Central Asian Journal of Medicine

HEPATOPROTECTIVE PROPERTIES OF LOW MOLECULAR WEIGHT CHITOSAN

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ABSTRACT

In 30 rats with acute toxic liver damage caused by the administration of CCl4 in olive oil over a span of four days, at a dosage of 2.5 milligrams per kilogram of body weight, biochemical indicators of liver damage were studied. To reproduce acute toxic liver damage, 30 rats were administered CCI4 4 times at a dose of 2.5 ml/kg body weight subcutaneously for 4 days. No mortality was observed. Pharmacotherapy of acute toxic liver damage was carried out 24 hours after the final administration of the toxicant. The animals were separated into the following groups: control group (10 rats) with ATH + placebo (H2O); comparison group (10 rats) with ATH + comparison drug Carsil at a dose of 100 mg/kg; main group (10 rats) with ATH + LMWC at a dose of 25 mg/kg. The drugs were administered as a suspension intragastrically through a special tube for 12 days. In the control group, the development of syndromes of cytolysis, cholestasis, mesenchymal inflammation, and hepatic cell failure, a decrease in catalase activity, and an elevation in the content of MDA, FASL, and NF-kB was established, which coincided with the morphological signs of hepatocyte destruction. Low-molecular chitosan obtained from Bombyx mori pupae to a certain extent restored the histiostructure of the liver of rats with ATH, reduced the processes of lipid peroxidation and apoptosis, cytolysis, cholestasis and hepatocellular failure. In terms of hepatoprotective action, it was not inferior to the classical hepatoprotector Carsil.

Key words: liver, carbon tetrachloride, low molecular weight chitosan, carsil.

INTRODUCTION

Chitosan belongs to the group of biocompatible and biodegradable polymers, absolutely safe, non-toxic. This is a cationic aminopolysaccharide of natural origin, a copolymer of glucosamine and N-acetylglucosamine, which is synthesized by partial deacetylation of chitin (β-(1–4)-Poly-N-acetyl-d-glucosamine) [15]. Based on molecular weight, chitosan can be divided into chitosan with a high molecular weight of 190 to 375 kDa, with a deacetylation degree (DD) of more than 75%, and chitosan with a low molecular weight of 20 to 190 kDa, with a deacetylation degree of less than 75%. A high level of deacetylation indicates a low rate of dehydration, respectively, a low degree of deacetylation indicates a high level of dehydration [3]. With increasing molecular weight, the activity of chitosan increases. But despite this, chitosan with low and medium molecular weight exhibits higher antimicrobial properties [1-17]. However, their hepatoprotective properties have not been studied.

Purpose of the research

To evaluate the liver-protecting effects of low molecular weight chitosan derived from pupae in a model simulating acute toxic liver injury.

Materials and Methods

Experimental studies were carried out following the requirements of the Declaration of Helsinki for the humane treatment of animals (Strasbourg, 1985) and the “Regulations on the use of laboratory animals in scientific research” atelier work and pedagogical process of Tashkent Medical Academy (TMA) and methods for implementing the requirements of biomedical ethics.” In order to address these issues, trials were conducted on 40 adult male rats weighing between 160-180 grams initially. These rats were maintained on a standard diet within the laboratory facilities of the Center for Biomedical Technologies (CBT) at TMA, specializing in pharmacology and toxicology. For the experiment, outbred white male rats of the same litter, the same age and body weight were used. They were kept in quarantine for a week and only after that were included in the experiments. The intact group consisted of 10 rats of the same litter. To reproduce acute toxic liver damage, 30 rats were administered CCI4 4 times at a dose of 2.5 ml/kg body weight subcutaneously for 4 days. No mortality was observed. Pharmacotherapy of acute toxic liver damage was carried out 24 hours after the final administration of the toxicant. The subjects were categorized into the following sets: a control group (consisting of 10 rats) receiving ATH+ placebo (H2O); a comparison group (with 10 rats) receiving ATH+ a reference drug, Carsil, at a dosage of 100 mg/kg; and a primary group (comprising 10 rats) receiving ATH+ LMWC at a dosage of 25

mg/kg. The medications were administered orally as a suspension through a specialized tube over a period of 12 days.

To assess the detoxifying function of the liver, an etaminal test was performed 24 hours after the last administration of drugs. The principle of the method is based on the fact that etaminal itself exhibits a hypnotic effect; after its metabolism in the liver by the cytochrome P450-dependent monooxygenase system, it loses its pharmacological properties. For this purpose, the animals were subcutaneously injected with etaminal sodium at a dose of 40 mg/kg and the time of falling asleep and waking up, the absence of the digestion reflex was recorded, and their difference (min) was calculated, which reflects the state of the detoxifying function of the liver. 24 hours after the test, the animals were decapitated under light ether anesthesia, and blood was collected for biochemical studies and liver for morphology. In the blood serum, the activity of alanine (ALT) and aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (GGTP), the content of total protein, albumin, glucose, cholesterol, bilirubin, urea, creatinine, uric acid was determined on a biochemical analyzer MINDRAY BA-88A (China) using special test kits from the company in addition, catalase activity [9, Koralyuk M.A.], the content of malondialdehyde (MDA) [1, Andreeva L.I.], as well as the content and by the enzyme immunoassay method on an enzyme immunoassay analyzer ELISA (country) using test kits from the company. For histological analysis, liver samples were preserved in a solution containing 10% formalin, ethyl alcohol, and acetic acid, followed by embedding in paraffin. Sections measuring 4–5 µm in thickness were prepared and stained with hematoxylin and eosin to evaluate the overall tissue structure.The preparations were visualized in Polyvar (Reichert-JUNG, Austria) and Leica DMRE (Leica Microsystems Wetzlar GmbH, Germany) microscopes with a digital video surveillance system and “Videotest-4” image analysis program. The data collected during the research underwent statistical analysis using Microsoft Office Excel-2012 software on a Pentium-IV personal computer, utilizing its integrated statistical processing functions.

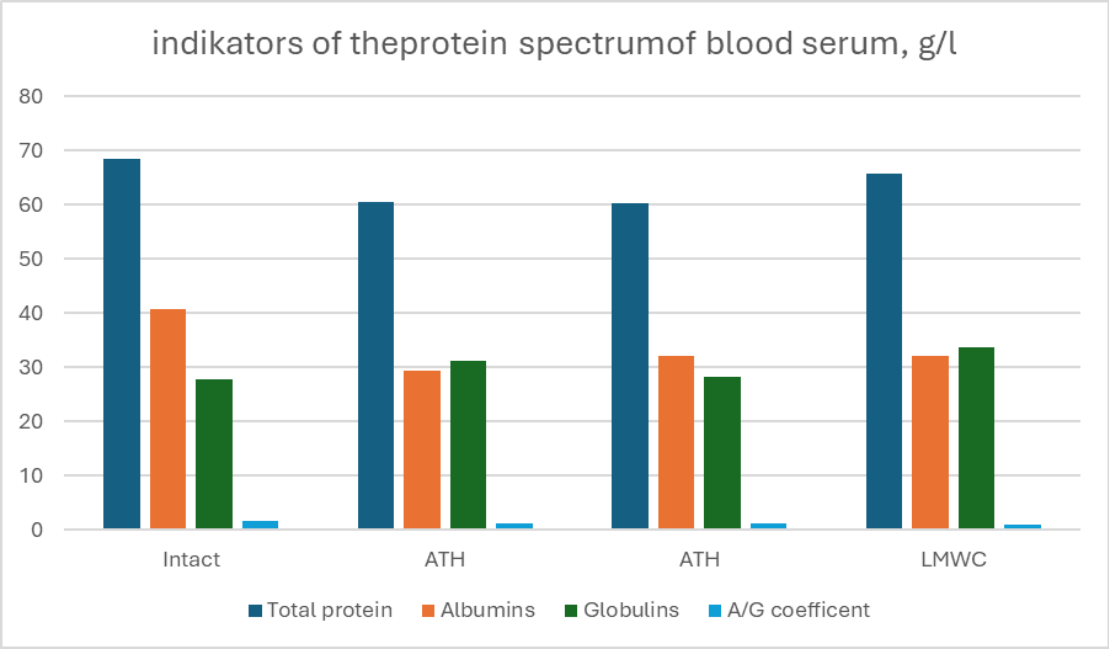
Results and Discussion

The studies showed that in the control group of rats, the duration of hexenal sleep was 180.33±5.97 minutes, while the value of this indicator in intact rats was 74.66±4.21, which exceeded the normative values by 2.42 times ($P<0.001$). In the comparison group (ATH+carsil), the duration of hexenal sleep was 95.17±4.47 minutes, which is 1.89 times ($P<0.01$) shorter than the indicators of the control group of animals, but 1.27 times ($P<0 .05$) longer than the values of intact rats. The use of LMWC at a dose of 25 mg/kg for 12 days contributed to a shortening of

sleep duration, amounting to 114.17±5.47 minutes. This was 1.58 times (P<0.01) shorter than the values of the control group, but still significantly exceeded the values of the comparison group and intact rats by 1.2 (P>0.05) and 1.53 times (P<0.05), respectively.

Thus, we can say that LMWC increases the pharmacometabolizing function of the liver of rats with ATH and its effect is comparable to the well-known hepatoprotector carsil.

Analysis of biochemical parameters showed that in rats of the control group with ATH in the blood serum, there was a tendency towards a fall in the content of total protein (see Table 1). NMH slightly increased its level, while carsil did not have such an effect. In ATH rats, the albumin content decreased statistically significantly by 1.39 times (P<0.05) relative to the values of intact rats. When treating ATH pharmacologically with the reference drug Carsil, we noted a slight inclination towards elevation in serum albumin levels compared to the untreated group. However, these levels remained statistically significantly lower, showing a decrease of 1.27 times (P<0.05) compared to the readings from healthy, untreated rats. The direction of changes in the content of albumin in the blood serum was the same in the group of rats receiving LMWC at a dose of 25 mg/g. This indicator remained lower by 1.27 (P<0.05) times relative to the values of the intact group of rats.



The content of globulins in the blood serum of rats with ATH tended to increase, indicating the presence of pronounced inflammatory processes in the liver parenchyma of experimental animals. The comparison drug carsil brought the studied indicator closer to the measurements of healthy rats, while when using LMWC its values remained at the level of the control group of animals. The

developing dysproteinemia contributed to a decrease in the albumin/globulin ratio. In intact rats, this indicator was 1.55±0.12. At the same time, this ratio in rats with ATH decreased by 1.43 times (P<0.05), indicating a fairly pronounced fall in the content of the synthetic function of hepatocytes against the background of activation of stellate cells and the presence of inflammatory processes in the liver of experimental animals. During experimental pharmacotherapy with the reference drug carsil, this indicator only tended to increase and remained below 1.32 times (P<0.05) relative to the measurements of the healthy group of rats. This indicates the persistence of structural changes in the liver parenchyma of rats in this group and was mainly associated with low levels of albumin. The use of LMWC did not have a noticeable effect on the A/G coefficient. This indicator remained within the measurements of the control group of animals and was significantly lower by 1.51 times (P<0.05) than the measurements of the healthy group of animals. This was mainly due to the persistence of high globulin values, indicating a weak effect of this drug in the correction of ATH.

The blood serum glucose levels in rats with ATH showed a tendency to increase in comparison to those of healthy rats. Treatment with ATH Carsil showed no significant impact on glucose levels. However, when LMWC was administered, we observed a reduction by 1.39 times (P<0.05) compared to the control group of rats and by 1.27 times (P<0.05) compared to healthy rats. We attribute this effect to its hypoglycemic properties [8, Konstantin S. M.]. The mechanism of its hypoglycemic effect is related to the regulatory role of the main pathways of glucose into the liver and muscles. The effectiveness of chitosan and its derivatives depends on their size and molecular weight. In the research of Naveed M. et al. (2019), Yu S.Y. et al. (2017) showed the expression of the glucose transporter 4 (GLUT4) gene in diabetic rats with increased glycogen production in the liver [16, Naveed M.; 17, Yu S.Y.].

The content of triglycerides in the blood serum of rats with ATH increased statistically significantly by 1.85 times (P<0.01) relative to the measuremetns of healthy rats. This is due to a decrease in VLDL in the liver, which transfers endogenous TAG to the blood and organs, and tissues. It should be said that all study drugs reduced high levels of TAG. Thus, with pharmacotherapy with carsil, this decrease was 1.92 times (P<0.001), with the use of LMWC - 1.56 times (P<0.01) relative to the indicators of the control group of animals. At the same time, LMWC showed a slight inferiority compared to carsil and came close the levels observed in intact rats.

In the blood serum of rats with ATH, the level of total cholesterol sharply increased, exceeding the measurements of the healthy group of rats by 2.08 times

(P<0.001). Indeed, according to literature data, the cholesterol content in blood serum increases in liver diseases, due to a decrease in the synthetic function of hepatocytes, in the endoplasmic reticulum of which primary bile acids are synthesized from cholesterol. On the other hand, with acute and chronic liver damage, due to compression of the bile ducts by edema of hepatocytes and inflammatory processes, intrahepatic cholestasis increases, which also leads to the development of hypercholesterolemia. Pharmacotherapy with ATH carsil for 12 days contributed to a significant decrease in total cholesterol in the blood serum of experimental animals by 1.92 times (P <0.001) and approaching the values of intact rats. This was due to the choleretic effect of silymarin, which is part of carsil. The effect of chitosan was manifested to a lesser extent. Thus, the content of total cholesterol in the blood serum of rats with ATH treated with LMWC statistically significantly decreased by 1.5 times (P < 0.05). Despite this decrease, the total cholesterol level in the blood serum of this group of animals remained 1.39 times higher than that of the healthy animals (P<0.05).

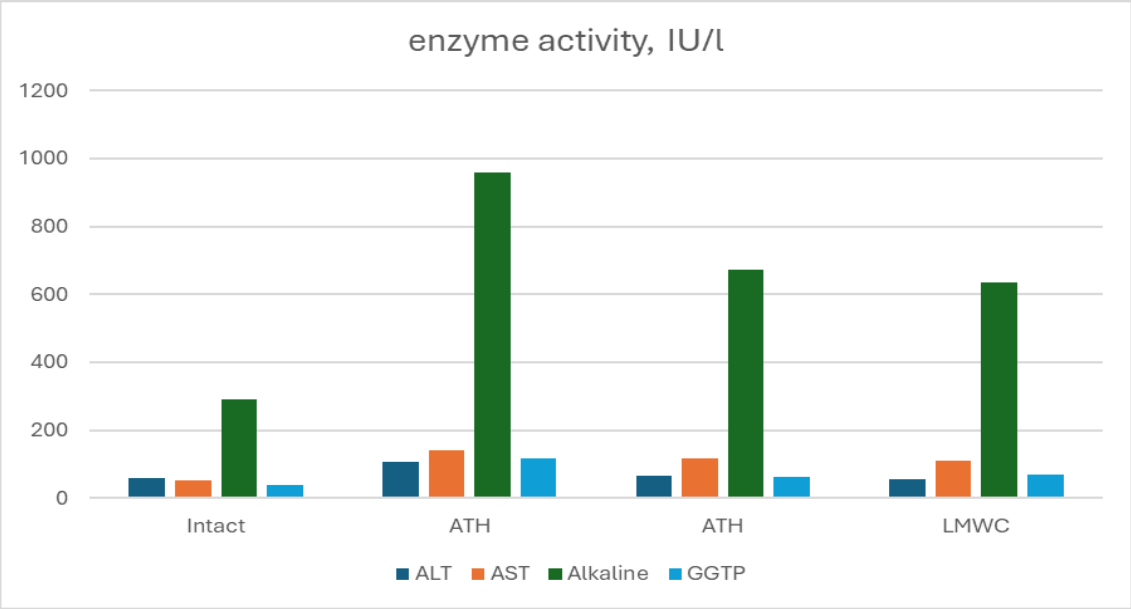
Thus, various chitosan derivatives reduced the severity of hyperbilirubinemia, hypertriglyceridemia, and hypercholesterolemia characteristic of rats with toxic liver damage. Studies by Inoyatova F.Kh., Milusheva R.Yu., Kutlikova G. (2016-2018) showed the effectiveness of chitosan and, especially its sulfated form, in reducing the risk of atherogenesis, which was confirmed by low values of total cholesterol and cholesterol in very low and low-density lipoproteins in rats with hypercholesterolemia [12, Kutlikova G.M.]. According to the authors, they suppressed high levels of endothelin-1, homocysteine, and CRP, and increased eNOS activity and nitric oxide content, which coincided with its pronounced lipid-lowering properties.

Pharmacotherapy with LMWC in rats with ATH for 12 days led to a significant decrease in urea and uric acid levels by 1.39 (P<0.05) and 1.63 times (P<0.01), without having a significant effect on creatinine levels. Despite such positive changes in the level of urea and uric acid in the blood serum of rats remained significantly 1.73 (P<0.01) and 1.81 times (P<0.01), respectively.

The next stage of our research was to assess the effect of drugs on the activity of liver enzymes (see Table 4). It was found that in rats with ATH, ALT activity increased statistically significantly by 1.83 times (P<0.01) relative to the values of the intact group of rats. An increase in the activity of this enzyme indicates cytolysis of hepatocytes since it reaches its highest concentrations in the liver. The degree of increase in aminotransferase activity indicates the severity of the cytolytic syndrome but does not directly indicate the depth of the violation of the organ function itself. AST activity in the blood serum due to acute toxic hepatitis

statistically significantly increased by 2.73 times (P<0.001), which is typical for carbon tetrachloride hepatitis, which mainly damages the mitochondria of hepatocytes. Alkaline phosphatase activity increased 3.31 times (P<0.001), indicating destruction of hepatocytes (hepatic cell mechanism) or disruption of bile transport (cholestatic mechanism). The hepatic cell mechanism of increasing alkaline phosphatase activity plays a leading role in toxic and drug-induced liver damage. In some cases, alkaline phosphatase activity increases due to the simultaneous action of both mechanisms of damage. In our opinion, with toxic hepatitis, is both due to hepatic and cholestatic mechanisms. GGTP activity in rats with ATH statistically significantly increased by 3 times (P<0.001) relative to the values in intact rats. This enzyme is mainly localized in the membranes of microsomes and the cytoplasmic membrane of cells, therefore, an increase in GGTP activity in the blood serum is of great diagnostic importance for diseases of the liver and hepatobiliary tract. As can be seen from the data presented, the ATH model we used is adequate and is characterized by all developing syndromes of liver damage.

Experimental pharmacotherapy with carsil in rats with ATH for 12 days led to a fall in the activity of the studied enzymes in the blood serum of rats. Thus, the activity of ALT, AST, ALP, and GGTP decreased by 1.62 (P<0.05); 1.2; 1.43 (P<0.05), and 1.84 times (P<0.01), respectively. While ALT activity showed some convergence with the measurements of healthy rats, AST, ALP, and GGTP levels remained higher, exceeding those of the intact rat group by 2.28 (P<0.001), 2.32 (P<0.001), and 1.64 times (P<0.01) respectively (refer to Table 4). This indicates the persistence of dystrophic changes in hepatocytes and cholestasis in experimental animals.



Experimental pharmacotherapy with LMWC for 12 days in rats with ATH led to a decrease in the activity of ALT, AST, ALP, and GGTP by 1.94 (P<0.001); 1.27 (P<0.05); 1.51 (P<0.05) and 1.69 times (P<0.01), respectively, relative to the values of the untreated group of rats. If ALT activity approached the values of healthy rats, the activities of AST, ALP, and GGTP remained above the values of the healthy group of rats by 2.15 (P<0.001); 2.2 (P<0.001) and 1.78 (P<0.01) times higher than them. These values showed no significant difference from those of the comparison group, suggesting the continued presence of inflammatory and infiltrative processes in the livers of the experimental animals.

Thus, in rats with ATH, hypoalbuminemia develops against the background of increased globulins, productive hyperazotemia, hypertriglyceridemia, hypercholesterolemia, and increased liver enzymes. The data obtained indicate the development of syndromes of cytolysis, cholestasis, mesenchymal inflammation, hepatic cellular failure, and a decrease in the detoxifying function of the liver. This is due to the toxic effect of trichloromethane radicals. Experimental pharmacotherapy with carsil and LMWC leads to a certain extent to an increase in albumin content, a decrease in the level of globulins, and residual nitrogen indicators. Along with this, the drugs reduced the severity of triglyceridemia, hypercholesterolemia, and hyperfermentemia.

To clarify some aspects of the mechanism of the hepatoprotective action of LMWC in blood serum, we studied the indicators of lipid peroxidation. Analysis of the MDA content in the blood serum of rats with acute toxic hepatitis showed a significant increase of 172.9% (P<0.001) compared to the measurements of healthy rats (see Table 5). It is established that the administration of CCl4 to rabbits results in the early degradation of cytochrome P-450 in liver microsomes, inhibition of the enzyme glucose-6-phosphatase, ultrastructurally detectable intense necrosis and fatty degeneration of the liver. Metabolized in the liver, carbon tetrachloride forms active metabolites that covalently bind to lipids, inducing lipid peroxidation. On the other hand, the covalent binding of carbon tetrachloride metabolites to cellular elements is more important in liver damage than lipid peroxidation.

It is well known that the function of catalase is to protect the body from reactive oxygen radicals and hydrogen peroxide. Analysis of the activity of this enzyme showed its decrease by 52.1% (P<0.01), indicating that a decrease in the activity of antioxidant enzymes plays an important role in the development of hyperlipid peroxidation. Indeed, the ratio of catalase activity to MDA level in intact rats was 19.10±1.57, while in rats with ATH, it was 3.15±0.21, which is 5.67 (P<0.001) times lower than the measurements in healthy rats. Consequently, under the influence of active carbon tetrachloride radicals, a fall in the activity of

antioxidant enzymes leads to the destruction of biomembranes and the development of cytolysis of hepatocytes.

Table 5

The levels of malondialdehyde and catalase activity in the serum of the experimental animals, M±m, n=10

Groups	MDA content, nmol/ml		Catalase activity, mkat/l	
	M±m	P_1/P_2	M±m	P_1/P_2
Intact	1,520±0,107		28,13±1,77	
ATH+H_2O	4,253±0,176	0,001	13,32±0,91	0,01
ATH+carsil	2,288±0,132	0,02 0,002	20,46±0,97	0,03 0,02
ATH+LMWC	2,675±0,153	0,02 0,01	17,98±1,56	0,02 0,05

Note: P1 represents the reliability of variances between the metrics of the intact and experimental groups, while P2 signifies the reliability of differences between the metrics of the treated and untreated groups.

Experimental pharmacotherapy with carsil in rats with ATH contributed to a fall in MDA content by 44.2% (P<0.002) compared to the measurements of the control group. Nevertheless, despite these favorable alterations, the MDA level remained elevated by 52.3% (P<0.02) compared to the readings of the intact rat group. Simultaneously, catalase activity showed a statistically significant increase of 51.1% (P<0.02) compared to the control group, yet it remained 27.7% lower (P<0.03) than the levels observed in the intact rat group. The index of catalase activity/MDA level was 9.15±0.21, which is 2.69 (P<0.001) times higher than the values of the control group, but remained lower than the measurements of the healthy group of rats by 2.11 times (P<0.001).

Pharmacotherapy with LMWC in rats with ATH contributed to a decrease in MDA content by 40.4% (P<0.01) relative to the values of the untreated group, but still exceeded the values of intact rats by 62.6% (P<0.02). Catalase activity increased by 29.9% (P<0.05) but remained below the values of the intact group of rats by 37.8% (P<0.02). The ratio of catalase activity/MDA content in this group was 6.44±0.59, which is 2.17 (P<0.001) times higher than in the control group. However, this indicator remained lower than the values of intact rats by 2.61 times (P<0.001). It should be said that the antioxidant effectiveness of LMWC was lower compared to the measurements of the comparison group.

Thus, in rats with ATH, hyperlipid peroxidation was established due to a decrease in catalase activity. The antioxidant effectiveness of LMWC was lower than that of the group of animals receiving carsil.

Fas ligand, known as the "death factor", binds to the Fas receptor and induces cell death. The Fas-FasL system initiates the destruction of autoreactive T cells and the development of hepatitis. The membrane-bound Fas ligand is converted into a soluble form by metalloproteinase. Taking into account the above, we determined the content of FasL in the blood serum of experimental animals using the enzyme immunoassay method. The studies showed that the FasL content in healthy rats was 2.30±0.10 ng/ml (Fig. 1). In rats with ATH, this measurement statistically significantly elevated by 4.35 ($P<0.001$) times and amounted to 10.01±0.43 ng/ml, indicating activation of the receptor-mediated apoptosis mechanism.

According to the literature, in viral hepatitis, the “external” pathway of apoptosis mainly predominates, due to the initiation of “death receptors” on the surface of hepatocytes and cholangiocytes, in which the Fas–FasL interaction plays a leading role [7, Karev V.E.; 10, Kremer A.E.]. It should be said that along with this, the “internal” path is also activated [11, Kurbanova N.N.]. Immunohistochemical studies have shown that on the surface of T-lymphocytes that form the inflammatory infiltrate in the liver in chronic hepatitis C (CHC), the expression of Fas ligand is increased, which can serve as an apoptotic signal for hepatocytes carrying the Fas receptor on their surface [4, Dmitrieva E.V.]. On the other hand, the accumulation of unrepaired DNA damage resulting from oxidative stress is one of the triggers of apoptosis [2, Antonova T.V.; 6, Hatano E.].

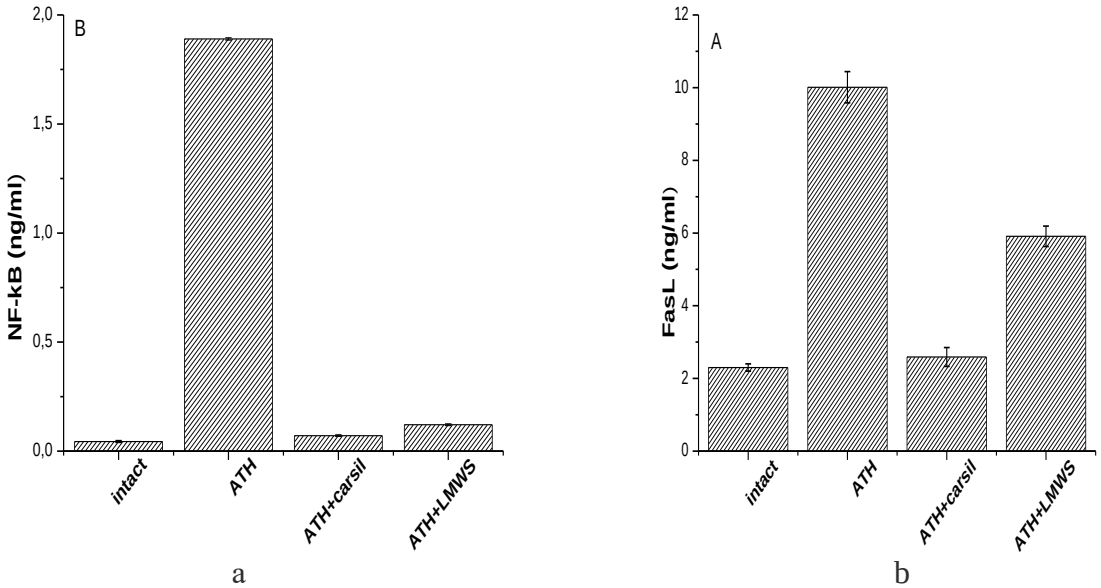


Fig. 1. The effect of carsil and low molecular weight chitosan on the content of FasL (ng/ml) (a) and NF-kB (ng/ml) (b) in the blood serum of rats with acute carbon tetrachloride hepatitis after 12 days of use.

Experimental treatment of ATH with the standard drug Carsil at a dosage of 100 mg/kg resulted in a reduction of the FasL content in the blood serum of the

experimental animals by 4 times (P<0.001) compared to the control group of rats, measuring 2.59±0.26 ng/ml. Concurrently, we observed a convergence of these values towards those of healthy rats. When using LMWC, we also observed a decrease in FasL content in the blood serum of experimental animals. Thus, the FasL content in the blood serum of experimental animals decreased to 5.91±0.28 ng/ml, which is 1.69 (P<0.02) times lower than the control group. However, these indicators were still significantly higher by 2.57 (P<0.001) and 2.36 (P<0.001) times the values of intact rats and rats of the comparison group.

NF-kB is one of the main transcription factors responsible for adaptive cell responses. NF-kB is a family of cytoplasmic proteins that when stimulated, become free, moving into the nucleus, where they are active by binding to the promoter regions of more than 100 genes responsible for inductive homeostasis. NF-kB is present in the cytoplasm in an inactive form, being in a complex with inhibitory IkB proteins. When stimulated, NF-kB undergoes phosphorylation and ubiquitination, which, after additional phosphorylation, can migrate into the cell nucleus, to the site of its action. Transcriptional activity of NF-kB appears within minutes after stimulation. NF-kB has one of the central positions in the regulation of the inflammatory process. Considering the above, it was of interest to study its content in the blood serum of experimental animals.

The research revealed that the NF-kB content in the blood serum of healthy rats measured 0.044±0.005 ng/ml (refer to Fig. 2). In rats with ATH, there was a significant spike in this factor's content, reaching 0.189±0.005 ng/ml, surpassing the levels observed in healthy rats by 4.3 times (P<0.001). It should be said that activation of NF-kB increases the expression of adhesive molecules, and accelerates the synthesis of pro-inflammatory cytokines and inducible enzymes (NO-synthase, cyclooxygenase-2, collagenase, etc.). This protein mediates inflammatory and immune responses, responses to viral infections, cell division, and regulation of apoptosis. Activation of NF-kB usually delays apoptosis, prolonging the life of effector cells at the site of inflammation.

Experimental treatment of ATH with the standard drug Carsil at a dosage of 100 mg/kg resulted in a reduction of the elevated NF-kB level by 2.66 times (P<0.001) compared to the control group, reaching 0.071±0.004 ng/ml. Despite this decrease, the level of this factor in the blood serum of rats was significantly 1.61 (P<0.05) times the value of the intact group of rats. Pharmacotherapy with LMWC at a dose of 25 mg/kg also contributed to a decrease in the high values of experimental animals by 1.56 (P<0.05) times, amounting to 0.121±0.004 ng/ml. These values were 1.7 (P<0.01) times lower than the values of the comparison group and 2.75 (P<0.001) times lower than the values of the intact group of rats.

Therefore, HMX chitosan reduces the level of high levels of NF-κB in the serum of ATH rats.

Drawing from the obtained results, the following conclusions can be inferred:

1. Low molecular weight chitosan obtained from pupae... to a certain extent restores the histiostructures of the liver of rats with ATH and is not inferior in morphological characteristics to the classical hepatoprotector carsil.

2. In the mechanism of the hepatoprotective action of LMWC, an important role is played by the activation of antioxidant defense enzymes, the slowdown of lipid peroxidation, and the reduction of FasL and NF-kB levels.

3. To a certain extent, restoration of the structure of biomembranes leads to activation of the pharmacometabolizing function of hepatocytes, a decrease in hyperenzymia and hyperbilirubinemia, and an increase in the protein-synthesizing function of hepatocytes.

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