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ANTIOXIDANT AND HEPATOPROTECTIVE EFFECT OF CHITOSAN VERSUS VITAMIN E IN EXPERIMENTAL CARBON TETRACHLORIDE-INDUCED LIVER INJURIES

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ABSTRACT. The aim of our study was to assess the hepatoprotective and antioxidant effects of chitosan as compared with vitamin E in experimental toxic liver injury induced by carbon tetrachloride. Blood and liver samples were collected in order to assess hepatocytolisis (AST, ALT), oxidative stress (MDA, carbonyl proteins, GSH, SH groups, SOD and CAT), and histopathology examination was performed in order to asses inflammation and fibrosis. Liver enzymes level showed a significant, progressive increase after repeated exposure to CCl_4, first in liver tissue, then in the blood. Malondialdehyde and carbonyl proteins significantly increased, and GSH progressively decreased. Chitosan increased the GSH in the liver tissue to a value superior to that of the control group and decreased the AST and MDA level both in the liver and in the blood to values comparable to that of control group. Chitosan decreased carbonyl proteins level in the liver but slightly increased them in the blood. Vitamin E had similar effects concerning liver function and lipid peroxidation, but paradoxically, it induced protein peroxidation both in blood and in liver tissue. Histological modifications support the observed biochemical changes.

Keywords: *chitosan, vitamin E, fibrosis, antioxidants*

INTRODUCTION

The liver plays an essential role in the metabolism of a numerous toxic substances that enter the organism through the gastro-intestinal tract [1, 2]. Chronic exposure to small doses of carbon tetrachloride (CCl_4) leads

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to formation of trichloromethyl and trichloromethyl peroxy radicals which, in turn, are able to oxidase the polyunsaturated fatty acids (PUFA) of the cell membrane and to induce oxidative injuries [3, 4]. Membrane injuries will lead to hepatocytes necrosis [5] and release of the liver enzymes. The oxidative stress will induce an increase in the activity of antioxidant enzymes (SOD, CAT, GPx) [5] and of the endogenous antioxidants level (GSH, SH), followed by a decrease, when the endogenous reserves will be depleted [6]. The use of synthetic and natural antioxidant substances have positive effects against oxidative stress induced liver injuries [3, 4, 7-9]. Vitamin E is capable to protect the PUFA against the reactive oxygen species (ROS) and can be used as an efficient antioxidant substance for the membranes [8, 9]. In toxic hepatitis induced by repeated experimental exposure to small amounts of carbon tetrachloride, vitamin E is able to scavenge free radicals, thus reducing the lipid peroxidation and protecting the endogenous antioxidants [10, 11]. Chitosan (CS) is a polymer obtained through the deacetylation of the chitin found in the exoskeleton of the marine shellfish. It has hemostatic [12], anti-inflammatory and antibacterial properties [13-15]. Recent studies also underlined its antioxidant and hepatoprotective properties and its ability to reduce the oxidative stress [16]. The aim of our study was to assess the antioxidant and hepatoprotective effects of chitosan as compared with vitamin E in experimental toxic liver injury induced by carbon tetrachloride.

RESULTS AND DISCUSSIONS

The mean values of the lipid and protein peroxidation products are presented in tables 1 and 2, of the non-enzymatic antioxidants in tables 3 and 4, and of the enzymatic antioxidants in table 5. Statistical significant differences ($p<0.05$) between groups are marked as follows: [a] (Ctrl-CCl_4), [b] (Ctrl-VitE), [c] (Ctrl-CS), [d] (CCl_4-VitE), [e] (CCl_4-CS), [f] (VitE-CS).

Table 1. Mean values of the malondialdehyde in plasma and liver tissue

Group	*Day 15*		*Day 30*	
	Plasma (nmol/ml)	Tissue (nmol/mg prot.)	Plasma (nmol/ml)	Tissue (nmol/mg prot.)
Ctrl	2.37±0.36[a]	0.061±0.017[a]	1.26±0.17	0.078±0.009[a]
CCl_4	2.82±0.31	0.092±0.015	1.30±0.60	0.101±0.023
CCl_4+VitE	2.81±0.55	0.072±0.022	1.40±0.20	0.086±0.015
CCl_4+CS	2.42±0.38	0.075±0.020	1.32±0.20	0.085±0.016

Table 2. Mean values of the carbonyl proteins in plasma and liver tissue

Group	*Day 15*		*Day 30*	
	Plasma (nmol/mg prot.)	Tissue (nmol/mg prot.)	Plasma (nmol/mg prot.)	Tissue (nmol/mg prot.)
Ctrl	1.13±0.32[b, c]	2.72±0.74[c]	1.21±0.14[b,c]	2.77±0.75
CCl_4	1.72±0.59	3.21±0.49[e]	1.44±0.45[d]	2.38±0.76
CCl_4+VitE	1.71±0.48	3.22±0.66	2.05±0.47	2.76±0.35
CCl_4+CS	2.10±0.50	1.75±0.61	1.73±0.34	2.82±0.84

Table 3. Mean values of the reduced glutathione (GSH)

Group	*Day 15*		*Day 30*	
	Plasma (nmol/ml)	Tissue (nmol/mg prot.)	Plasma (nmol/ml)	Tissue (nmol/mg prot.)
Ctrl	17.62±3.43[a,b]	0.63±0.28[a,b,c]	6.13±1.53[b,c]	1.42±0.36[b,c]
CCl_4	14.12±2.60[d]	2.20±0.70[e]	5.66±0.79[d,e]	0.93±0.51[d,e]
CCl_4+VitE	5.90±0.81[f]	2.41±1.09	10.16±1.64	2.16±0.34
CCl_4+CS	14.85±5.44	3.98±1.45	10.48±1.18	1.97±0.42

Table 4. Mean values of Sulfhydryl Groups (SH)

Group	*Day 15*		*Day 30*	
	Plasma (µmol/ml)	Tissue (µmol/mg prot.)	Plasma (µmol/ml)	Tissue (µmol/mg prot.)
Ctrl	0.081±0.015[c]	0.056±0.007	0.132±0.029[a,c]	0.078±0.013[a,c]
CCl_4	0.088±0.040[e]	0.052±0.007	0.084±0.022[d]	0.054±0.012[e]
CCl_4+VitE	0.069±0.028[f]	0.066±0.029	0.158±0.036[d]	0.062±0.013
CCl_4+CS	0.015±0.003	0.060±0.019	0.064±0.026	0.043±0.007

Table 5. Mean values of the enzymatic antioxidants plasma

Group	SOD (U/ml)		CAT (U/ml)	
	Day 15	*Day 30*	*Day 15*	*Day 30*
Ctrl	6848.57±984.86[a,b]	11344±1721.08[a,c]	5.26±0.49[a]	8.98±0.96
CCl_4	10418.16±1463.55	13606±1520.50[d,e]	6.53±0.40	8.52±0.43
CCl_4+VitE	9259.53±1046.89	10310±684.79[f]	7.62±1.46	8.23±1.10
CCl_4+CS	8897.06±2608.63	9306±576.22	6.56±1.75	8.57±0.46

After CCl_4 administration, our study identified a significant increase in MDA level both in plasma (day 30) and in liver tissue (day 15 and 30), which suggests the onset of the oxidative stress first in the liver and then in the blood. CCl_4 administration first stimulated the antioxidant enzymes levels (SOD, CAT) and induced a decreased of endogenous antioxidants (GSH,

SH). Good correlations between MDA and GSH have been identified (ρ=0.57). After vitamin E administration, our studied identified a significant increase of GSH level in the liver tissue to a level superior to that of the control group. After chitosan administration, a significant GSH increase was observed (day 15) in the liver tissue as compared with the CCl_4 exposed rats, and the level remained increased until the end of the experiment (day 30). Our results identified biochemical changes similar to those described in the literature [5, 17-21]. Both chitosan and vitamin E are able to decrease the oxidative stress level.

Carbon tetrachloride also induced an increase in carbonyl proteins level in the plasma, but not in the liver tissue. Neither vitamin E, nor chitosan protected the proteins against oxidative stress. Paradoxically, both substances demonstrated a slightly pro-oxidant effect on the proteins. Vitamin E administration induced protein peroxidation in the plasma, while the liver tissue values were not significantly modified as compared with the control group, changes that can be explained by the dual effect of vitamin E [11, 22, 23]. This effect was less visible concerning chitosan; its administration decreased protein oxidation in the liver tissue, while the plasma levels remained slightly increased. Anraku *et al.*, using a low molecular weight chitosan, also observed a decrease in plasma albumin peroxidation [24]. It is possible that the antioxidant effect on the proteins might be related to the molecular weight of the chitosan.

Table 6. Mean values of the liver enzymes

Group	AST (U/ml)		ALT (U/ml)	
	Day 15	*Day 30*	*Day 15*	*Day 30*
Ctrl	$42.93\pm8.37^{a,b,c}$	$58.46\pm16.15^{a,b,c}$	$35.88\pm3.92^{a,b,c}$	$45.48\pm13.10^{a,b,c}$
CCl_4	153.69 ± 18.55^{e}	$229.82\pm41.09^{d,e}$	172.10 ± 20.28^{e}	$220.61\pm54.68^{d,e}$
CCl_4+VitE	155.33 ± 27.58^{f}	186.37±18.95	147.00±34.39	152.24±19.45
CCl_4+CS	124.19±16.46	181.99±26.69	129.87±17.54	173.57±35.26

After the oxidative injuries, transaminases (AST, ALT) are released in the serum. AST is much more specific for liver injuries [25]. Our research identified a significant increase in liver enzymes levels after CCl_4 administration during the entire experiment, proportional with the duration of the exposure to CCl_4. AST serum level correlates with MDA level both in plasma (ρ=0.792) and in liver tissue (ρ=0.493). After vitamin E administration, some studies identified a decrease in the AST and ALT levels. We also identified a gradual decrease in AST level after vitamin E treatment, without arriving to values similar to those of the control group. The use of natural antioxidants in toxic liver injuries has hepatoprotective and antioxidant effects. Previous studies identified the antioxidant capacity of the chitosan in toxic liver pathology [16, 26]. Our study identified a decrease in AST levels after chitosan administration. Our results are

consistent with those found in the literature. Vitamin E and chitosan, administered simultaneously with CCl_4, have different effect of the oxidative stress and liver injuries. Both substances have the ability to decrease the transaminase levels, but none of them, in the used doses, completely protects against the action of CCl_4. In the context of our experiment, chitosan has superior antioxidant effects as compared with the vitamin.

Microscopic examination of the liver sections from animals of the control group revealed normal liver architecture. In the experimental groups at both sampling intervals, the most obvious lesions were represented by vacuolar dystrophy and hepatocellular necrosis, mainly in the central areas. Degenerated hepatocytes from the central areas presented clear cytoplasmic vacuoles of different size. Some of the hepatocytes presented numerous small vacuoles, the cytoplasm having a foamy aspect, other hepatocytes suffer hydropic change, characterized by hypertrophy and ballooning of the hepatocytes with a centrally located nucleus. Some hepatocytes presented granular degeneration. In some areas diffuse dilatation of the sinusoids was also evident (figures 1 and 2).

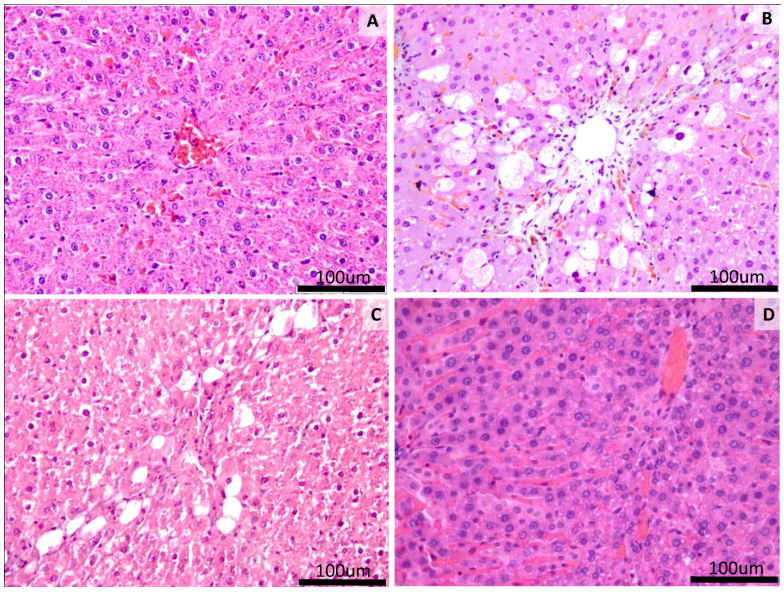

Figure 1. Histological changes in different experimental groups on the 15th day. Control group (A): central area, normal hepatic architecture; CCl_4 group (B): vacuolization of the hepatocytes from central areas; CCl_4+VitE group (C): hepatocellular vacuolization and necrosis; CCl_4+Chitosan group (D): discrete microvesicular steatosis, hepatocytes with a foamy aspect near the central areas, HE stain, original magnifications of 200×, Scale bar=100 μm.

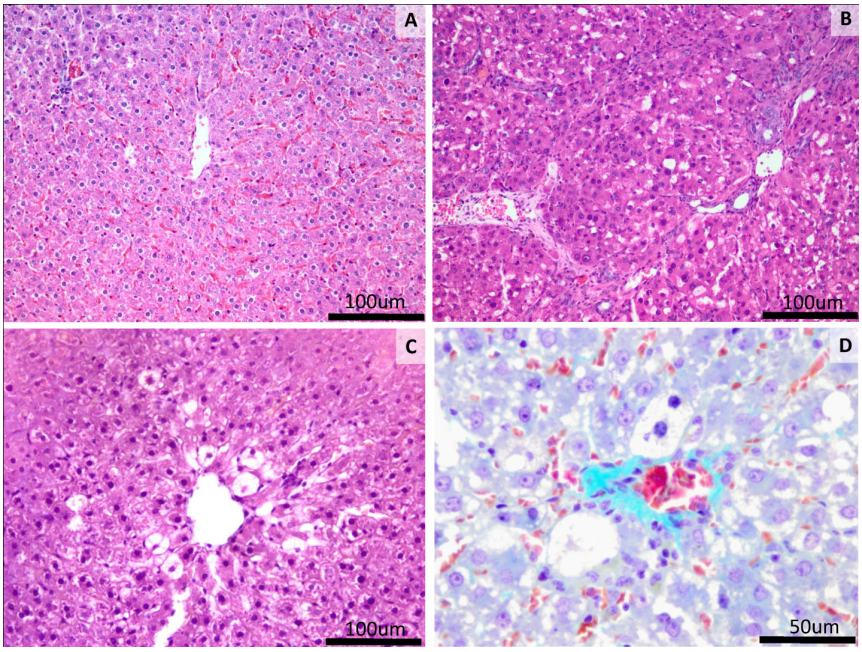

Figure 2. Histological changes in different experimental groups on the 30th day. Control group (A): central area, normal morphology, slight congestion; CCl_4 group (B): vacuolization of the hepatocytes from the central areas, centro-central bridges of degenerated and necrotic hepatocytes, oval cell hyperplasia; CCl_4+VitE group (C): hepatocellular vacuolization and necrosis in the central areas; CCl_4+Chitosan group (D): microvesicular steatosis and ballooning degeneration of the hepatocytes from central areas; HE stain (A,B,C), Masson's trichrome stain (D) original magnifications of 200× (A,B,C), 400x (D); Scale bar=100μm (A,B,C), Scale bar=50μm (D).

CONCLUSIONS

Our study confirmed the antioxidant effects of chitosan. Chitosan is able to decrease lipid peroxidation and to protect the endogenous antioxidant systems against the toxic effects of CCl_4. Only discrete histological liver injuries have been observed after chitosan administration. Vitamin E also demonstrates antioxidant properties, but the severity of the histological liver injuries was higher as compared to chitosan. In the used dose, the chitosan has a superior antioxidant and hepatoprotective effect as compared to vitamin E.

EXPERIMENTAL SECTION

Sixty four female Wistar rats, randomly distributed into four equal groups, have been used. The animals have been maintained at 23±2°C in the biobase of the Department of Physiology, Iuliu Haţieganu University of Medicine and Pharmacy Cluj-Napoca, Romania. The animals received standard food and water *ad libitum.* The experiment took place with the approval of the Ethical Committee of Iuliu Haţieganu University of Medicine and Pharmacy, Cluj-Napoca, and respected the Directive 86/609/EEC. Group I (Ctrl) received, by gavage, 0.9 ml/kg b.wt. sunflower oil, twice a week, for 30 days. Group II (CCl_4) received, by gavage, 1.2 ml/kg b.wt. CCl_4 25%, diluted in sunflower oil, twice a week, for 30 days. Group III (CCl_4+vit E) received, in addition to the CCl_4, 5 mg/kg b.wt. vitamin E (Sicomed®, 30 mg/ml vials) daily, intramuscular injections. Group IV (CCl_4+CS) received, in addition to the CCl_4, daily intraperitoneal injection with 3 mg/kg b.wt. chitosan (Sigma-Aldrich®, molecular weight 190-310 kDa, deacetylation degree 75-85%). After 15 and 30 days, eight animals from each group were anesthetized with ketamine. Blood samples were taken from the retroorbitary sinus. Animals were euthanized by cervical dislocation. Liver samples were taken for biochemical assessment and a portion of the liver was fixed in formaldehyde for histological examination.

Malondialdehyde levels in the plasma and in the liver were assessed using the method of Conti [27]. Protein peroxidation was estimated through the measurement of the protein carbonyl groups using the method of Reznick [28]. The total amount of proteins was assed using Bradford method [29]. SOD activity was assessed using the method described by Flohe [30], and CAT activity using the method described by Pippenger *et al.* [31]. Sulfhydryl groups (SH) and reduced glutathione (GSH) were assessed using the method of Hu [32, 33]. Liver function was evaluated using the activity of ALT and AST enzymes in the blood with commercial assay kits (Diagnosticum Zrt. Budapest) [34].

Liver tissue samples were fixed in 10% buffered neutral formalin, embedded in paraffin; the sections were made at 4 micrometers with a microtome Leica RM 2125 RT and stained by Haematoxiline-Eosine and Masson's trichrome methods. The slides were examined under a microscope Olympus BX 51. The images were taken with Olympus DP 25 digital camera and processed by a special image acquisition and processing program: Olympus Cell B. Sections were scored by an independent observer blinded to the experimental protocol. The following lesions were scored according to Knodell Histological Activity Index (HAI): portal inflammation, periportal/bridging necrosis, intralobular degeneration/focal necrosis and fibrosis (Knodell) [35].

Statistical analysis was performed using non-parametric tests (Mann-Whitney, Kruskal-Wallis, Wilcoxon, Spearman non-parametric correlation). The analysis was done for every moment of the experiment and also concerning the dynamic of the parameters, using MedCalc 14.0 sofware. The results were expressed as mean±SD. *P* values were considered significant if <0.05.

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