Research Article

Study of the Effects of Various Polyphenols on Proliferative Processes and the Functional State of Liver Mitochondria of Experimental Toxic Hepatitis

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ABSTRACT

Background: Toxic hepatitis is considered one of the most serious concern of health care. Because, would be reason for developing liver cirrhosis and primary liver cancer. **Materials and Methods:** In this work, models of toxic hepatitis animals prepared and effects of various polyphenols on the liver mitochondria of the model animals were studied. For this, liver mitochondria of the animals were isolated and the lipid peroxidation, the respiration rate and parameters of oxidative phosphorylation, activity of enzymes of the respiratory chain, and mitochondrial proteins synthesis were determined. **Results:** It was defined that studied polyphenols of plant origin, which are rutan and hetasan showed antioxidative properties. It was shown that these polyphenols express stabilization influence on decreased functional conditions of liver mitochondria in the case of toxic hepatitis by stimulating energy-producing function, activity of oxidative chain enzymes and mitochondria proteins synthesis. During the investigations of polyphenols' antiproliferative abilities, it was defined that rutan is characterized by considerable antifibrotic activity. **Conclusion:** The plant polyphenols rutan and hetasan are hepatoprotectors with pronounced therapeutic effects in toxic hepatitis. In this study, it was determined that the therapeutic effect of these polyphenols includes an improvement of energy metabolism, a decrease of the process of lipid peroxidation of mitochondrial membranes, as well as the proliferation of fibrous tissue in the liver parenchyma. It may be occurs do to antioxidant activity of the polyphenols.

Keywords: Functional state, Polyphenols, Proliferative processes, Toxic hepatitis.

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INTRODUCTION

Toxic hepatitis is considered one of the most serious and urgent problems of modern health care. It has been established that the risk of developing liver cirrhosis and primary liver cancer is extremely high with toxic hepatitis.[1-3]

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It is known that toxic hepatitis is accompanied by a wide range of metabolic changes and various symptoms. The leading manifestation of liver pathology is the proliferation of connective tissue.[3,4] The biochemical mechanisms of the proliferation of connective tissue in the liver have not been reliably established. It is assumed that the synthesis of collagen and glycosaminoglycans is stimulated by lipid peroxidation products - malondialdehyde (MDA) and Schiff base, released from necrotic hepatocytes.[5-7] For the treatment of toxic hepatitis, hepatoprotective agents are used that prevent the development of metabolic, functional and structural disorders in hepatocytes. The effectiveness of numerous

hepatoprotective agents, despite their positive effect on the main syndromes of toxic hepatitis remains low.

This situation gives the order to the need to search for effective domestic hepatoprotective agents for the treatment of toxic liver diseases.

From this point of view, polyphenolic compounds of plants are presented great interest. Polyphenols are an extensive group of biologically active substances in the composition of the molecules that contain two or more phenolic groups. Polyphenols have a variety of biological activities with low toxicity. They are used in medical practice as broad-spectrum drugs. Polyphenols are also used as hepatoprotectors in the treatment of liver pathologies of various etiologies.[8-10]

The aim of the study is the investigation of the effect of several plant polyphenols (rutan, hetasan, euphorbin, providin, punitan) on the proliferation of liver connective tissue, as well as on the process of lipid peroxidation, functional state, the activity of polyenzyme systems and protein synthesis in the mitochondria of liver cells during experimental toxic hepatitis caused by carbon tetrachloride.

MATERIALS AND METHODS

Causing toxic hepatitis in animals

The study was carried out on white male rats weighing 200-220g, corresponding to an age of approximately 10 weeks and before the experiment acclimatized for 1 week in defined standard living conditions, placed in 12 hr light/dark cycle, constant temperature (20 ± 2°C) and fed with pellet food and water. All experimental procedures were approved by the rules of the "European convention for the protection of vertebrate animals used for experimental and other scientific purposes" (Strasbourg, 1986) and the Institutional ethical committee in compliance of the Republic Uzbekistan legislation.

Experimental toxic hepatitis was caused by subcutaneous injection of an olive oil solution (1:1) of carbon tetrachloride (Cat No. AC148170025, Thermo Scientific Chemicals) at the rate of 0.5 ml per 100 g of animal weight 2 times a week for 2 months.[11]

Isolation of mitochondria

Mitochondria were isolated by generally accepted method of differential centrifugation as a described[12] with some minor modification. For isolation of mitochondria, the animals were euthanized by cervical dislocation, the liver was taken out, homogenized and re-suspended in isolation buffer (300 mM sucrose, 2 mM EDTA, and 5 mM Tris–HCl, pH 7.4). Mitochondria were isolated by differential centrifugation at +1°C temperature. Nuclei and intact cells were centrifuged for 12 min at 600×g. The resulting supernatant was centrifuged for 18 min at 6000×g. Mitochondria (resulting pellet) were re-suspended in 500 µl of isolation buffer without EDTA and put-on ice.

Protein content was measured by the Lowry method[13] with bovine serum albumin (HiMedia) as a standard.

Determination of lipid peroxidation

The rate of lipid peroxidation (LPO) was determined by the formation of malondialdehyde (MDA) in mitochondria.[14] For the correction of the detected abnormalities in the blood serum and liver mitochondria, the following plant polyphenols were used: rutan, hetasan, euphorbin, punitan, providin. These compounds were administered subcutaneously to the hepatitis rats for 30, 45 and 90 days with the following doses: rutan - 65 mg, hetasan - 85 mg, euphorbin - 100 mg, providin - 70 mg and punitan - 58 mg per kg of bodyweight of the animal. Vitamin E, a well-known natural antioxidant, was used as a reference drug.

Determination of respiration rate and parameters of oxidative phosphorylation

The respiration rate and parameters of oxidative phosphorylation were recorded by the polarographic method using a rotating platinum electrode as described[15] with some modifications. Respiration and oxidative phosphorylation of mitochondria were evaluated by the rate of oxygen consumption. The rate of oxygen consumption of the liver mitochondria of the experimental rats was measured using a high-precision respirator Strathkelvin Instruments in constantly stirred and thermostated (26°C) closed cell with a volume of 1 ml. The incubation medium of mitochondria consists of 120 mM KCl, 5 mM NaH_2PO_4, 10 mM Tris NCl, 5 mM glutamate, 5 mM malate and 14 mM MgCl2 (pH = 7.4). The volume of mitochondrial protein was 3-4 mg of protein in 0.05 ml of mitochondrial suspension.

The following parameters were used to assess the condition of the mitochondrial respiratory chain:

The respiration coefficient of mitochondria (V_3 / V_4) was determined by the Chans method. In addition, the ADF/O (R / C) coefficient was also used to assess the ATP synthesis activity of mitochondria. In this case, the ratio of the amount of consumed oxygen was measured when all the ADP added to the mitochondria was fully synthesized to ATP.

a) V_4 - high content of substrates in the incubation medium - 5 mM glutamate and 5 mM malate

(complex substrates I) without the presence of ADP;

b) The conditions present in the determination of V_3 - V_4 are only 200 μM in the presence of ADP (in this case, the respiratory chain is considered as a determining factor of the respiratory rate).

It was taken into account in the experiments that the amount of oxygen in 1 ml of incubation culture at 26C is 500 ng-atoms. Moreover, respiratory rate of mitochondria in different metabolic states was established by measuring of the oxidation of 1 ng-atomic oxygen per 1 min of substrate relative to 1 mg of mitochondrial protein.

Determination of activity of enzymes of the respiratory chain

The activity of enzymes of the respiratory chain - NADH-, succinate- and cytochrome-c-oxidase was determined after a single freezing-thawing of mitochondria.[16] For this, recently isolated mitochondria are placed in a freezer with a temperature of -15 to -20°C. Before the experiment, mitochondria were thawed at room temperature to a liquid consistency, then this liquid was kept at 0°C. When the mitochondria freeze, the inner membrane is damaged, and it allows to pass NADH and cytochrome c. NADH- and succinate-oxidase activity was detected in 0.03 M phosphate buffer containing $5x10^{-4}$ M EDTA at pH 7.4. Cytochrome c-oxidase activity was detected in 0.001 M EDTA and 0.2 M phosphate buffer containing 0.02 M ascorbate at pH-7.2. The final concentration of NADH in the measuring cell was 3 μmol/ml; succinate was 10 μmol/ml; cytochrome c was 1.5 mg/ml. The specific activity of enzymes was expressed in 1 min at a temperature of 25°C relative to 1 mg of mitochondrial protein.

Additionally, determination of free and bound hydroxyproline in blood serum was carried out by the oxidation of hydroxyproline by chloramine B (T) and condensation of its oxidation products with para-dimethylaminobenzaldehyde.[17]

Determination of mitochondrial proteins synthesis

The synthesis of mitochondrial proteins was established by the incorporation of C^{14}-leucine into mitochondrial proteins.[18] The proses was conducted as a follows: mitochondria were isolated and incubated with 200 μl protein-synthesizing mixture in the presence of partially purified protein synthesis modulator proteins for 10 min at 30°C. Then, [^{14}C] leucine was added, and the incubation was continued for 40 min at 30°C. After the reaction, unlabeled L-leucine was added to the incubation medium, and incubation was further continued for 10 min at 30°C. After this incubation, the tubes were chilled in an ice bath, and the reaction mixture was centrifuged at 10,000 g for 10 min at 0°C. The precipitate was washed two times with washing buffer A and the mitochondrial pellet was suspended in 1.5 ml buffer B and then incubated at 30°C for 10 min. The mixture was shaken and 100 μl of 50% trichloroacetic acid was added. The mixture was centrifuged at 10,000 g for 30 min at 0°C. The pellet was washed twice with 2.0 ml 5.0% trichloroacetic acid. The final pellet was dissolved with 300 μl 88% formic acid and transferred to a counting flask containing 10 ml scintillation liquid, and radioactivity was measured.

Statistical analysis

Statistical analysis and exponential curve fitting were performed using Origin 8.6 software (Microcal Software Inc., Northampton, MA). Results were expressed as mean ± S.E.M. To determine the statistical significance of the results One-Way ANOVA and two-tailed *t*-test were performed.

RESULTS

The first stage of our research was to study the content of MDA, a secondary product of peroxidation (LPO) in liver mitochondria of toxic hepatitis and treatment with various polyphenols for 30, 45 and 90 days. It was found that long-term administration of hepatotoxin led to a decrease in the survival rate of animals to 77%, causing toxic hepatitis, characterized by an increase of the rate of MDA formation in the liver mitochondria up to 224% (ascorbate dependence, Figure 1).

The initiation of a chain reaction of lipid peroxidation (LPO) by free radicals leads apparently to a structural and functional alteration of the biological membranes of hepatocytes, an increase of their permeability for ions with the subsequent dissociation of oxidative chains and damages to the enzyme systems of the cells.[19,20] The injection of vitamin E to the hepatitis animals caused a significant decrease of MDA to 69%. Figure 1 shows that all the studied polyphenols at 30, 45 and 90 days of administration inhibited in different degrees of the process of lipid peroxidation in liver mitochondria. The effect of polyphenols was quite comparable to the influence of a natural antioxidant - Vitamin E. However, it was found that among the studied polyphenols, rutan significantly exceeded the antioxidant effect of Vitamin E. Thus, monthly administration of rutan to experimental rats reduced the process of lipid peroxidation nearly to the control level. As toxic hepatitis progresses, possibly due to a

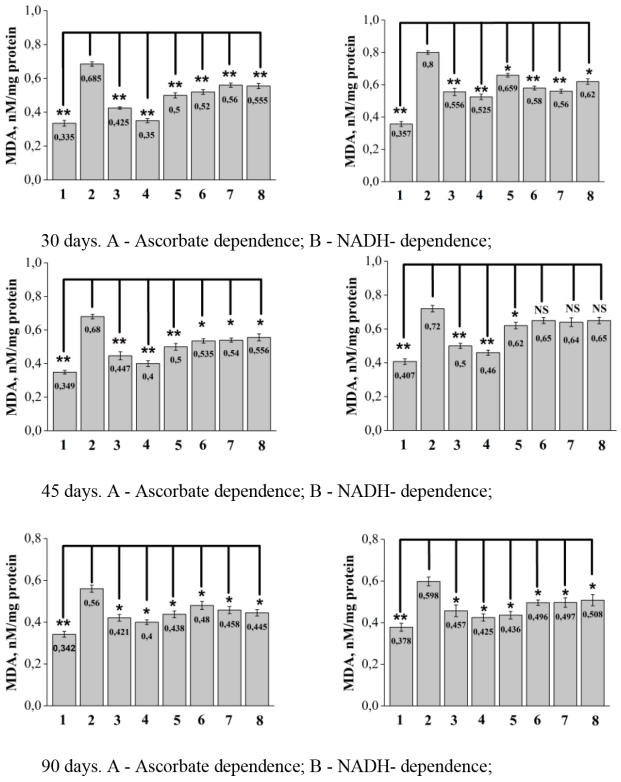

Figure 1: Changes for NADH-dependent and ascorbate-dependent MDA in the liver mitochondria of rats with toxic hepatitis for 30, 45 and 90 days and the effect of various polyphenols on it.

decrease in oxidation substrates or the proliferation of connective tissue elements in the liver, noted that there is a tendency to decrease the process of lipid peroxidation in mitochondria. Therefore, on the 45th day of the experiment, the MDA content was 80% higher than in intact animals and much lower compared to the 30th day of the course of hepatitis. Under these conditions, the antioxidant effect of vitamin E and other polyphenols was retained. As in the previous series of studies, polyphenol rutan, in comparison with other polyphenols, significantly reduced the content of MDA, although its effect did not exceed the antioxidant effect of Vitamin E.

The discovered pattern persisted even after the 90th day administration of polyphenols to hepatitis rats. The increase of the MDA content was 59%. The antioxidant effects of Vitamin E and rutan were 43.4% and 48.5%, respectively. The rest of the polyphenols also reduced the process of lipid peroxidation, although the effect of the latter was much less than the effect of Vitamin E and rutan.

Thus, in this series of studies, we found that carbon-tetrachloride hepatitis causes an increase in the process of lipid peroxidation, which decreases with the injection of various polyphenols.

Obviously, the mechanism for increasing the intensity of lipid peroxidation in toxic liver damage is injure of the mitochondrial membranes. It is known that, in various biological membranes, including mitochondrial, due to increased lipid permeability, permeability for various ions, non-electrolytes and macromolecules is induced. This effect of loss of barrier functions by membranes underlies the development of toxic forms of hepatitis. As a result of an increase of lipid peroxidation, changes in the properties of such membrane-bound enzymes like Ca^{2+}-ATPase, Na+ / K+-ATPase, cytochromes P-450, b5, cytochrome c, glucose-6 phosphatase, monoamine oxidase, phospholipase, etc. The inactivation of Ca^{2+}-ATPase slows down the "pumping out" of Ca^{2+} ions from the cell and, at the same time, accelerates the entry of calcium to the cell. This is accompanied by an increase in the intracellular concentration of Ca^{2+} ions and cell damage.[21,22] Various antioxidants are widely used to prevent this situation.

In our experiments, rutan and hetasan had the highest antioxidant activity, containing up to 6 and 8 hydroxyl groups in their molecules. It is characteristic that the general picture of the normalizing influence of polyphenols on LPO is similar to the effect of the classical antioxidant vitamin E, but the effectiveness of rutan exceeds the action of Vitamin E.

Thus, the conducted studies allow us to conclude that the studied polyphenolic compounds of plant origin have antioxidant activity, and some of them (rutan and hetasan) are prefer in efficiency to the well-known antioxidant Vitamin E.

It is known from the literature that practically all phenolic compounds have antioxidant activity. In particular, when they interact with oxidative radicals, semiquinoid radicals and radical ions are formed. In the presence of the latter, the intensity of peroxidation decreases. In this case, the activity of polyphenolic compounds depends on the number of hydroxyl groups in the molecule.[23] Currently, extensive experimental material has been accumulated, indicating a violation of bioenergetic mechanisms in mitochondria during the development of various forms of hepatitis: uncoupling of respiration and oxidative phosphorylation, a decrease of the rate of electron transfer along the respiratory chain, activation of proton ATPase, etc.[21-23] Based on these studies, it was established that the violation of the energy functions of mitochondria is a consequence of an increase in the generation of reactive oxygen species. Consequently, the search for compounds possessing antioxidant properties that can directly influence some mitochondrial processes is considered urgent.

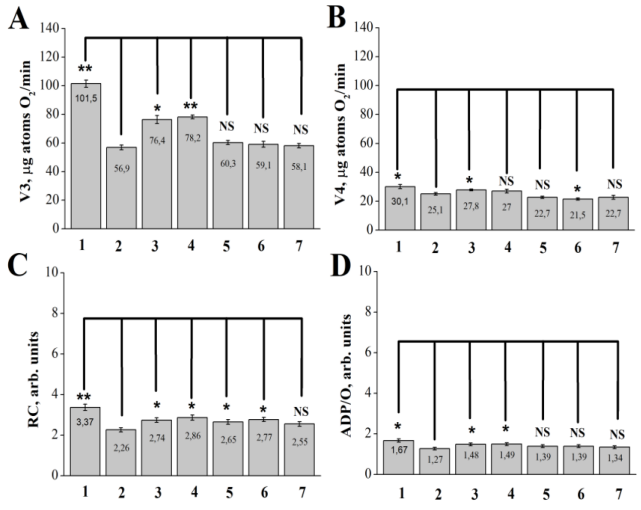

Figure 2: Influence of the injection of various polyphenols during 10 days on the respiration rate of mitochondria and oxidative phosphorylation of the liver of rats in toxic hepatitis (*n*=10; M±m).

Note: In the numerator, the oxidation substrate is succinate;

Based on this, the next stage of our research was to study the effect of various polyphenols on respiration and oxidative phosphorylation of rat liver mitochondria in toxic hepatitis. From the data presented in Figure 2, it can be seen that the mitochondria of intact rats are characterized by a high conjugation and efficiency of the processes of oxidation and phosphorylation during the oxidation of succinate. In animals with acute liver damage, there was a slowdown in respiration, a decrease of the efficiency in phosphorylation and respiratory control. Thus, the rate of oxygen consumption in the active state was reliably reduced by 44%. At the same time, noted that a less significant decrease in oxygen consumption in the regulated state (in 30%). These changes decrease respiratory control and ADP/O.

The introduction of polyphenols in experimental animals under these conditions caused various changes in the studied parameters. Among the used compounds, rutan, and hetasan were found as the most effective. It was found that, under the influence of rutan, the rate of oxygen consumption in active and regulated states increased by more than 20 and 15%, respectively, when succinate was used as an oxidation substrate. As a result, respiratory control and ADP/O increased. The introduction of hetasan also improved the functional and metabolic parameters of mitochondria in hepatitis rats, but the effect of the compound on the respiration rate and indicators of oxidative phosphorylation was low pronounced in comparison with the effect of rutan. The remaining polyphenols used in this series of studies —providin, punitan, and euphorbin — caused statistically insignificant changes in the rate of oxygen consumption and oxidative phosphorylation of mitochondria.

Thus, our studies have shown that toxic hepatitis caused by prolonged administration of carbon tetrachloride leads to impaired respiratory and phosphorylating functions of mitochondria and is accompanied by inhibition of oxidation of NAD-dependent substrates. Probably, an increase of the LPO rate observed in chronic liver damage leads both to a direct effect of lipid peroxidation products on the lipid matrix of mitochondrial membranes and to an indirect effect of LPO metabolites on the respiratory function of mitochondria. It is known that hydroperoxides, unsaturated aldehydes and MDA formed during LPO are mutagens and have pronounced cytotoxicity, suppress glycolysis and oxidative phosphorylation in mitochondria.[22-25] Disturbances from oxidative phosphorylation and energy metabolism of the liver can occur already in the early stages of the development of hepatitis when there are still no noticeable changes in the membrane structures of cells. This is evidenced by the data obtained on the 30th day of the development of pathology. Later, as the pathological process progressed, the functional and metabolic parameters of mitochondria during the oxidation of NAD-dependent substrates were even more disturbed, involving the membrane structures of hepatocytes in this process.

The 10-day administration of plant polyphenols to hepatitis animals gradually improved the parameters of energy metabolism of mitochondria. Among the studied polyphenols, rutan and hetasan were the most effective. Showing a powerful antioxidant effect, these compounds stimulated the functional and metabolic parameters of mitochondria, restoring the respiratory function of mitochondria.

Obviously, in conditions of toxic hepatitis caused by the introduction of carbon tetrachloride, the polyphenols rutan and hetasan influence the main pathogenetic links of hepatitis in addition to the antioxidant effect by directly protecting the mitochondrial respiratory function.[22-26]

After the detection of a violation of the respiratory and phosphorylating activity of mitochondria during the oxidation of NAD-dependent substrates in the liver mitochondria of rats with toxic hepatitis, it was suggested that carbon tetrachloride may inhibit the activity of mitochondrial enzymes. However, the question of how deeply hepatotoxin penetrates certain enzymatic spaces remained unclear in the area of localization of NAD- and FAD-dependent enzymes. The main interest was the study of the effect of polyphenol compounds on the activity of enzymes damaged by carbon tetrachloride.

In this regard, the studies were carried out to investigate the activity of enzymes in the mitochondrial respiratory chain. We selected three links of the respiratory chain. The first link was the dehydrogenation of substrates controlled by enzymes containing nicotinamide coenzymes (NAD and NADP). The second link was controlled by the flavin-dependent enzyme system. The third link was the oxidation of reduced forms of flavin coenzymes. In the final link, the biological oxidation of hydrogen is accepted by molecular oxygen to form water. We studied the activity of the following enzymes: NADH dehydrogenase, succinate dehydrogenase and cytochrome oxidase - which are located in the inner membrane of mitochondria and are involved in the transfer of electrons in all three links of the respiratory chain.

The study of the activity of NADH - dehydrogenase of rat liver mitochondria showed that the introduction of carbon tetrachloride inhibits the activity of the enzyme. Polyphenols used as mitochondrial stabilizing agents had different effects on the activity of NADH dehydrogenase. Figure 3 shows that 10 days of administration of all studied polyphenols increased the reduced activity of the enzyme. The most effective activators of NADH dehydrogenase were rutan, and hetasan. However, under the influence of these polyphenols, the activity of NADH dehydrogenase was not completely restored. The polyphenols euphorbin, punitan and especially providin had a slight stimulating effect on enzymatic activity.

The regularity of changes of the activity of NADH-dehydrogenase, found after 30-day administration of polyphenols, persisted after a longer administration of these compounds for 45 days (Figure 3), with the only difference that the stabilizing effect of polyphenols

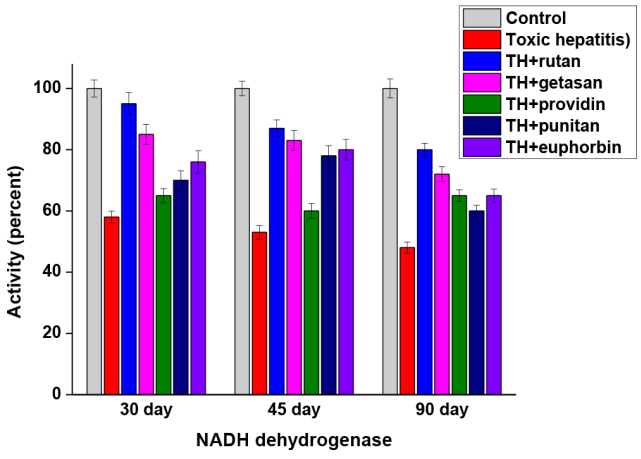

Figure 3: Effect of polyphenols on the activity of NADH-dehydrogenase of rat liver mitochondria in toxic hepatitis (*n* =10).

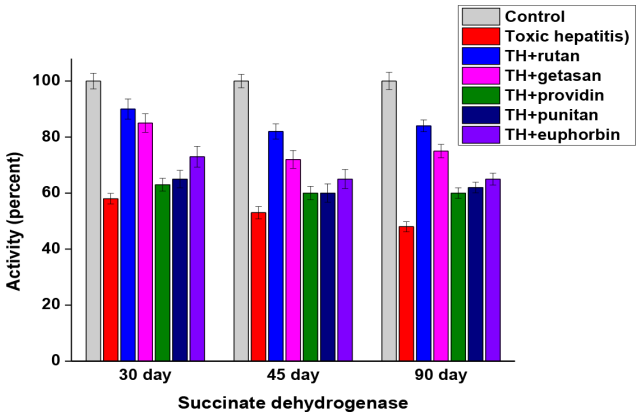

Figure 4: Effect of polyphenols on the activity of succinate dehydrogenase of rat liver mitochondria in toxic hepatitis (*n* =10).

was less pronounced. Even longer administration of polyphenols for three months also led to a slight decrease in the effectiveness of polyphenols on the activity of NADH dehydrogenase.

In the next series of studies, the activity of succinate dehydrogenase in the liver mitochondria of hepatitis rats was studied and it was found that the activity of the enzyme significantly decreases to 36% in this pathology (Figure 4).

The introduction within 30 days of polyphenols – rutan and hetasan were a positive stabilizing effect on succinate dehydrogenase, although a complete recovery of enzymatic activity was not observed. The rutan was the most effective, and providin was the least effective among all the studied polyphenols.

The obtained results after polyphenols administration for 30 days showed that long-term administration of polyphenols has a lesser stabilizing effect on the activity of succinate dehydrogenase. Therefore, 45 days and 90 days of administration of all the studied polyphenolic compounds did not change the general picture.

Our subsequent studies were devoted to the determination of the activity of rat liver cytochrome oxidase with the introduction of carbon tetrachloride. According to Figure 5, in chronic liver intoxication with carbon tetrachloride, the activity of this enzyme decreases almost by 48%.

Cytochrome oxidase is the final component of the respiratory enzyme chain, which transfers electrons from cytochrome C to molecular oxygen. Only cytochrome oxidase is capable of reacting directly with oxygen among all the carriers of the electron transport chain. Cytochrome oxidase is a complex protein, the molecule which includes two hems, two copper atoms, and 20-30% of the lipid component. Significant inhibition of enzymatic activity found in our experiments is

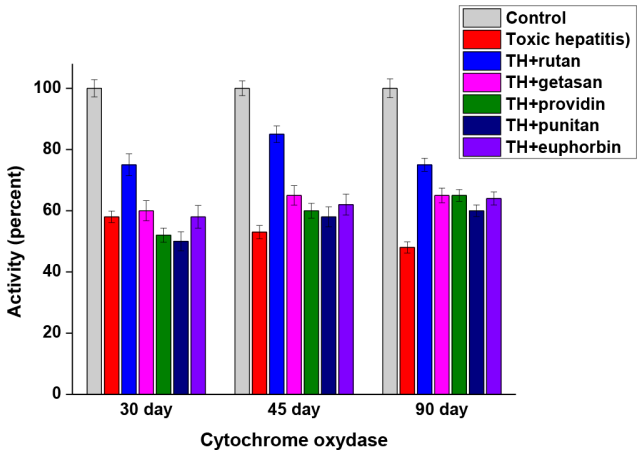

Figure 5: Effect of polyphenols on the activity of cytochrome oxidase of rat liver mitochondria in toxic hepatitis (*n* =10).

associated with damages to the lipid component and a change in the region containing hems and copper atoms.[27,28] It is known that when copper is separated from cytochrome oxidase, the enzyme is completely inactivated. The decrease of the activity of cytochrome oxidase is played a certain role in the interaction of LPO products with the enzyme molecule, leading to the inactivation of the last.

Polyphenol administered within 30 days restored enzyme activity only by 18%. Hetasan and euphorbin restored the enzymatic activity even less - by 8%. Euphorbin and providin had almost no effect on cytochrome oxidase.

The introduction of the above polyphenols for 45 days had a better stabilizing effect of the enzymatic activity: rutan and hetasan increased the activity of cytochrome oxidase by 22% and 16% respectively. The rest of the polyphenols also showed more effectiveness in comparison with the previous period of administration. The situation that was observed with a 45-day administration of drugs was persisted in a three-month administration of polyphenols.

Thus, the results of the studies showed that intoxication with carbon tetrachloride leads to inhibition of the enzymes of the mitochondrial respiratory chain. Probably, the detected inhibition of enzyme activity by hepatotoxin is associated not only with the violation of mitochondrial oxidation and inhibition of conjugated phosphorylation but also with the direct effect of carbon tetrachloride on the structure of the respiratory chain enzymes. The used polyphenols have a stabilizing effect on enzymatic activities.

Many natural and artificial origin polyphenols are used in medical practice for the complex therapy of acute and toxic hepatitis, liver cirrhosis. One of the mechanisms of influence of polyphenolic compounds, in addition to the antioxidant effect, is the ability to restore the function of the respiratory chain and associated phosphorylation.[21,22,29-31] Probably, polyphenolic compounds are involved in the transfer of electrons along the respiratory chain. They have high antiradical activity, prevent the development of free radical oxidation reactions and the formation of lipid peroxides and can shunt the transport of electrons in the respiratory chain of mitochondria. Rutan and hetasan were effective in our experiments so apparently cause a similar effect on the polyenzyme complex of liver mitochondria. They have the possible optimal redox potential and have conformational accessibility for interaction with inhibited respiratory enzymes and carrying out both one- and two-electron transfer.

After establishing the stimulating effect of some polyphenols on the enzymes of the mitochondrial respiratory chain and on the energy-producing processes occurring in the liver of rats with toxic hepatitis, it was necessary to find out whether the discovered effect of polyphenols is associated with the regulation of the latter of mitochondrial protein synthesis. It is known that mitochondria have an autonomous transcription and translation system.

To clarify this issue, a whole series of studies were carried out in which the incorporation of labelled H^3-leucine into the proteins of isolated mitochondria of the liver of rats with toxic intoxication, as well as from the liver of hepatitis rats after administration of various polyphenols for 30, 45 and 90 days.

Figure 6 presents the results on the effect of various polyphenols on the biosynthesis of proteins of mitochondria in the liver of hepatitis rats with the introduction of polyphenols for 30, 45 and 90 days.

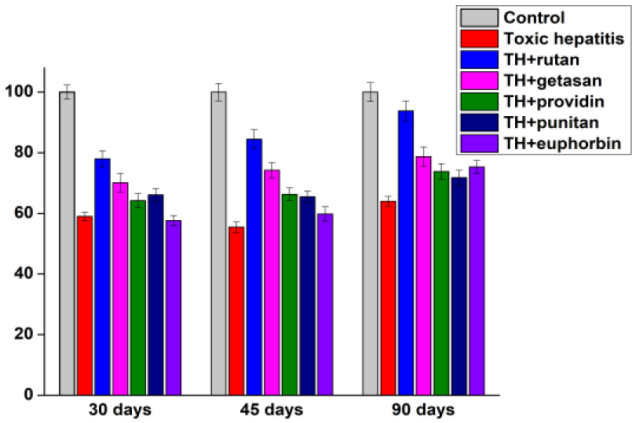

Figure 6: Influence of various polyphenols on the incorporation of C_{14}-leucine into mitochondrial proteins of the liver of rats in toxic hepatitis.

The presented data show that carbon tetrachloride intoxication after 30 days of pathology development causes a significant decrease (41%) in the incorporation of labelled leucine into mitochondrial proteins, which is consistent with the existing opinion about the specific damage of not only nuclear but also mitochondrial genomes by this hepatotoxin.[22,23,32]

The administration of various polyphenols to hepatitis rats did not induce a complete restoration of the protein-synthesizing system of mitochondria, although some polyphenols increased the biosynthesis of mitochondrial proteins. Thus, the polyphenol rutan increased the incorporation of the label into the mitochondrial proteins of hepatitis animals by 19%.

Long-term administration of polyphenols for 45 days had a more pronounced positive effect on the biosynthesis of mitochondrial proteins. Thus, in rats treated with rutan and hetasan, the increase of the incorporation of labelled leucine into mitochondrial proteins were 29.0% and - 20% respectively. In addition, punitan, providin, and euphorbin are ineffective after 30-day administration; they also, to varying degrees, growth the biosynthesis of mitochondrial liver proteins in hepatitis animals.

These data are consistent with the published results on the stimulation of the mRNA level by polyphenols, which is responsible for the synthesis of cytochrome oxidase subunits,[27] as well as with data obtained using other models. Thus, *in vivo* administration of polyphenols increased the maximum respiration rate achieved in the presence of the substrate ADP and phosphate, which was caused by an increase of the synthesis of mitochondrial proteins by polyphenols.

Thus, on the basis of the experiments carried out to study the effect of polyphenols on the synthesis of mitochondrial proteins, data were obtained indicating that the polyphenols rutan and hetasan have the ability to stimulate translation processes in the mitochondria of rat liver during intoxication with carbon tetrachloride.

The results obtained in our experiments on the restoration of the biosynthesis of mitochondrial proteins in liver cells of hepatitis rats under the influence of various polyphenols were indicated the importance of using such compounds for the treatment of toxic liver damage.

According to the known mechanisms of fibrogenesis, an antifibrotic drug must combine a number of properties: it must contain antiviral activity, an anti-inflammatory effect, influence the immune response to prevent and reduce the activation of hepatic stellate cells, and regulate the apoptosis of these cells.[33] At present, great importance is attached to the study of the processes of fibrotization of the liver tissue in hepatitis and cirrhosis. The ability of hepatoprotectors to reduce the leading manifestation of toxic liver pathology - the proliferation of connective tissue is great importance. The molecular mechanisms of proliferation of connective tissue in the liver during the development of hepatitis have not been reliably established. It is assumed that the synthesis of collagen and glycosaminoglycans is stimulated by lipid peroxidation products - malondialdehyde and Schiff bases, released from necrotic hepatocytes.[34,35]

For the confirm this assumption, in the next series of experiments, we studied the content of oxyproline, a marker of connective tissue proliferation, in the liver of rats with carbon tetrachloride hepatitis, as well as the effect of various polyphenols on this indicator.

The data presented in the Figure 7, indicate that toxic hepatitis is accompanied by an increase in the content of free and bound hydroxyproline by 36% and 37%, respectively, compared with the control. The administration of polyphenols to sick animals caused, to varying degrees, a pronounced decrease in both forms of hydroxyproline. The most effectives were rutan and hetasan. However, despite the fact that these polyphenols, especially rutan, decreased the serum hydroxyproline content of experimental rats, the concentration of this compound was still higher than in intact rats.

The further course of toxic hepatitis caused to an increase of free and bound forms of hydroxyproline in the blood of experimental animals. The content of free and bound hydroxyproline in this group of rats were increased by 50 and 52%, respectively, compared with intact animals. Among the polyphenols used, as in previous experiments, rutan had the greatest reducing effect. The effect of hetasan on the oxyproline content was less pronounced than rutan. The rest of the polyphenols, although they caused a decrease in the content of hydroxyproline, the obtained results were unreliable. In the next series of experiments,

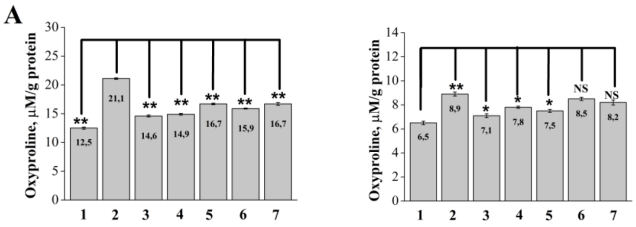

Figure 7: Changes of content of hydroxyproline in the blood serum of rats with toxic hepatitis and after administration of various polyphenols for 30, 45 and 90 days (*n* = 6; M ± m).

Note: A. Free oxyproline; B. Bounded oxyproline;

similar results were obtained. With the progression of the pathological process in the liver on the 90th day of the development of toxic hepatitis the content of the studied forms of oxyproline increased even more (by 55.5% and 59%, respectively) in the blood serum. These data indicate the stimulation of the proliferation of connective tissue, leading to an increase in liver fibrosis.

DISCUSSION

Pharmacotherapy with polyphenols contributed to a decrease of the level of hydroxyproline in the blood of hepatitis rats, and the result found in the study of rats remained on 30 and 45 days of the experiment: rutan had the highest hydroxyproline-lowering ability, while hetasan was less effective. As a result of our studies, we have established that rutan and hetasan among all the studied polyphenols have a very important property for the hepatoprotector - antifibrotic effect.

Therefore, the obtained data in our study show that, as a result of the development of toxic hepatitis under the action of carbon tetrachloride, the initiation of a chain reaction of lipid peroxidation (LPO) by free radicals is observed, which apparently leads to a structural and functional alteration of biological membranes of mitochondria of hepatocytes, an increase in them permeability to ions with subsequent dissociation of oxidative chains, damage to the enzyme systems of the cell.[19,20]

All studied polyphenols (rutan, hetasan, providin, punitan, and euphorbin) exhibit antioxidant properties in different degrees. A comparative study of the effect of these polyphenols on the content of the lipid peroxidation product, MDA, showed that rutan and hetasan are able to significantly suppress the formation of MDA, in contrast to providin, punitan, and euphorbin at all studied periods. It should be noted that the antioxidant effect of rutan was superior to the natural antioxidant Vitamin E. These results are consistent with the existing literature that polyphenol compounds as antioxidants play an important role in the prevention of structural and dysfunction of the liver in various pathological conditions by accelerating regeneration and restoring functional activity.[6,36-42]

The biological role of polyphenols in an animal cell is associated with their ability: first, to form strong chelate complexes with various metal ions; secondly, to interact with free radicals; thirdly, to participate in the transport of electrons; fourthly, to bind with various enzymes, changing their activity. In clinical practice, vast experience has been accumulated in the use of drugs based on polyphenols as immunotropic, antiproliferative, lipid-correcting and antioxidant compounds.

Currently it is established that LPO products can directly affect the lipid matrix of mitochondrial membranes, as well as change the functional state of mitochondria using various indirect mechanisms. It was found that the intensification of lipid peroxidation can initiate the opening of the mega-channel, cyclosporin A, of the sensitive pore of the inner mitochondrial membranes, which, can lead to the development of various pathological processes.[40-43]

On the other hand, it is known that incubation of mitochondria in the presence of LPO inducers activates mitochondrial phospholipids,[44,45] most of which is phospholipase A. Activation of this enzyme leads to the accumulation of lysophospholipids and free fatty acids in the mitochondrial membrane, which increase the permeability of the inner mitochondrial membrane, thereby affecting the synthesis and activity of membrane-bound enzymes and the process of oxidative phosphorylation. The last plays an important role in the energy metabolism of the cell and its insufficiency in toxic hepatitis leads to the suppression of NAD-dependent dehydrogenases of the Krebs cycle and FAD-dependent succinate dehydrogenase, as well as cytochrome oxidase. The polyphenols used in our experiments contributed to the restoration of the energy deficit of the cell. Rutan and hetasan significantly increased the oxidative phosphorylation of mitochondria, as well as the activities of NADH - dehydrogenase, succinate dehydrogenase and cytochrome oxidase. The basis of the positive effect of these polyphenols on the formation of energy in mitochondria and stimulation of the activity of enzymes in the respiratory chain is apparently the participation of polyphenols in the transfer of electrons along the respiratory chain and an increase of oxygen supply to mitochondria.

Carbon tetrachloride is used in experimental studies as a model of acute toxic liver damage based on metabolic activation of cytochrome P-450,[46,47] Developing liver failure associated with the intensification of peroxidation processes, the production and accumulation of highly toxic metabolites, leads to necrosis of hepatocytes and liver fibrosis. As a result of our studies, we found that under the influence of rutan in the blood of hepatitis rats, the content of oxyproline, the main marker of the growth of liver connective tissue, decreases. The introduction of rutan for 45 and especially 90 days significantly reduced the amount of hydroxyproline, and therefore had an effect on the process of liver fibrosis. The rest of the studied polyphenols, including hetasan had a less pronounced antifibrotic effect.

CONCLUSION AND SUMMARY

Therefore, based on the conducted studies, we assume that the plant polyphenols rutan and hetasan are hepatoprotectors with pronounced therapeutic effects in toxic hepatitis. The therapeutic effect of these polyphenols includes an improvement of energy metabolism, a decrease of the process of lipid peroxidation of mitochondrial membranes, as well as the proliferation of fibrous tissue in the liver parenchyma. Probably, in the mechanism of inhibition of collagen neoplasm by these compounds, both the antioxidant effect of polyphenols and the direct effect of the last on the content of oxyproline are of primary importance.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

MDA: malondialdehyde; **EDTA:** ethylenediaminetetraacetic acid; **LPO:** lipid peroxidation; **ATP:** adenosine triphosphate; **NADH:** nicotinamide adenine dinucleotide; **PMSF:** phenylmethylsulfonyl fluoride; **FAD:** flavin adenine dinucleotide; **ANOVA:** analysis of variance;

Authors' Contribution

Authors SD, GM, SK, MG, and GU did experimental design work. SK, GM, MY, NKh, DT, SKh, FH, FE and GU conducted experiments. Authors SD, GM, SK, FH, and FE performed the statistical analysis, wrote the protocol, managed the analyses of the study, and wrote the first draft of the manuscript. All authors read and approved the final manuscript.

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