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HEPATOPROTECTIVE PROPERTIES OF NANOSULFATE CHITOSAN ON THE MODEL OF ACUTE TETRACHLORMETHANE HEPATITIS

Zulfiya I. Galieva[1], Firuza Kh. Inoyatova [2], Vazira Rakhmanova [3], Rakiya Yu. Milusheva [4], Sayora Sh. Rashidova [5]

1 basic doctoral student of biological chemistry of Tashkent Medical Academy, Tashkent, Uzbekistan

2 DSc, B.D., Associate Professor, Head of the Department of biological chemistry of Tashkent Medical Academy, Tashkent, Uzbekistan

3 PhD, Associate Professor, Head of the Institute of Polymer Chemistry and Physics Academy of Science of the Republic Uzbekistan, Tashkent, Uzbekistan

4 PhD, Associate Professor, Head of the Institute of Polymer Chemistry and Physics Academy of Science of the Republic Uzbekistan, Tashkent, Uzbekistan

5 DSc, Ch.D., Associate Professor, Head of the Institute of Polymer Chemistry and Physics Academy of Science of the Republic Uzbekistan

ABSTRACT

The effects of low molecular weight chitosan (LMWC) and nanosulfate chitosan (NSC) were studied in comparison with the classical hepatoprotector carsil on the model of tetrachlormethane acute toxic liver injury (ATLI). The optimal hepatoprotective doses of LMWC 25 μg and NSC 10 mg/kg were established for oral administration for 12 days after reproducing the ATLI model. The drugs in these concentrations restored the detoxifying function of hepatocytes, reduced the severity of cytolysis, cholestasis, mesenchymal inflammation, hepatocellular insufficiency syndromes, activated antioxidant activity and reduced high values of lipid peroxidation and apoptosis.

Key words: acute toxic liver injury, hepatoprotectors, carsil, low molecular weight chitosan, nanosulfate chitosan, cytolysis, cholestasis, mesenchymal inflammation syndromes, lipid peroxidation, apoptosis, histology.

INTRODUCTION

According to the Global Hepatitis Report 2024 published by the World Health Organization (WHO), the number of victims of this disease is constantly growing

[12, 16]. Viral hepatitis is the second leading infectious killer worldwide, accounting for 1.3 million deaths per year, the same as tuberculosis, the leading infectious killer. New data from 187 countries show that the estimated number of deaths from viral hepatitis has increased from 1.1 million in 2019 to 1.3 million in 2022, with 83% due to hepatitis B and 17% due to hepatitis C. Every day, 3,500 people die from hepatitis B and C viruses worldwide. Pharmacotherapy for toxic hepatitis is mainly aimed at protecting hepatocytes from free radicals, stabilizing hepatocyte membranes, detoxifying, reducing transaminases, and immune regulation [5, 9, 14]. There is a large group of hepatoprotectors that differ in chemical nature and structure. Therefore, it is necessary to approach the choice of drugs differentially, since they are characterized by a certain specificity and have characteristic side effects. Chitosan belongs to the group of biocompatible and biodegradable polymers, absolutely non-toxic. It is a cationic aminopolysaccharide of natural origin, a copolymer of glucosamine and N-acetylglucosamine, which is obtained by partial deacetylation of chitin (β-(1–4)-poly-N-acetyl-d-glucosamine) [7, 8].

Purpose of the research

In our previous studies, the effectiveness of chitosan, and especially its sulfated form, in reducing the risk of atherogenesis was demonstrated [6]. It had low toxicity, no cumulative, resorptive, irritating, embryotoxic and teratogenic properties. The optimal dose of the drug is 25 mcg/kg. It should also be noted that there are broad prospects for the practical application of chitosan sulfate in medicine, associated with the possibility of forming nanoparticles of various structures [7, 10]. In this regard, it was of interest to study the hepatoprotective properties of this drug in comparison with low-molecular chitosan and the classical hepatoprotector carsil.

Material and Methods

The staff (Milusheva R.Yu., Rakhmanova V.) of the Institute of Chemistry and Physics of Polymers of the Republic of Uzbekistan kindly provided samples of low-molecular chitosan (LMC) and nanochitosan sulfate chitin (NSC) to us.

Experimental studies were carried out in accordance with the requirements of the Helsinki Declaration on the Humane Treatment of Animals (Strasbourg, 1985). To solve the set tasks, experiments were carried out on 50 sexually mature male rats with an initial weight of 160-180 g, kept on a standard diet in the laboratory of pharmacology and toxicology of the Tashkent Medical Academy (TMA), under the supervision of prof. A.Kh. Rakhmanov. The animals were divided into 5 groups: Group 1 - intact, Group 2 - control (ATLI + H_2O in a volume of 0.5 ml / 100 g),

Group 3 (ATLI + carsil at 100 mg / kg), Group 4 (ATLI + LMWC at 25 mg / kg), Group 5 (ATLI + NSC at 10 mg / kg).

To reproduce acute toxic liver injury (ATLI), 40 animals were administered CCI_4 at a dose of 2.5 ml / kg, subcutaneously for 4 days. There was no mortality by the end of the toxicant administration. Pharmacotherapy of acute liver injury was carried out 24 hours after the final administration of toxicants for 12 days.

At the specified time, the animals were taken out of the experiment under light ether anesthesia, after which blood was collected and pieces of liver were taken for morphological studies. The blood was centrifuged, the serum was separated and the following biochemical parameters were determined: the content of total protein, albumin, bilirubin, cholesterol, glucose, as well as the activity of alanine (ALT), aspartate aminotransferase (AST), gamma-glutamyl transpeptidase (GGT), alkaline phosphatase (ALP) on a MINDRAY BA-88A biochemical analyzer (China) using reagents from CYPRESS Diagnostics (Belgium), malondialdehyde (MDA) [1], catalase [4], the content of antibodies to NF-kB and FASL proteins by the enzyme immunoassay on an ELIZA enzyme immunoassay analyzer using reagents from this company. Together with pathologists, identical areas of the liver were selected from each animal for a mixture of 10% formalin for histological studies. The obtained data were statistically processed on a Pentium-IV personal computer using the Microsoft Office Excel-2012 software package. The significance level of $P<0.05$ was taken as statistically significant differences.

Results and Discussion

The conducted studies showed that the used drugs LMWC and NSC slightly increased the low level of total protein in the blood serum, while carsil did not have such an effect (see Table 1). During pharmacotherapy of ATLI with the comparison drug carsil, we observed only a tendency to increase the low level of albumins in the blood serum relative to the values of the untreated group. The same direction of changes in the content of albumins in the blood serum was noted in the group of rats receiving LMWC at a dose of 25 mg/g, while when using NSC, the synthetic function of the liver increased, as evidenced by an increase in the content of albumins in the blood serum by 1.18 times relative to the values of the control group of animals. The comparison drug Carsil and NSC brought the studied parameter closer to the values of intact rats, while when using LMWC, its values remained at the level of the control group of animals. During experimental pharmacotherapy with the comparison drug Carsil and LMWC, the albumin/globulin ratio only slightly increased, while when using NSC, it increased by 1.24 times ($P < 0.05$) relative to the values of the control group, and was higher than the comparison group. This was due to both an increase in the albumin level

and a decrease in the globulin content in the blood serum of rats in this group. As can be seen from the data provided, NSC is more effective in maintaining the protein spectrum of blood serum, which even exceeds the effectiveness of Carsil.

Table 1

Effect of hepatoprotectors on protein metabolism parameters in the blood serum of rats with acute tetrachloromethane hepatitis, M ± m, n = 8

Groups	Blood serum protein spectrum indicators, g/l			
	total protein	albumins	globulins	A/G coefficient
intact	68,48±2,92	40,63±0,47	27,84±2,90	1,55±0,12
ATLI+H_2O	60,49±1,83	29,28±2,23[a]	31,21±3,48	1,08±0,19 [a]
ATLI+Carsil	60,33±2,74	32,01±2,16 [a]	28,31±1,95	1,18±0,13 [a]
ATLI+LMWC	65,66±2,85	32,03±2,16 [a]	33,64±3,00	1,03±0,15 [a]
ATLI+NSC	62,83±1,85	34,54±1,78 [a,b]	28,29±1,90	1,28±0,13 [b]

Note: a – significance of differences relative to the intact group parameters; b – reliability of differences relative to the control group parameters; (P<0.05)

Pharmacotherapy of ATPP with carsil resulted in reliable decrease of high content of total bilirubin in rats' blood serum by 3 times (P<0.001) and approaching the values of intact rats (see Table 2). This was due to the presence of silymarin in carsil. Its mechanism of action is associated with stabilization of hepatocyte membranes, acceleration of reparation processes, neutralization of free radicals, activation of systemic processes, and slowing down of penetration of some hepatotoxins into liver cells. When using LMWC, content of total bilirubin in blood serum of rats with ATPP statistically significantly decreased by 2.31 times (P<0.001) relative to the control group parameters, but remained higher than the values of the intact group of rats by 1.58 times (P<0.01). NSC had a more pronounced hypobilirubinemic effect. Thus, with its 12-day use at a dose of 10 mg / kg, by the final date the high level of bilirubin decreased by 2.72 times (P<0.001). At the same time, it did not differ significantly from the values of the comparison group. The glucose content in the blood serum of rats during ATPP pharmacotherapy did not change significantly, when using NMC, we observed its decrease by 1.39 times (P<0.05) relative to the values of the control group of rats, and we did not observe such a pronounced hypoglycemic effect with NSC. Hypertriglyceridemia in rats with ATPP during pharmacotherapy with carsil decreased by 1.92 times (P<0.001), when using NMC - by 1.56 times (P<0.01), when using NSC - by 1.64 times (P<0.01) relative to the values of the control group of animals. At the same time, LMWC and NSC were slightly inferior to carsil, and approached the values of intact rats. The same dynamics were noted in the content of total cholesterol. Analysis of nitrogen metabolism indices in the blood serum of rats with ATPP showed the development of hyperazotemia, manifested by an increase in the amount of urea, creatinine and uric acid.

Pharmacotherapy of ATPP with carsil contributed to a decrease in the content of urea and uric acid by 1.83 (P<0.01); 1.13 and 1.79 times (P<0.01), LMWC - by 1.39 (P<0.05) and 1.63 times (P<0.01) urea and uric acid, did not have a significant effect on the level of creatinine; NSC - by 1.74 (P<0.01) and 2.41 times (P<0.001), relative to the values of the control group of animals. NSH was not inferior to the classic drug Carsil in its hypoazotemic action.

Table 2

Effect of hepatoprotectors on biochemical parameters of blood serum in rats with acute tetrachloromethane hepatitis, M±m, n=8

Groups	Biochemical parameters of blood serum			
	Total bilirubin, μmol/l	Glucose, mmol/l	Triglycerides, mmol/l	Total cholesterol, mmol/l
intact	9,25±0,52	5,04±0,27	0,54±0,07	2,60±0,23
OTG+H_2O	33,81±2,41 [a]	5,54±0,29	1,00±0,06 [a]	5,40±0,40 [a]
OTG+carsil	11,31±1,09 [b]	5,18±0,35	0,52±0,04 [b]	2,81±0,26 [b]
OTG+LMWC	14,65±1,54 [a,b]	3,97±0,29 [a,b]	0,64±0,06 [b]	3,61±0,17 [a,b]
OTG+NSC	12,38±1,96 [b]	5,19±0,58	0,61±0,05 [b]	3,49±0,17 [a,b]

Notes are the same as in Table 1.

The drugs reduced high values of liver enzyme activity (see Table 3). Experimental pharmacotherapy of ATPP with carsil for 12 days led to a decrease in the activity of ALT, AST, ALP and GGT: the decrease was 1.62 (P<0.05); 1.2; 1.43 (P<0.05) and 1.84 times (P<0.01), respectively. The use of LMWC for 12 days in rats with ATPP led to a decrease in the activity of the above enzymes by 1.94 (P<0.001); 1.27 (P<0.05); 1.51 (P<0.05) and 1.69 times (P<0.01), respectively. The use of NSC reduced their activity by 1.84 (P<0.01); 1.62 (P<0.01); 1.82 (P<0.01) and 1.73 times (P<0.01), respectively, relative to the values of the untreated group of rats. NSH was not inferior to carsil in its activity.

Table 3

Effect of hepatoprotectors on the activity of serum enzymes in rats with acute tetrachloromethane hepatitis, M±m, n=8

Groups	Enzyme activity, IU/l			
	ALT	AST	ALP	GGTP
intact	58,26±2,81	51,10±5,06	289,25±23,93	38,38±2,19
ATLI+H_2O	106,68±6,36 [a]	139,44±3,02 [a]	957,5±72,6 [a]	115,50±9,26 [a]
ATLI+Carsil	65,70±5,64 [b]	116,55±6,81[a,b]	671,2±67,2 [a,b]	62,88±3,99 [a,b]
ATLI+LMWC	55,04±4,30 [b]	110,13±5,72[a,b]	635,9±80,2 [a,b]	68,38±3,98 [a,b]
ATLI+NSX	58,01±4,22 [b]	85,79±7,07[a,b,v] v - p=0.007	58,01±4,22[a,b,v] v- p=0.131	85,79±7,07[a,b,v]

Notes are the same as in Table 1

Therefore, of the studied chitosan derivatives, NSC was the most effective, but we did not reveal complete restoration of biochemical parameters.

To clarify some aspects of the mechanism of hepatoprotective action of chitosan derivatives in blood serum, we studied the parameters of lipid peroxidation (LPO) and apoptosis. Experimental pharmacotherapy of ATLI with carsil contributed to a decrease in high MDA values by 44.2% relative to the values of the control group (see Table 4). However, despite such positive shifts, the MDA level remained higher by 52.3% compared to the parameters of the intact group of rats. At the same time, catalase activity statistically significantly increased by 51.1% relative to the values of the control group, but was still lower by 27.7% than the parameters of the intact group of rats. LMWC contributed to a 40.4% decrease in MDA content relative to the untreated group, but still exceeded the intact rats by 62.6%. Catalase activity increased by 29.9%, but remained below the intact rats by 37.8%. It should be noted that the antioxidant efficiency of LMWC was below the comparison group. LMWC contributed to a 45.5% decrease in MDA content relative to the untreated group, but still exceeded the intact rats by 48.6%. Catalase activity increased by 58.6%, but remained below the intact rats by 24.1%. It should be noted that the antioxidant efficiency of LMWC was above the comparison group.

Table 4

LPO and apoptosis indices in the blood serum of experimental animals, M±m, n=8

Groups	MDA content, nmol/ml	Catalase activity, mkat/l	NF-kB, pg/ml	FASL, pg/ml
Intact	1,520±0,107	28,13±1,77	0,044±0,005	2,30±0,10
ATLI+H_2O	4,253±0,176a	13,32±0,91 [a]	0,189±0,005 [a]	10,01±0,43 [a]
ATLI+Carsil	2,288±0,132 [a,b]	20,46±0,97 [a,b]	0,071±0,004 [a,b]	2,50±0,26 [b]
ATLI+LMWC	2,675±0,153 [a,b]	17,98±1,56 [a,b]	0,121±0,004 [a,b]	5,91±0,28 [a,b]
ATLI+NSX	2,222±0,141 [a,b]	21,54±0,82 [a,b]	0,092±0,007 [a,b]	2,59±0,19 [b]

Notes are the same as in Table 1.

Therefore, the antioxidant efficiency of LMWC was lower than that of the animals treated with Carsil, while the effect of LMWC exceeded the values of the comparison group.

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. Membrane-bound FAS ligand is converted into a soluble form by metalloproteinase. Taking into account the above, we determined the FASL content in the blood serum of experimental animals using the enzyme immunoassay. The studies showed that the FASL content in rats with ATLD increased statistically significantly by 4.35 times ($P < 0.001$), indicating the activation of receptors mediated by the 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 [2, 3]. It should be noted that, along with this, the “internal” pathway is also activated. Immunohistochemical studies have shown that the expression of FAS ligand is increased on the surface of T-lymphocytes that form an inflammatory infiltrate in the liver in chronic hepatitis C (CHC), which can serve as an apoptotic signal for hepatocytes carrying the Fas receptor on their surface [3]. On the other hand, the accumulation of unrepaired DNA damage resulting from oxidative stress is one of the triggers of apoptosis [2, 11]. Experimental pharmacotherapy of ATLI with the comparison drug carsil at a dose of 100 mg/kg contributed to a 4-fold decrease in the FASL content in the blood serum of experimental animals relative to the values of the control group of rats and brought them closer to the values of intact rats. When using LMWC, this indicator decreased by 1.69 times ($P < 0.01$), but was still significantly higher by 2.57 ($P < 0.001$) and 2.36 ($P < 0.001$) times than the values of intact rats and the comparison group rats. At the same time, when using LMWC, the FASL content decreased by 3.86 times ($P < 0.001$) relative to the values of the control group of rats, approached the values of the intact group of rats and did not differ from the comparison group indicators.

NF-kB is one of the main transcription factors responsible for adaptive cellular responses. NF-kB is a family of cytoplasmic proteins that, when stimulated, become free and move to the nucleus, where they become 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, in a complex with inhibitory IkB proteins. When stimulated, NF-kB undergo phosphorylation and ubiquitination, which, after additional phosphorylation, are able to migrate to the cell nucleus, to the site of their action. The transcriptional activity of NF-kB manifests itself within minutes after stimulation. NF-kB plays a central role in the regulation of the inflammatory process. The studies showed that the NF-kB content in the blood serum of ATLI rats increased sharply by 4.3 times ($P < 0.001$). It should be noted that NF-kB activation increases the expression of adhesion molecules, accelerates the synthesis of proinflammatory cytokines and inducible enzymes (NO synthase, cyclooxygenase-2, collagenase, etc.). This protein mediates the inflammatory and immune response, the reaction to viral infections, cell division and regulation of apoptosis. NF-kB activation usually delays apoptosis, prolonging the life of effector cells in the inflammation focus.

Experimental pharmacotherapy of ATLI with the comparison drug carsil at a dose of 100 mg / kg led to a decrease in the high level of NF-kB by 2.66 times (P < 0.001) relative to the values of the control group. Despite such a decrease, the level of this factor in the blood serum of rats is significantly higher by 1.61 times (P < 0.01) than the value of the intact group of rats. LMWC contributed to the decrease of this index by 1.56 times (P<0.01), but was 1.7 (P<0.01) and 2.75 (P<0.001) times higher than the values of the comparison group and the intact group of rats. LMWC reduced the high level of NF-kB by 2.05 times (P<0.001) relative to the values of the intact group of rats. These valuesslightly exceeded the valuesof the comparison group rats and 2.09 times (P<0.001) – the values of the intact animals. Consequently, chitosan derivatives lead to the decrease of the high level of NF-kB in the blood serum of rats with ATLD. The most effective in this regard is LMWC, its activity is not inferior to Carsil.

Conclusion

To confirm the hepatoprotective properties of the studied drugs, we conducted a histological study of liver tissue. Thus, in the control group of animals with ATLD, treated with a placebo for 12 days, the morphological picture was characterized by the presence of diffuse periportal mesenchymal-cellular infiltration, lysis and fatty transformation of hepatocytes with displacement of nuclei. In several lobes of the liver, dust-like fatty degeneration of hepatocytes was detected under low magnification. In rats with ATLD, treated with the drug carsil at a dose of 100 mg / kg for 12 days, signs of tissue hypoxia and congestion were preserved. In the group of rats with ATLD, treated with LMWC at a dose of 25 mg / kg also for 12 days, signs of hypoxia and activation of the mesenchymal cellular reaction were morphologically detected, indicating the initial stage of sclerotic changes in the liver. The results of the study of a group of rats with ATPP treated with NSC at a dose of 10 mg / kg for 12 days indicated the greatest effectiveness of this drug compared to other groups. The revealed morphological changes indicated an increase in regenerative processes in the liver tissue with the formation of dust-like obesity, which was resorbed, with an insignificant cellular reaction. At the same time, in the control group of rats with ATPP, where a placebo was used, a diffuse mesenchymal-cellular reaction was noted, which was an irreversible process ending in the synthesis of collagen fibers, sclerotic changes with subsequent pathological regeneration and the formation of false lobules, which was subsequently aggravated by portal hypertension. The studies indicate the possibility of widespread use of the drug NSC in practical medicine. Analyzing the positive effect of NSC, it should be said that the size of nanoparticles and the uniformity of their size distribution are essential immunomodulatory elements for

antigen stabilization and cellular absorption [13]. The smaller size of nanoparticles provides a larger surface area, which ensures enhanced interaction with mucin and improved mucoadhesion, and ensures better localization of the antigen [15]. Chitosan nanoparticles have greater oral absorption than chitosan itself due to their small size and high zeta potential. Apparently, these properties of NSC had the most pronounced positive effect. Based on the obtained results, the following conclusion can be made: in the model of ATLI with tetrachloromethane, the optimal hepatoprotective doses of chitosan derivatives are low-molecular chitosan - 25 mg / kg and nanosulfate chitosan - 10 mg / kg. The drugs in these concentrations restored the detoxifying function of hepatocytes, reduced the severity of cytolysis syndromes, cholestasis, mesenchymal inflammation, hepatocellular insufficiency, activated antioxidant activity and reduced high values of lipid peroxidation and apoptosis.

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