATP content of 15-20% results in decrease of energy-dependent functions of cell to 75-80% and this process, in turn, leads to multi-systemic diseases' development: disorders of synthetic processes in CNS, heart, liver, kidneys and other organs.[17]

It is known that calcium regulates many cellular processes, including energy production in mitochondria. It is generally accepted that the synthesis of ATP in mitochondria is regulated by Ca^{2+} due to activation of several dehydrogenases in Krebs cycle.[18] Evtodienko Yu.V. and others[19] studied the Ca^{2+} accumulation in mitochondria in the physiological range (10^{-8}-10^{-6}M) at synthesis and hydrolysis of ATP in rat liver. After adding of Ca^{2+} (5×10^{-7}M) to breathing mitochondria it has been observed maximum synthesis and hydrolysis. If Ca^{2+} added to mitochondria reduced to 10^{-8}M or increases to 5×10^{-6}M it occurs inhibition of oxidative phosphorylation and ATP hydrolysis.

At heliotrine hepatitis NAD.PH dehydrogenase (quinone), NAD.PH oxidase, succinate dehydrogenase, succinate oxidase, cytochrome c oxidase and ATP synthase activities are reduced, located in respiratory chain of liver mitochondria.[20, 2, 21, 22, 23, 24, 25, 26] As a result of these changes efficiency of oxidative phosphorylation reduces and ATP synthesis slows down.[20, 27, 28, 2, 23, 30, 31, 32, 33]

Thermopsis (*Thermopsis alterniflora* Regel et Schmalh) (family of legumes - Fabaceae) is a medicinal plant, and total flavonoid - flavosan (luteolin, chrisoeriol, apigenin, Cynaroside and formononetin) of this plant has a low toxicity properties. Flavosan treatment of dogs at doses

of 5000 mg / kg orally caused no side effects and no animals died.[34] Flavosan has a strongly marked cardioprotective properties and normally affects lipid metabolism and range of lipoprotein at hyperlipidemia and atherosclerosis[34], and decreases total bilirubin and alanine aminotransferase levels in serum at hepatitis and lipid peroxidation in liver tissue. Flavosan has a tangible cardioprotective effect at hyperlipidemia and atherosclerosis, it acts effectively in disorders of lipid metabolism and lipoprotein spectrum and has antiprotector and antiatheromatosis effect.[34] Flavosan possesses antitoxic and anti-hypoxic properties.[35, 36]

At investigation the effect of flavonoids included in flavosan on mitochondria isolated from rat liver following changes were detected: luteolin is considered to be an inhibitor of respiration and oxidative phosphorylation in mitochondria[37], chrisoeriol no effect on oxidative phosphorylation of glutamate, but dose-dependently increases mitochondrial respiration at 2 and 4 states, the result of which is reduction of respiratory state of Chance.[37; 38] The ratio of ADP/O is not subject to change. Chrisoeriol dose-dependently inhibited oxidation of succinate and dinitrophenyl-dependent OXPHOS. This leads to a decrease in respiratory state of Chance.

It was found that apigenin has no effect on succinate oxidation in mitochondria, but dose-dependently reduces the rate of oxidative phosphorylation of glutamate.[37; 38] Cynaroside accelerates glutamate oxidation in mitochondria.[38] Thus indicators of oxidative phosphorylation slightly increases with glutamate and conversely decreases with succinate.

However, until now influence of flavosan on reactive oxygen species formation, oxygen accumulation, mitochondrial respiration and oxidative phosphorylation, activity of rotenone sensitive and insensitive NAD.PH oxidase is studied insufficiently.

Therefore, we set a goal to investigate, firstly influence of heliotrine on accumulation of calcium, reactive oxygen species and energy formation in mitochondria, and secondly influence of flavosane on aforecited processes at heliotrine hepatitis.

2. MATERIALS AND METHODS

For the experiment, white male rats weighing 160-180 g were used and they divided into two groups. Rats of the first group served as a control and physiological saline solution was injected subcutaneously into their bodies and rats of the secong group were injected pyrrolizidine alkaloid - heliotrine prepared in physiological saline solution at 50 mg per kg of animal body mass twice a week for two months.[2] After 2 months hepatic animals were divided into 2 groups. On per kg of animal body mass of the first group were administered 100 mg flavosane orally once a day for 30 days. The animals of the second group took physiological saline solution equal to flavosan. The third group consisted of healthy animals. Animals of all groups received food and water in a sufficient amount.

Mitochondria were isolated from rat liver.[39] The rate of respiration in different metabolic states (V2 - a state of rest prior to the addition of ATP, the V3 - after the addition of ATP, i.e. the state of phosphorylation, V4 - a condition in which ADP added to the cell turned into ATP, V_{DNP} - state after the addition of dinitrophenol) was recorded a rotating platinum electrode by using the polarographic method. Composition of measuring medium: sucrose - 0.25 M KH_2PO_4 - 5 mM Tris-HC1 - Buffer - 10 mM (pH 7.4). As substrates oxidation 10 mM of glutamate (pH 7.4) was used. The experiment started with the addition of ADP - 200 µM 2,4 - dinitrophenol (DNP) - 5×10^5 M. Respiration state of Chance and ADP (V3: V4) was calculated by the method of Chance & Williams. The rate of oxidation of substrates in different metabolic states expressed in nanograms of oxygen per minute per mg of mitochondrial protein.

Absorption of calcium cation process is one-way on a gradient of electrochemical potential, depending on the ion content in the measurement environment. The driving force of the absorption of cations is a difference in electrical potential in inner mitochondrial membrane, regulated by respiratory chain activity or a gradient of potassium ions ("diffuse potential").[14]

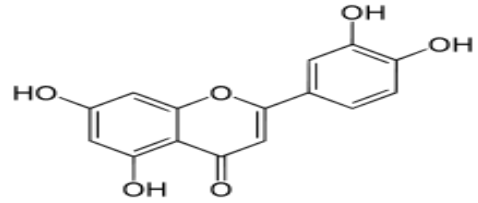
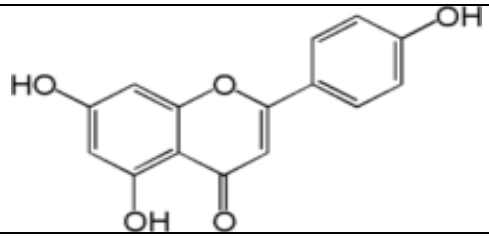
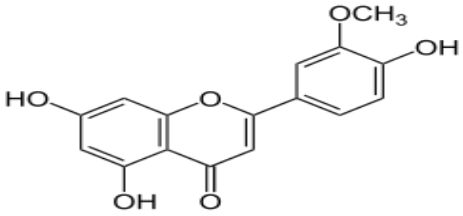
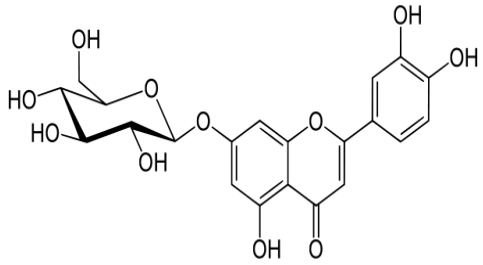
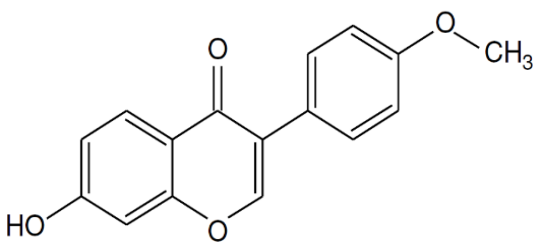
Transport of calcium through mitochondrial membrane measured by the method of pH-metry, based on exchange of $2H/Ca^{2+}$ in mitochondria. Energy-dependent process of calcium absorption is carried by uniporter mechanism irrespective of the movement of other ions. Such an accumulation of calcium in mitochondria called uniporter. By adding calcium chloride to mitochondrial suspension it was observed spontaneous calcium release accumulated within mitochondria. This release of calcium causes the activation of endogenous phospholipase and lesion of the integrity of mitochondrial membrane. This leads to change in conduction and opening of mitochondrial cyclosporin A sensitive pores (holes). So voluntary accumulation of a large number of calcium by mitochondria increases endurance of mitochondrial membrane structures to harmful effects of calcium. Calcium stoichiometric exchange on protons can be explored. At participation of phosphate as anion exchange $2H^+/Ca^{2+}$ stoichiometry is constant and equal to 1. The measurement environment for the experiment: KCl- 120mmol, tris - buffer NC1 – 10 mmol (pH 7.4), succinate - 5mmol, rotenone - 1mkg / ml phosphate (KH_2PO_4) - 1 mmol. The system is calibrated by known content of HCl.

To measure NAD.PH-oxidase systems' activity thawed mitochondria were used. Oxidase systems activity in mitochondrial membrane was measured by the polarographic method by measuring oxygen consumption rate. For this purpose, measurement

medium, consisted 0,66M of sucrose, 0.05 M Tris-HCl (pH 7,4) and 0.005 M histidine was used. Activity of NAD.PH oxidase was identified by the addition of 3 mol NAD.PH in cell with a volume of 1 ml.

Lipid peroxidation process was determined by micromethod of Y.A. Vladimirov and A.I. Archakov. Incubation medium in a mixture of 1 ml was: 0.2 mM $Na_4P_20_7{\cdot}10H_20$; 0.8mm ascorbic acid; 0.012 mM Mohr's salt ($FeS0_4(NH_4)_2{\cdot}6H_20$); 50 mM Tris-HCl buffer (pH 7.4) and 50 µl of a suspension of mitochondria. All components of medium besides ascorbic acid were added to control sample. The mixture was incubated for 20 minutes at 37^0C with constant stirring. The reaction was stopped by adding of 1 ml of 30% trichloroacetic acid and after centrifugation 6000 g/min for 10 minutes, precipitate was removed. Then 0.3 and 1.2 ml of thiobarbituric acid were added to the pellet (0,12M and 0.6 M, respectively). To enhance the formed color tube was placed in a water bath and incubated at 100°C for 10 min. The intensity of the color formed is measured spectrophotometrically at 535 nm. Formed malondialdehyde was calculated at a molar absorbance equal to 1.56/10 cm.

The rate of lipid peroxidation reaction was shown in nM malondialdehyde/mg of protein per minute. The protein content of mitochondria was determined by the method of Lowry et al.[40] Flavosan was kindly provided by Prof. Z.A. Khushbaktova and V.V. Syrov from the Institute of Chemistry of Plant Substances.

NAMES and Structures of Flavonoids, Included in Flavosan	
	**Luteolin.** 2-(3,4-Dihydroxyphenyl)- 5,7-dihydroxy-4-chromenone. Other names: Luteolol, Digitoflavone, Flacitran Chemical formula – $C_{15}H_{10}O_6$. Molar mass – 286.24 g/mol^{-1}.
	**Apigenin.** (4',5,7-тригидроксифлавон) IUPAC name: 5,7-Dihydroxy-2-(4-hydroxyphenyl)-4*H*-1-benzopyran-4-one Other names: Apigenine; Chamomile; Apigenol; Spigenin; Versulin; 4′,5,7-Trihydroxyflavone; C.I. Natural Yellow 1 Chemical formula – $C_{15}H_{10}O_5$. Molar mass – 270.24 g/mol^{-1}.
	**Chrysoeriol** IUPAC name: 5,7-dihydroxy-2-(4-hydroxy-3-methoxyphenyl)chromen-4-one Other names: Chrysoeril, Chrysoriol, Chrysoeriol, AC1NQXE5, CHEMBL214321 Chemical formula – $C_{16}H_{12}O_6$ Molar mass – 300.26 g/mol
	**Cynaroside** IUPAC name: 2-(3,4-dihydroxyphenyl)-5-hydroxy-7-[(2*S*,3*R*,4*S*,5*S*,6*R*)-3,4,5-trihydroxy-6-(hydroxymethyl)oxan-2-yl]oxychromen-4-one Other names: Glucoluteolin**;** Luteoloside**;** Cinaroside**;** 7-Glucoluteolin**;** 7-Glucosylluteolin**,** Luteolin 7-glucoside**;** Luteolin-7-glucoside; Luteolin 7-O-glucoside**;** Luteolin-7-O-glucoside. Chemical formula – $C_{21}H_{20}O_{11}$. Molar mass – 448.37 g/mol
	**Formononetin** IUPAC name: 7-hydroxy-3-(4-methoxyphenyl)chromen-4-one Other names Biochanin B, Formononetol, 7-Hydroxy-4'-methoxyisoflavone, 4'-O-methyldaidzein Chemical formula – $C_{16}H_{12}O_4$. Molar mass – 268.26 g/mol

RESULTS AND DISCUSSION

Changes in lipid peroxidation in liver mitochondria after injection of flavosan into the body of hepatic animals shown in Table 1.

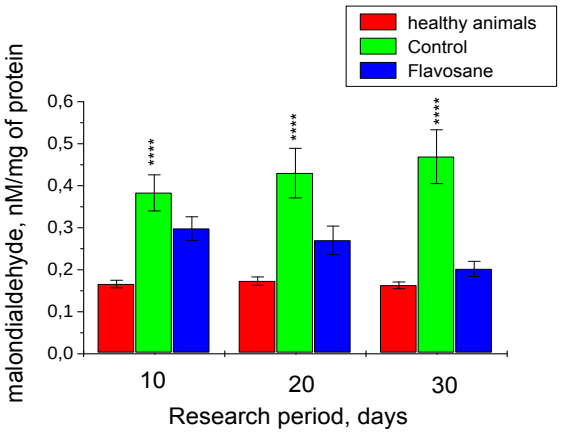


Fig. 1: Influence of flavosane on lipids peroxidation processes in liver mitochondria of hepatic animals (M ± м; n = 6-7)

Note: here and other tables confidence factor is shown as: $^{*}P<0,05$; $^{**}P< 0,02$; $^{***}P<0,01$; $^{****}P<0,001$.

If in the 10, 20 and 30 days of experiment lipid peroxidation process increased up to 2.30, 2.49 and 2.88 as compared with the healthy animals, in the animals administered flavosan these indexes increased up to 1.89; 1.65 and 1.24 as compared with healthy animals. The results showed that flavosan reduces the rate of lipid peroxidation in liver mitochondria and this process is accelerated with the duration of the experiment. The results, obtained on the effect of flavosane on calcium transport in mitochondria at heliotrine hepatitis are shown in Table 2

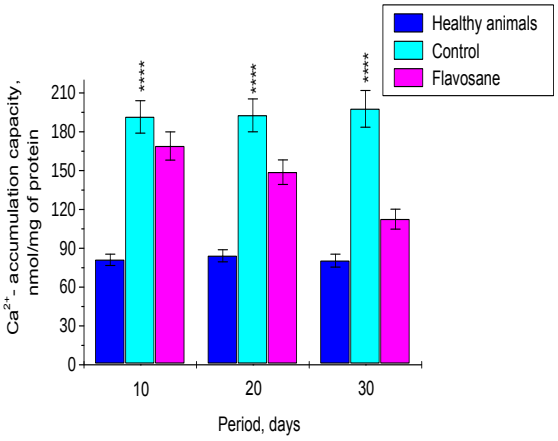


Fig. 2: Correction of Ca^{2+} accumulation change by flavosane in liver mitochondria at heliotrine hepatitis (M±м; n = 10-12).

If in animals receiving flavosane ability of mitochondria to calcium accumulation was 2.08 as compared to the control, to the 20 and 30 days was 1.77 and 1.40, respectively.

Flavosane injection into the body of hepatic animals reduces the ability of mitochondria to calcium accumulation, and this process is accelerated in accordance with the timing of the introduction of flavosane into the body. Changes in mitochondrial respiration and oxidative phosphorylation in liver mitochondria by flavosane in animals susceptible to hepatitis influenced by heliotrine shown in Table 3.

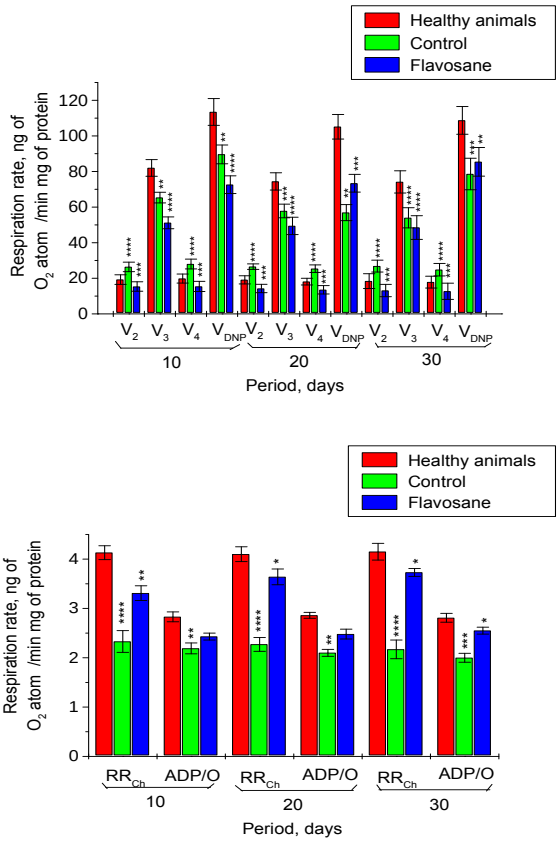


Fig. 3: Influence of flavosane on glutamate oxidation in liver mitochondria at heliotrine hepatitis (M+м; n =12-18).

In the 10 day experience glutamate oxidation in V2 and V4 states increased up to 36.9 and 41% 2 compared to healthy animals in a metabolic V3 state, and conversely decreased respiratory steady state of Chance and ADP/O ratio to 12, 4%. Glutamate oxidation in liver mitochondria of hepatic animals, receiving flavosane decreased to 35.7% compared with indexes of healthy animals, oxidation in V2 and V4 states decreased to 20.7 and 22.2%, respectively. As a result, respiratory state increased to 19.9%, ADP ratio to 13.7% and came close to that of healthy animals. It means, that flavosane for 10 days reduces glutamate oxidation in liver of hepatic animals and substantially recovers respiratory steady

state of Chance and ADP/O ratio. Thus, glutamate oxidation in liver mitochondria of hepatic animals influenced dinitrophenol decreased to 21%, while under the influence of flavosane decreased to 36%. So flavosan reduces the transfer of electrons in the respiratory chain of the NAD-dependent substrates to oxygen molecules.
Results, obtained from the 20-day of experiment almost consistent with the results obtained in the 10-day of experiment. By the 30 day of experiments glutamate oxidation in liver mitochondria of hepatic animals in V3 state decreased to 22.3% compared to the control, while under the influence of flavosane decreased by 33.6%. On the background of these changes flavosane significantly increasing respiratory steady state of Chance and ADP/O ratio, brought them to the indicators of healthy animals. However, at hepatitis flavosane has not affect on glutamate oxidation under the influence of dinitrophenol in mitochondria. So treatment of chronic heliotrine hepatitis by flavosane at the beginning of the experiment decrease of glutamate oxidative phosphorylation accelerated, but respiratory steady state of Chance and ADP/O ratio begins to recover. At continuation of the experiment flavosane gradually restores oxidative phosphorylation of glutamate and enhances rates of oxidative phosphorylation recovery. Thereof it can be concluded that effect of hepatitis treating by flavosane observed on the background of reduction of electrons transport from substrates through respiratory chain to oxygen molecule.

Calcium transport within the cardiomyocyte and across the sarcolemma is vital in regulating two essential process in the heart; intracellular Ca^{2+} is known to be the central regulator of cardiac contractility and it is becoming increasingly clear that calcium is a key second messenger in signal transduction pathways in the heart.

Cells lose viability in response to hypoxia, ische- mia, exposure to toxic chemicals, and withdrawal of growth factors. Cell death in response to these stim- uli can occur within minutes or take many hours to develop. Very rapid onset of cell death typically leads to tissue necrosis. Such necrotic cell death is the con- sequence of acute disruption of cellular metabolism, leading to ATP depletion, ion dysregulation, mito- chondrial and cellular swelling, activation of degra- dative enzymes, plasma membrane failure and cell lysis.[1] Acute cytotoxicity causing necrotic cell death di¡ers from apoptosis or programmed cell death.[2] Apoptosis takes several hours or more than a day to develop fully. In apoptosis, metabolism is not severely impaired, and cells shrink rather than swell. Indeed, apoptosis often requires the expression of speci¢c genes. In its purest form, apoptosis repre- sents a special form of cellular di¡erentiation that leads to the orderly resorption of unneeded or un- wanted cells. Apoptosis occurs frequently during em- bryonic development and is essential in the forma- tion and sculpting of body parts.[3]

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At heliotrine injection into the body it was observed the same process: the oxidation of NAD-dependent substrates ATP synthesis slows down, content of cellular ATP, phosphatidic capacity and energy charge of Atkinson decreases, i.e. with increase of proton conductivity in inner mitochondrial membrane is observed "slight" cleavage from oxidative phosphorylation.

Reducing of adenine nucleotide translocase activity in mitochondria leads to decrease in ATP synthesis. Reduced ATP content enhances lipid peroxidation. From the literature, it was known that in mitochondria without adenine nucleotides, opened pores inhibited at oxidative stress. Initially, the opening of pores is inhibited by ATP. On the other hand, at prevention of the pores induction

influence of ATP is more effective than other adenenine nucleotides.

The differences in influence of the induction process on the changes in conductivity does not depend on the mediation adenine nucleotide translocase to ADP and ATP. This means that various components are based on the effect of pore components of adenine nucleotides. Perhaps influence of ATP on the opening of pores depends on the cooperation of the kinase in outer mitochondrial membrane. Opening of Ca^{2+} channels, and change in conductivity and form of membrane by endogenous and exogenous oxidants of cells were showed by a number of authors.

Restricting of calcium entering from environment reduces the toxic effect of peroxides and increase of calcium entering into the cell accelerates cell death. Amplification of calcium entry into the cell leads to disruption of the cell and its organelles, and membranes in resulting deepens hepatocyte necrosis and cirrhotic disorders.

With heliotrine injection into the body and enhancement of liver intoxication mitochondrial swelling is strengthened and leads to rupture of outer membrane and release of 'suicide protein'. With the increase of such mitochondria in the cell, the contents of "suicide" proteins is increased to a critical level. One peculiar feature of apoptosis is considered to decrease the inner membrane potential. This is due to the opening of the pores and increased non-specific induction of the conductivity of the mitochondrial membrane. These changes cause mitochondrial swelling, outer membrane damage and release of cytochrome c and other apoptosis-causing substances from the intermembrane space.

Prolonged administration of heliotrine to the animal organism leads to release of calcium and other ions, including those leading to mitochondrial apoptosis of liver cells. As a result cell necrosis begins. At necrosis dies not only one cell, but also standing side-by-side cells and neighboring cells in the tissues. In contradistinction to apoptosis in necrosis the regulatory cell system is not able to control the processes, and thus begins chaos in metabolic processes and hydrolytic activity of lytic enzymes - lipases, phospholipases, proteases and other sharply increases and the cell will not be able to stop the pathological process.

Data on influence of flavosane on NAD.PH oxidase activity in liver mitochondria of hepatic animals are given in Table 4.

At hepatitis in the 10, 20 and 30 days of experiment rotenone sensitive NAD.PH oxidase activity decreased to 33.4; 31.3 and 39.3%, respectively. After the 20-day of treatment by flavosane rotenone sensitive NAD.PH oxidase activity has not changed, but by day of 30 almost close to normal performance. In the 10, 20 and 30 days of experiment rotenone insensitive NAD.PH oxidase activity increased to 56.2; 67.5 and 79.9%, while under the influence of flavosane 44.4; 31.5 and 22%.

So at hepatitis in liver mitochondria flavosane gradually increases rotenone sensitive NAD.PH oxidase activity and vice versa lowers rotenone insensitive NAD.PH oxidase activity and by 30 day experiment brings to the standards.

Transport of electrons from outer membrane and to inner membrane and from inside to outside at oxidation of exogenous NAD.PH in liver mitochondria occurs through cytochrome c.

Previously, scientists assumed that NAD.PH oxidation in mitochondria, broken and damaged by externally added cytochrome c is carried out only with the participation of cytochrome oxidase. Subsequently, it was shown electron transport in an intact, i.e. in a normally functioning mitochondria from outer membrane to inside. The authors concluded that in the contact areas of internal and external membranes of the mitochondria there is electron transport chain. This chain consists of complex III, NAD.PH-b5 reductase, exogenous cytochrome c and cytochrome oxidase, and they direct electrons from the surface of outer membrane and inner membrane matrix on the contrary. The activity of this path depends on the activity of the respiratory chain of mitochondria and intactness.

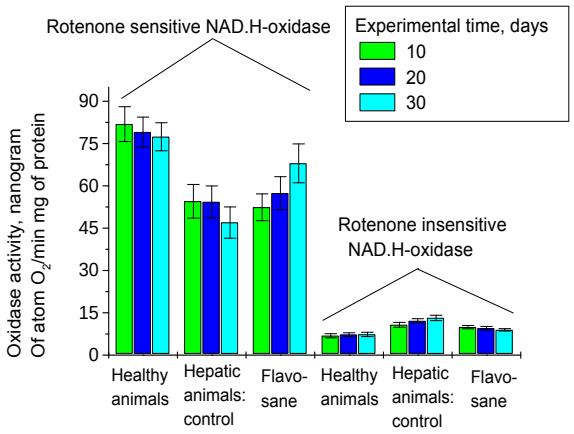

Fig. 4: Changes in the activity of NADPH oxidase at hepatitis in the liver mitochondria exposed by flavosan (M ± m; n = 6 - 7)

In consequence of the toxic effects of heliotrine on the liver tissue calcium intake in liver cells increases, the processes of calcium entry into mitochondria of cells and the formation of reactive oxygen species in mitochondria accelerates.

As a result structure and function of mitochondria is disturbed. As a result of cytochrome c release from inner membrane to the intermembrane space rotenone insensitive NAD.PH oxidase activity is reduced and it is observed gradual increase in activity of rotenone

sensitive NAD.PH oxidase. In such circumstances, it occurs junction of cytochrome b and cytochrome oxidase systems. In addition, inhibition of rotenone sensitive NAD.PH oxidase may be due to electron transfer reduction of flavin mononucleotide (FMN) to KoQ.

In our opinion, one of the causes of this process is lesion of the integrity of the blood vessels of the liver and a sharp decline in oxygen transport to the cells at hepatitis. The literature confirms the strengthening of lesion of the blood vessel system integrity and decrease oxygen transport in hepatocytes. Hypoxia in turn leads to a change in the pH of the cells, the excessive increase of calcium ions and reactive oxygen species. As a result, there are noticeable changes in structure and function of mitochondrial reticulum. This increases the size of mitochondria, reduced the number of cristae and gigantic mitochondria appear in the cell. According to the authors, at such changes in mitochondria first of all hypoxia occurs and it leads to development of cirrhosis. The rate of ATP synthesis plays an important role in the process of oxidative phosphorylation in mitochondria with an effective implementation of the various functions of hepatocytes.

So mitochondrial respiration and oxidative phosphorylation related disorders begin at heliotrine hepatitis. The therapeutic effect of flavosane at heliotrine hepatitis observed in increasing the efficiency of mitochondrial oxidative phosphorylation. Flavosane's effect on hepatitis by reducing the transfer of electrons along the respiratory chain is biologically desirable, because increased oxygen consumption in chronic hepatitis can lead to a deeper hypoxia in the tissues. In our opinion, such a useful property of flavosane as economical oxygen consumption indicates that it has a membrane stabilizer, antioxidant and antihypoxic properties. From these facts it transpires that flavosan can be an effective medicine in the treatment of hepatitis.

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