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ORIGINAL ARTICLE

Protective effect of chitosan treatment against acetaminophen-induced hepatotoxicity

Eda Ozcelik [a,*], Sema Uslu [b], Nilufer Erkasap [c], Hadi Karimi [b]

[a] *Department of Nutrition and Dietetics, School of Healthy Sciences, Artvin Coruh University, Artvin, Turkey*
[b] *Department of Biochemistry, School of Medicine, Eskisehir Osmangazi University, Eskişehir, Turkey*
[c] *Department of Physiology, School of Medicine, Eskisehir Osmangazi University, Eskişehir, Turkey*

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Abstract Acetaminophen (APAP) is the most commonly reported toxic ingestion in the world. Severe liver injury resulting from overdose or chronic use of APAP remains a significant clinical problem. In recent years, the mechanisms underlying liver injury caused by APAP have become much better understood. We have studied the protective effect of chitosan supplementation against APAP-induced hepatotoxicity with respect to changes in the levels of total and lipid-bound sialic acid in the serum and in the liver tissue and changes in the activity of diagnostic marker enzymes, lipid peroxidation, and ceruloplasmin oxidase enzyme in normal and experimental groups of rats. During the experimental period, chitosan (200 mg/kg body weight per day) was administered to APAP + chitosan-treated rats by oral gavage. Results showed that treatment with APAP induced a significant increase in the serum alanine aminotransferase and alkaline phosphatase activities, in total and lipid-bound sialic acids levels, and in the liver lipid peroxide content. The administration of chitosan significantly prevented APAP-induced alterations in the levels of diagnostic marker enzymes, total sialic acid, lipid-bound sialic acid, and malondialdehyde in the experimental groups of rats. Furthermore, chitosan administration increased the activity of ceruloplasmin oxidase. In conclusion, our results suggest that chitosan has a protective effect on APAP-induced hepatic injury in rats. The study sheds light on the therapeutic potential of chitosan in an APAP-induced hepatotoxicity model.

Conflicts of interest: All authors declare no conflicts of interest.

* Corresponding author. Department of Nutrition and Dietetics, School of Healthy Sciences, Artvin Coruh University, Cayağzı Road, Liflevha District, Artvin, Turkey.
E-mail address: eda_ozcelik@yahoo.com (E. Ozcelik).

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Introduction

Acetaminophen (APAP) is a widely used analgesic with few side effects when taken in therapeutic doses. Hepatotoxicity is a common consequence of APAP overdose. Hepatotoxicity may be predictable or unpredictable. Predictable reactions typically are dose-related and occur in most people shortly after reaching the threshold for toxicity. Acetaminophen is a fairly predictable hepatotoxin, as are chemicals that are no longer used as drugs such as carbon tetrachloride, phosphorus, and chloroform [1,2]. In APAP hepatotoxicity, biochemical signs of liver damage become apparent within 24—48 hours after the time of overdose and produce dose-related centrilobular necrosis in the liver [2,3]. Early diagnosis of APAP hepatotoxicity is essential because rapid deterioration is common, and current treatments are very effective in preventing morbidity and mortality [1,2].

The liver is a key organ that is responsible for many critical functions within the body; if it becomes diseased or injured, the loss of these functions can cause significant damage to the body [4]. Several investigators have previously demonstrated that antioxidants prevent hepatotoxicity by inhibiting lipid peroxidation [5] and by suppressing the activity of AST and ALP [6]. Sialic acid (SA), a derivative of neuraminic acid, is attached to the carbohydrate chains of glycoproteins and glycolipids. The attachment of SA to carbohydrate chains occurs in the liver. Therefore, it has been hypothesized that liver function influences SA concentrations [7]. Studies have led to a better understanding of the biological and pathological importance of SA. Increased SA concentrations in various body fluids, especially in the blood serum, are observed in many pathological processes in which there is tissue damage, tissue proliferation, and inflammation [8—10]. This shift occurs because of the location and the ubiquitous distribution of SA. Much research suggests that measuring the level of SA in blood serum can predict the risk of various diseases [11,12]. A significant increase in SA concentrations has also been observed during diabetes, cardiovascular diseases, chronic liver disease, and liver malignancies. The mechanisms underlying the elevated SA concentrations in different diseases are unclear [13,14].

In recent years, several studies have examined the effects of antioxidants for supporting normal liver function and for treating liver diseases. Antioxidants have recently attracted much attention as useful alternative medicines for treating or preventing lifestyle-related disorders; however, relatively little knowledge is available on their mode of action [4]. Chitosan (CS), an important polysaccharide of marine origin, is prepared from the shells of crustaceans. It has attracted much attention as a biomedical material, owing to its antitumor [15], antiulcer [16], immunostimulatory [17], and antibacterial activities [18]. In recent years, the antioxidant activity of CS has attracted the most attention. Studies show that CS has hepatoprotective effects related to antioxidant activity [19,20].

Hepatoprotective effects related to the antioxidant activity of CS on APAP-induced hepatic injury have not previously been demonstrated. The present study investigated the hepatoprotective effects of CS against APAP-induced liver toxicity in rats by measuring lipid peroxidation; alkaline phosphatase (ALP), alanine aminotransferase (ALT), ceruloplasmin (Cp) oxidase enzyme activities; and total sialic acid (TSA) and lipid-bound sialic acid (LSA) levels as key indicators of hepatic detoxification.

Materials and methods

Chemicals

Acetaminophen, chitosan, N-acetylneuraminic acid, and o-dianisidine dihydrochloride were obtained by Sigma Chemical Co (St. Louis, MO, USA).

Animals

Spraque-Dawley male rats weighing 200—250 g were used for the experiment. The animals were housed in polypropylene cages under hygienic conditions and maintained at normal room temperature. The animals were allowed to have food and water *ad libitum*. The experiment was conducted in accordance with the guidelines of the Experimental Research and Application Center of Medical Sciences, Eskisehir Osmangazi University (Eskisehir, Turkey) and approved by the Institutional Animal Ethics Committee.

Experimental Design

The experimental animals were divided into four groups of nine animals each. Group I rats (i.e., normal control) were only administered the standard diet and water. In Group II rats (i.e., APAP group), hepatotoxicity was induced by 14 days of oral gavage with APAP (250 mg each/kg body weight/day) [21]. In Group III rats (i.e., CS group), the animals were treated by 14 days of oral gavage with CS (200 mg/kg body weight/day) [19]. In Group IV rats (APAP + CS), the animals were treated for 14 days by oral gavage with APAP (250 mg each/kg body weight/day) and 3 hours later with CS (200 mg/kg body weight/day).

Preparation of serum and tissue samples

At the end of the experiment, the rats were anesthetized with xylasin-ketamine and sacrificed 24 hours after the last administration. Their blood was collected into biochemical tubes by heart puncture. Blood samples were centrifuged for 10 minutes at 3500 rpm (4000*g*) to obtain a clear serum, which was stored at −80°C. Serum samples were used to determine the activities of ALT, ALP, and Cp oxidase, and the levels of TSA, LSA, and total protein. The liver tissue was excised immediately and washed with ice-cold saline. A portion of liver tissue was homogenized with 0.1 M Tris—HCl at pH 7.4, and the homogenate was used to determine lipid peroxidation. A portion of liver tissue was homogenized with 0.1 M phosphate buffer at pH 7.4. The homogenates were used to determine the TSA and LSA levels.

Biochemical analysis

Determination of ALT and ALP levels

Hepatotoxicity was assessed by quantifying the serum activities of ALT and ALP. Serum ALT and ALP levels were measured using a modular autoanalyzer (Roche, Mannheim, Germany). Serum ALT and ALP activities were expressed by units per liter (U/L).

Determination of TSA levels

Serum and tissue TSA levels were detected, as described by the method of Sydow et al. [22]. In brief, 400-μL samples were treated with 3 mL of 5% perchloric acid for 5 minutes at 100°C, and then centrifuged at 1400*g* for 4 minutes. Two milliliters of the supernatant were mixed with 400 μL of Ehrlich reagent (i.e., 5 g p-dimethylaminobenzaldehyde, 50 mL HCl, and 50 mL distilled water). After incubating at 100°C for 15 minutes, 2 mL of distilled water were added to the sample and a spectrophotometer (Shimadzu, UV-1601, Kyoto, Japan) was used to read the optical density at a wavelength of 525 nm [23].

Determination of LSA levels

Serum and tissue LSA levels were measured, as described by Katopodis et al. [24]. In brief, 50-μL samples were extracted with chloroform:methanol (2:1 v/v) maintained at 4°C. The lipid extract was separated with 0.5 mL of distilled water. The aqueous layer was precipitated with phosphotungstic acid. The precipitates were resuspended in 1 mL of distilled water, and the presence of LSA in the suspension was determined with resorcinol reagent. The LSA contents were calculated using standard curves obtained for various concentration of N-acetyl neuraminic acid.

Determination of Cp oxidase activity

Serum Cp oxidase activity, using o-dianisidine dihydrochloride as the substrate, was determined spectrophotometrically by one technician, as described previously by Schosinsky et al. [25]. All measurements were performed in duplicate. The enzymatic activity of Cp was expressed in international units (IU) in the substrate consumed by the formula:[26]

$$\text{Cp oxidase activity} = (A15 - A5) \times 6.25 \times 10^2 \text{ U/L}$$

Determination of malondialdehyde levels

The tissue level of malondialdehyde (MDA) was measured in accordance with the method of Ohkawa et al. [27].

Protein measurement

Protein was assayed by the method of Bradford [28] (1976) using serum bovine albumin as the standard.

Statistical analysis

All data analyses were performed by using SPSS Statistics 20 (SPSS Inc, Chicago, USA) and SigmaStat 3.5 (Chicago, IL, USA). The descriptive statistics were demonstrated by *n* (i.e., the sample size) and by the mean and standard deviation for continuous variables. Continuous normally distributed measurements were compared across the groups by using one-way analysis of variance (ANOVA) with the Tukey method and the Student-Newman-Keuls method multiple comparisons. A $p < 0.05$ was accepted as significant.

Results

In the APAP group, the levels of ALT, ALP, TSA, LSA, and MDA were significantly higher ($p < 0.001$), compared to the control group and the CS-treated groups (Table 1). The Cp oxidase activity was decreased significantly ($p < 0.001$) in the APAP-treated group, compared to the control group and the CS-treated group. In the APAP + CS group the levels of ALT, ALP, TSA, LSA, and MDA were significantly lower ($p < 0.001$), compared to the APAP group. However, the activity of Cp oxidase was statistically higher ($p < 0.001$) in the APAP+CS group than in the APAP group (Table 1). Treatment with CS offered a protective effect against increased levels of ALT, ALP, TSA, LSA, MDA induced by APAP.

Discussion

In this study, we defined hepatotoxicity as injury to the liver that is associated with impaired liver function caused by exposure to APAP. The liver is an important organ in the body because it provides protection from potentially injurious exogenous and endogenous compounds. It is affected through this process. Thus, the protective mechanisms of the liver are of special concern [20]. Researchers are investigating natural products to explore their pharmacological features such as hepatoprotective, antioxidant, and anti-inflammatory activities. Several natural antioxidants reduce oxidative stress and protect from hepatic damage. Chitosan has attracted much attention as a biomedical material, owing to its antitumor, antiulcer, immunostimulatory, antibacterial, and other unique biological activities. Chitosan's antioxidant activity has recently attracted the most attention [15,16,19,29].

Acetaminophen-induced hepatic damage is the second leading cause of liver transplantation and accounts for considerable levels of morbidity and mortality.

The advantage of this model is that experiments are technically easy to perform because APAP is a dose-dependent toxicant and, most importantly, it is clinically relevant [30]. When the membrane of a liver cell is damaged, a variety of cytosolic enzymes are released into the bloodstream. The estimation of enzymes in the serum is a useful quantitative marker of the extent and the type of hepatocellular damage [31]. In this study, abnormally high activities of serum ALT and ALP after APAP administration is an indication of the development of hepatic damage, which is responsible for the leakage of cellular enzymes into the blood. When liver plasma membranes become damaged, a variety of enzymes that are normally located in the cytosol are released into the circulation. The present study suggested the ameliorative effect of CS on the activity of ALT and ALP against APAP-induced changes in rats. The observed reduction in the levels of liver function enzymes as a result of CS administration may result, in part, from the presence of antioxidant compounds that aid in reducing APAP-induced liver damage. This effect was possibly because of the

Table 1 Biochemical characteristics of the groups.

	Control ($n = 9$)	Chitosan ($n = 9$)	APAP ($n = 9$)	APAP + CS ($n = 9$)
ALT (U/L)	28.89 ± 7.08	33.33 ± 7.29[c]	174.33 ± 15.77[a]	83.44 ± 14.32[b]
ALP (U/L)	157.78 ± 15.77	150.00 ± 11.03[c]	480.67 ± 57.32[a]	254.00 ± 34.27[b]
Serum TSA (μg/mg protein)	39.11 ± 8.45	25.36 ± 6.34[d]	84.88 ± 8.96[a]	37.06 ± 5.81[b]
Serum LSA (μg/mg protein)	19.95 ± 3.63	16.20 ± 2.14[c]	52.33 ± 3.69[a]	21.64 ± 5.57[b]
Tissue TSA (mg/dL)	8.18 ± 1.61	8.47 ± 2.19[c]	31.21 ± 6.89[a]	19.76 ± 2.50[b]
Tissue LSA (mg/dL)	6.28 ± 1.94	4.51 ± 2.31[c]	21.66 ± 3.60[a]	12.89 ± 4.20[b]
Ceruloplasmin oxidase (U/L)	38.19 ± 7.61	33.45 ± 11.73[c]	20.04 ± 8.70[a]	40.50 ± 12.55[b]
MDA (nmol/mg protein)	7.73 ± 3.58	8.62 ± 3.11[c]	36.55 ± 4.71[a]	19.98 ± 2.45[b]

Normally distributed values are expressed as the mean ± standard deviation ($n = 9$ in each group).
ALP = alkaline phosphatase; ALT = aspartate aminotransferase; APAP = acetaminophen; CS = chitosan; LSA = lipid bound sialic acid; MDA = malondialdehyde; TSA = total sialic acid.
[a] $p < 0.001$, significantly different from the control group.
[b] $p < 0.001$, significantly different from the APAP group.
[c] $p > 0.05$, not significantly different from the control group.
[d] $p < 0.05$, significantly different from the control group.

ability of CS to reduce oxidative stress and enhance the endogenous antioxidant defense status [6,13,32].

Lipid peroxidation is regarded as an important initiation event in the toxicity mechanism of APAP. It is well documented that the liver tissue contains a relatively high content of polyunsaturated fatty acids (PUFAs), which are sensitive to peroxidative damage. The toxicity of APAP depends on the conversion of APAP into the toxic metabolite N-acetyl-p-benzoquinone imine (NAPQI) by cytochrome P540, which leads to hepatocellular damage. Lipid peroxidation may be a major pathway of disease initiation and proliferation. Increased lipid peroxidation and impaired antioxidant enzyme function in the liver tissue are characteristic observations in APAP-treated rats [33,34]. In the present study, increased lipid peroxidation—as evidenced by elevated levels of MDA in hepatic tissues after the administration of APAP—could be expected owing to the depletion of endogen antioxidant stores. Recent studies show that CS has a scavenging effect. The scavenging effect of CS on hydroxyl radicals inhibits lipid peroxidation of phosphatidylcholine and linoleate liposomes. Chitosan could prevent liver damage. In this study, the concentration of MDA decreased significantly after CS administration. Chitosan moreover decreased the ALT and ALP enzyme activities. Lipid peroxidation changes membrane integrity, thereby impairing major metabolic functions that depend on membrane structure and integrity. These results clearly showed that the antioxidant action of CS significantly reduces APAP-induced liver damage. Furthermore, CS has an important effect on inhibiting lipid peroxidation [19].

The liver secretes into the circulation numerous glycoproteins, all of which are sialylated on the termini of their glycans. As mentioned earlier, the addition of these sialic acids assures the survival of these serum proteins, and their removal can result in a rapid clearance that is mediated by hepatic receptors that recognize the underlying sugar chain. This is also of relevance in biotechnology in that many biotherapeutic agents must be produced as glycoproteins, which require adequate SA capping of their glycans to avoid rapid clearance [24].

The attachment of SA to carbohydrate chains occurs in the liver. Therefore, it has been hypothesized that liver function influences SA concentrations [7]. Several research studies show that SA concentrations are elevated in pathological states in which there is tissue damage, tissue proliferation, and inflammation; an increase in SA levels may reflect an acute phase response [35,36]. In this study, serum and tissue levels of TSA and LSA were significantly increased only in the APAP group of rats.

Chitosan administration significantly prevented the increase in the TSA and LSA levels. Based on these results, we believe that lipid peroxidation develops as a result of tissue damage in APAP-induced toxicity. At the same time, the increased production of LSA from the surface of the cell membrane results from increased sialoprotein synthesis in the liver. As a result, the TSA levels rise. Chitosan is highly positively charged; it may adhere to cell membranes and evoke some biological change in the cell membrane [37]. The present findings suggest that CS does not induce lipid peroxidation. Therefore, CS could interact with the cell membrane to decrease SA levels, and CS treatment could reduce the development of liver damage.

Ceruloplasmin is a copper-containing protein and is primarily synthesized in the liver. It is a ferroxidase enzyme. It plays a role in the transport of copper, modulation of coagulation, inactivation of biogenic amines, and defense against oxidative stress. Ceruloplasmin prevents the formation of oxygen-free radicals and lipid peroxidation; thus, it blocks free radical damage in tissue and in plasma [38,39]. Our results revealed that the activity of Cp oxidase was significantly decreased in the APAP-induced toxicity group, compared to the control group. The present findings suggest that Cp oxidase activity is inhibited in liver damage. Decreased Cp oxidase activity may contribute to the induction of oxidative stress. In this study, Cp oxidase activity was also increased in the serum after CS treatment. We have observed the stimulatory effect of CS treatment on Cp oxidase activity.

Based on the aforementioned results, it may be that CS has a significant protective effect on APAP-induced liver damage, it reduces lipid peroxidation and SA levels, and it improves endogenous antioxidant defense systems. This study showed biological evidence that supports using CS in the treatment of APAP-induced hepatotoxicity. Hence, CS could be used as an effective protector against APAP-induced damage.

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