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COMPARATIVE EFFECT BETWEEN CHITOSAN AND CHITOSAN-Cu COMPLEX ON CARBON-TETRACHLORIDE (CCL_4) INDUCED LIVER DAMAGE IN RATS

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ABSTRACT

BACKGROUND: Carbon tetrachloride (CCl_4) is a toxic material known to induce lipid peroxidation and liver damage.

The possible protective roles that involved by chitosan or chitosan-Cu complex against CCl_4 induced liver intoxication were investigated in male rats.

RESULTS: CCl_4 administred at dose 20 mg/kg body weight i.p., exceed malondialdehyde (MDA) and protein carbonyle (PC), depleted superoxide dismutase (SOD) and glutathione (GSH), in concomitant with marked increase in investigated liver function parameters , alanine aminotransferase and aspartate aminotransferase (ALT, AST), impaired serum and liver total protein , albumin and globulin. An elevation in serum and hepatic total lipids, total cholesterol, triglycerides and serum LDL and VLDL levels as well as a low level of HDL were recorded. In the same time, there was a significant increase in sodium and iron contents in the serum while a significant decrease in potassium and zinc contents were recorded.

Animals pretreated with chitosan (200 mg /kg body weight) orally by stomach tube for 21 consecutive days prior to CCl_4 challenge significantly attenuated most of the tested parameters, strengthen antioxidant defense system, ameliorated liver function effectively. Chitosan-Cu complex has a protective effect by a higher degree than that of chitosan only.

CONCLUSION: These findings suggest that pretreatment with chitosan-Cu complex has higher hepato-protective effects than that of only chitosan against CCl_4 induced toxicity in rat.

Key words: Carbon tetrachloride (CCl_4) – Chitosan – lipid peroxidation – liver functions.

INTRODUCTION

Liver disease is considered to be a serious health problem, as the liver is an important organ for the detoxification and deposition of endogenous and exogenous substances (Yang *et al.*, 2008).

Single administration of carbon tetrachloride (CCl_4) can rapidly lead to both oxidative stress via the excessive production of free radicals and acute liver injuries such as centrilobular necrosis and steatosis in rats **(**Weber *et al.*, 2003**).**

Chitosan, is a polysaccharide of marine origin which is prepared from the shells of crustaceans (Sini *et al*., 2005). Chitosan has attracted much attention as a biomedical material, owing to its antitumer, antiulcer, immunostimulatory, antibacterial and other unique biological activities (Xue *et al.*, 2001**).** Chiang *et al.*, (2000), showed that the scavenging effect of chitosan on hydroxyl radicals inhibits lipid peroxidation (LPO) of phosphatidyl-choline and linoleate lyposomes *in vitro*.

Le Houx and Grondin (1993) showed that chitosan maintained adequate cholesterol homeostasis in rats, despite a greatly intake of cholesterol. Chitosan can form complexes with many metal ions because it contains multiple amino, hydroxyl and acetamide groups (Varma *et al*., 2004).

Various copper complexes have been tested for antibacterial and antitumer properties **(**Hirano, 1995). The copper complexes interact with DNA, leading to chemically

induced cleavage of DNA and thus have antitumer activity (Liang *et al.*, 2003).

In the present study, we investigated which of either chitosan or chitosan – Cu complex has more protective effect against CCl_4-induced oxidative stress and hepatotoxicity in rats.

MATERIAL AND METHODS

Adult male Albino rats (Rattus rattus) weighing 120±8 g purchased from Eye Bank, Giza, Egypt, were kept in stainless steel bottom cages within an air-conditioned animal house at 23±2 ºC. Rats were fed commercial diet and allowed water *ad libitum* for a week, then randomly classified into six groups, each with five rats. Group I was kept normal and served as a control, each of the rats of group II was administered a single oral dose of CCL_4 (1.5 ml / kg body weight) .Group III received chitosan daily 200 mg/kg for 21 days (water soluble chitosan with M.W.$6x10^5$ and degree of deacetylation more than 85%, purchased from Alderich) . Group IV received orally chitosan-Cu complex (prepared locally by Reicha and his Co-workers, Physics Departement, Faculty of Science, Mansoura University, Egypt) at the same dose and period of chitosan . Group V received chitosan for 21 days followed by CCL_4 and group VI received chitosan-Cu complex for 21 days followed by administration of CCL_4. Twenty four hours after receiving CCL_4 rats were sacrificed by decapitation, blood was collected in nonheparinized tubes, allowed to centrifuge after 15 mints and sera were separated.

Animals were dissected, livers were removed, weighed, and part of each liver was excised and homogenized in cold distilled water 10% (w/v). Assay for lipid peroxidation product malondialdehyde (MDA) in liver tissue was performed spectrophotometerically at 512 nm (Ohkawa *et al.***,** 1982). The assessment of protein oxidation was performed by a spectrophotometric method (Smith *et al.*, 1991**).**The level of reduced glutathione (GSH) and the activity of liver superoxide dismutase (SOD) were determined as described by Nishikimi *et al.* (1972) and Prins and Loose (1969) respectively.Serum and liver ALT & AST activities were estimated by the methods of Reitman and Frankle(1957). Total protein content and albumin level were determined by using Diamond diagnostic kit according to the technique reported by Henry (1964) and Doumas *et al.* (1971) respectively. Serum and liver total lipids and total cholesterol were estimated according to the technique described by Zollner and Kirsch (1962**)** and Ratliff and Hall (1973) respectively, high density lipoprotein (HDLc) was determination according to (Gordon *et al*., 1977). Serum mineral contents (sodium, potassium, iron and zinc) were assessed using atomic absorption spectrophotometery (Zettner and Seligson 1964).

All data were expressed as mean ± S.E. The data analysis was performed by one-way ANOVA test. P value of ≤ 0.05 was considered significant.

RESULTS

CCL_4 administration to the rats significantly elevated hepatic MDA and PC levels while GSH and SOD activities were decreased significantly (table 1) , it was noticed that chitosan and chitisan-Cu complex administration in concomitant with CCL_4 significantly affect these estimated parameters positively.

Table (2) shows that serum and liver ALT & AST, were significantly increased, but the total protein content, albumin and globulin were decreased in CCL_4 administered group .Pretreatment with chitosan or chitosan-cu complex markedly attenuated most of these measured liver functions parameters compared to that treated only with CCL_4

Serum and liver total lipids, triglyceride, total cholesterol and serum LDL were elevated significantly while serum HDL and VLDL were decreased in CCL4 treated rats, pretreatment with chitosan or chitosan-cu complex improved most of these parameters (table 3).

A decline in the level of serum Na and Zn but K and Fe levels were increased in CCL4 treated group, amelioration noticed when the rats treated with chitosan or chitosan-cu complex prior to CCL4 (table 4) .

Table (1): Liver MDA, PC, GSH and SOD.

Animal Estimated parameters	(I) Control	(II) CCl_4	(III) Chitosan	(IV) Chitosan-Cu	(V) Chitosan + CCl_4	(VI) Chitosan- Cu + CCl_4
MDA (n mol/g) % of change	159.76±14.2	253.38±18.70[a] +58.60	136.0±15.00[b] -14.87	151.3±13.8[b] -5.29	191.4±14.5 +19.80	203.2±11.2 -27.19
PC(µ molNPH/g) % of change	0.162±0.019	0.276±0.023[a b] +70.37	0.166±0.009[b] +2.47	0.162±0.012[b] 000	0.22±0.007 [abc] +35.80	0.198±0.004[b] +22.22
GSH (mg/g) %of change	0.25±0.02	0.104±0.02[a] +58.4	0.256±0.015[b] +2.4	0.238±0.03[b] -4.8	0.148±0.013 [ac] -40.8	0.158±0.014[a d] -36.8
SOD (µ/g) %of change	34.40±1.57	18.60±1.36[a] -45.93	36.40±1.03[b] +5.81	36.20±1.66[b] +5.23	25.60±1.63 [abc] -25.58	23.80±1.50[ad] -30.81

a = Significant compared to control (I).
b = Significant compared to CCl_4 (II).
c = Significant compared to Chitosan(III).
d = Significant compared to Chitosan-Cu (IV).

Table (2): Serum and liver ALT, AST, total protein and serum albumin.

Animal Estimated parameters	(I) Control	(II) CCl_4	(III) Chitosan	(IV) Chitosan-Cu	(V) Chitosan + CCl_4	(VI) Chitosan- Cu + CCl_4
S. ALT (U/L) % of change	39.48± 1.00	71.86±3.13[a] +82.02	40.98±2.50[b] +3.80	41.64±2.30[b] +5.47	63.84±1.46[ac] +61.70	60.76±1.36[abd] +53.90
S. AST (U/L) % of change	33.16± 1.2	42.30±0.59[a] +27.56	36.48±1.05[b] +10.01	34.08±0.63[b] +2.77	40.28±1.2[a] +21.47	39.04±0.56 [ad] +17.73
S. total protein (g/dl) %of change	7.74±0.52	5.02±0.17[a] -35.14	7.60±0.68[b] -1.81	7.32±0.66[b] -5.43	6.34±0.10 -18.09	6.62±0.24 -14.47
S. Albumin (g/dl) %of change	3.78±0.09	2.66±0.07[a] -29.63	3.56±0.4[b] -5.82	3.30±0.13[b] -12.70	3.22±0.08 -14.81	2.98±0.08 -21.16
Liver ALT (u/g) % of change	33.76±1.12	39.12±0.34[a] +15.88	34.90±0.24[b] +3.37	33.32±1.00[b] +1.3	37.36±0.37[a] +10.66	36.82±0.82[d] +9.89
Liver AST (U/G) % of change	34.78± 0.26	41.26±0.89[a] +18.63	35.22±0.94[b] +1.26	35.60±0.97 [b] +2.36	37.78±0.25[b] +8.62	38.06±0.75[a] +9.43
Liver total protein (mg/g wet tis.) % of change	29.52±1.7	22.36±1.5 -24.25	26.92±1.2 -8.81	30.52±1.4[b] +3.39	22.44±1.7 -23.98	24.94±2.14 -15.51

a = Significant compared to control (I).
b = Significant compared to CCl_4(II).
c = Significant compared to Chitosan(III).
d = Significant compared to Chitosan-Cu(IV).

Table (3): Serum total lipids, triglycerides, total cholesterol and HDL. Liver total lipids, triglycerides, total cholesterol and HDL.

Animal group Estimated parameters	(I) Control	(II) CCl_4	(III) Chitosan	(IV) Chitosan-Cu	(V) Chitosan + CCl_4	(VI) Chitosan-Cu + CCl_4
S. total lipids (mg/dl) % of change	966.08±22.5	1125.02±9.90[a] +16.45	918.04±18.36[b] -4.97	877.22±36.4[b] -9.19	1043.4±20.6[c] +8.00	980.6±35.5[b] +1.50
S. triglycerides (mg/dl) % of change	90.66±2.30	125.20±5.2[a] +38.10	68.56±4.9[b] -24.37	80.48±10.8[b] -11.23	105.54±8.8[c] +16.41	94.58±5.4[b] +4.32
S. total cholest. (mg/dl) %of change	108.54±5.7	183.58±9.5[a] +69.13	106.3±5.5[b] -2.06	118.52±7.2[b] +9.19	137.88±4.8 [abc] +27.03	145.6±6.9[ab] +34.14
S. HDL (mg/dl) %of change	36.22± 1.6	19.64±1.2[a] -45.77	30.16±1.7[b] -16.73	34.34±1.9[b] -5.19	22.54±1.5 [ac] -37.77	26.88±1.7[abd] -25.77
L. total Lipids (mg/g) % of change	53.44±2.3	69.82±3.2[a] +30.65	47.87±1.0[b] -10.42	54.70±1.8[b] +2.36	62.76±2.2[c] +17.44	63.30±1.6[a] +18.45
L. triglycerides (mg/g) % of change	59.72±7.2	114.0±5.7[a] +90.89	59.90±3.6[b] +0.30	66.70±2.8[b] +11.69	74.30±4.6[b] +24.41	94.68±0.6[ad] +58.54
L. total cholesterol(mg/g) % of change	23.78±0.9	40.72±1.2[a] +71.24	24.24±2.9[b] +1.93	28.34±0.9[b] +19.17	34.04±1.4 [abc] +43.14	32.58±1.0[ab] +37.0

a = Significant compared to control (I).
b = Significant compared to CCl_4(II).
c = Significant compared to Chitosan(III).
d = Significant compared to Chitosan-Cu(IV).

Table (4): Serum Na, K, Fe and Zn.

Animal Estimated parameters	(I) Control	(II) CCl_4	(III) Chitosan	(IV) Chitosan-Cu	(V) Chitosan + CCl_4	(VI) Chitosan-Cu + CCl_4
Serum Na (mg/dl) % of change	157.8±1.3	88.2±1.7[a] -44.11	156.5±2.6[b] -0.82	162.3±3.8[b] +2.85	128.6±2.7[abc] -18.50	125.1±5.8[abd] -20.72
Serum K (mg/dl) % of change	6.02±0.08	8.82±0.40[a] +46.51	5.84±0.26[b] -2.99	5.46±0.35[b] -9.30	6.98±0.23[b] +15.94	7.72±0.31[ad] +28.24
Serum Fe (µg/ml) %of change	33.33±0.33	52.00±2.8[a] +56.01	33.23±0.8[b] -0.29	31.17±0.32[b] -6.49	47.5±0.79 [ac] +42.51	46.23±1.8[ad] +38.71
Serum Zn (µg/100 ml) %of change	15.5±0.16	9.90±0.13[a] -36.13	13.6±0.28[ab] -12.26	13.37±0.45[ab] -13.71	10.97±0.21 [ac] -29.19	10.35±0.31[ad] -33.22

a = Significant compared to control (I).
b = Significant compared to CCl_4(II).
c = Significant compared to Chitosan(III).
d = Significant compared to Chitosan-Cu(IV).

Discussion

Carbon tetrachloride (CCl_4) is a potent hepatotoxic agent; metabolism of CCL_4 is initiated by cytochrome P450 mediated transfer of an electron to the C-Cl bond to form an anion radical that eliminates chloride resulting in the trichloromethyl radical. Co-treatment with chitosan and CCl_4 significantly reduce these harmful effects on MDA and PC formation, probably by its antioxidant nature preventing the damage caused by free radical attack, and / or the inhibition of the deleterious actions of reactive oxygen species that damage lipids, DNA and proteins (Eidelman *et al*., 2002), inhibitory malondialdehyde formation triggered by CCL_4 (Yan *et. al*., 2006) and / or antiox-idant effect (Teselkin *et al*., 2000). And / or the scavenging effect of chitosan on hydroxyl radicals as reported by (Chiang *et al*., 2000). Increasing antioxidant enzyme activities may also lead to these improvements.

The reduction in liver SOD and GSH levels in CCl_4 treated animals reveal the deleterious effect on the antoxidative status, a result which concurs with the finding of Lee *et al.* (2007), and may be attributed to the inhibition of GSH reductase activity as reported by Ohta *et al*. (1997). The resulted attenuation in lipid peroxidation products and antioxidant enzymes activities obtained in rat groups treated with CCl_4 with chitosan or chitosan-Cu complex are in agreement with Yan *et al.* (2006). The rapid chitosan absorption by intestinal cells and the rapid distribution to other tissues in the body, can protect the hepatic tissue from CCL_4 toxicity (Zeng *et al*., 2007).

In the present study, the induction of CCl_4 caused increases in estimated liver enzymatic activities, in concomitant with decline in serum and liver protein reflected liver dysfunction, indicating their undesirable effects, these results agree with El-habibi and Amer (2000) and may be attributed to oxidative and reductive biotransformation and initiate biochemical events leading to liver cell necrosis (Weber *et al*., 2003 and Bhadaauria *et al.*, 2007).

Obtained decline in serum and liver total protein in CCl_4 treated group agree with previous report of El-Habibi and Amer (2000), may be attributed to the inactivation and degradation of CYP2E1, by CCL_4 where protein synthesis was blocked (Dai and Cederbaum, 1995). Obtained amelioration in serum and liver enzymes activities especially in group treated with CCl_4 and chitosan-Cu complex may be due to the suppressing ALT and AST activities by their antioxidant activity as reported by Lin and huang (2000) who showed that ALT and AST activities suppressed by antioxidant.

Obtained elevation in serum and liver ALT and AST are presumptive markers of CCl_4 induced hepatic injury that are in agreement with Popovic *et al.* (2007).These results may be due to loss in phospholipids membrane stability leading to the release of the enzymes as reported by Ahmed *et al*. (2000).

Significant hyperlipidemia, triglyceridemia and cholesterolemia also recorded in CCL_4 treated rats, and the restore in lipid content after chitosan and CCL_4 administration may be attributed to its antilipidemic property as mentioned by Xing *et al.* (2005), and / or to inhibitory action of chitosan on fat absorbtion. In addition to its ability to inhibit the increased accumulation of lipids in the systemic circulation (Choi *et al*., 2002) . These attenuation may be also as a result of excess food intake, where Le Houx and Grondin(1993) showed that rat fed chitosan ingested more food.

Co-administration of CCl_4 and chitosan or chitosan-Cu complex reduced the elevation of total cholesterol, and the maintained HDLc near the normal level, liver cholesterol content was decreased significantly, but did not reach control values. This indicates that under such conditions, the chitosan group had nearly reached the quilibrium , but not completely.

The elevated cholesterol in CCl_4 treated

group may be attributd to impaired utilization in sterodgenesis (Lin *et al.*, 1995).

Obtained amelioration in blood levels of total cholesterol, triglycerides, LDLc and VLDLc in chitosan treated group may be attributed to ability of chitosan in depressing of these parameters , as reported by Geremias *et al.* (2006), and may be attributed to catabolic derangement in lipoprotein metabolism Santhosh *et al.* (2006) and/or through the capability of chitosan for increasing the fecal excretion of cholesterol, a view which in line with Yao and Chiang (2002). These results may be also due to increased uptake of LDL from the blood by tissue as mentioned by Kissler *et al.* (2005). Also, this ameliorative effect of chitosan may be due to stop the passive exchange between plasma lipoprotein and the cell membrane (Brown and Goldstain, 1986). Obtained increase in serum and liver triglycerides in CCl_4 treated group may be attributed to increased lypolysis of adipose tissue stores as reported by Kruger *et al.* (1967), and /or the uptake of free fatty acids (FFA) from adipose tissue by the liver, leading to the hypertiglyceridemia, a view which in accordance with Stenberg (1976).

The resulted attenuation in serum and liver triglycerides levels in chitosan and chitosan-Cu complex treated animals may be as result of reduction in cholesterol and FFA levels by the hypolipidemic effect of chitosan as mentioned by Xing *et al.* (2005) and / or stimulating metabolic process by antioxidants that prevent formation of free radicals as mentioned by Eidelman *et al* . (2002).

Yonekura *et al.* (2004) reported that 1%dietary chitosan could increase zinc absorption in rats by formation of stable complexes with phytic acid. Zinc is very important in insulin synthesis activating anabolic process decreasing lipid deposition and increase protein synthesis. In addition chitosan residues have amino groups indeed, nitrogen atoms hold free electrons doubles that can react with metal cations (Varma *et al.*, 2004). However, the amino groups are easily protonated in acidic solutions, facilitating protein synthesis.

The resulted disturbances in Na and K levels in CCl_4 treated animals may be attributed to the degradation of membrane phospholipids in liver and to the loss of membrane fluidity, as lipoprotein levels of long-chain PUFA, were significantly decreased by CCl_4 (Moody *et al* ., 1981).

The water solubility of chitosan was dependent on the pH of solution. The free – NH_2 translated into $–NH_3^+$ which had weak chelating ability with metal cations, stimulating Na-K stability, hence nearly restored Na and K levels (Zeng *et al.*, 2007).

Conclusion

It seems that chitosan maintained normal liver functions in rats despite acute CCL_4 intoxication, attention should be paid to the possible effect of chitosan-Cu complex on the bioavailability and its pertinent clinical benefits, due to its highly hypolipidimic and strengthen antioxidant system.

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التاثير المقارن لمركب الكيتوزان و الكيتوزان مع النحاس ضد سمية الكبد المستحثة برابع كلوريد الكربون

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الرابع كلوريد الكربون سميه تحدث اكسده فوقيه وتلف بالكبد وقد تم دراسه الدور الوقائي المحتمل لمركب الكيتوزان والكيتوزان مع النحاس ضد السميه الكبديه في ذكور الجرذان .

تم الحقن بجرعه حاده بين الغشاء البريتوني برابع كلوريد الكربون (20 مجم / كجم من وزن الجسم), وقد احدث ذلك زياده في مالون ثنائي الألدهيد (MDA) وكربونيل البروتين (PC) وانخفاض في فوق انزيم الديسميوتيز (SOD) والجلوتاثيون (GSH) بانسجه الكبد وصحب ذلك زياده في انزيمات وظائف الكبد المقاسه (ALT&AST) مع انخفاض في مستوي البروتين في المصل والكبد. كما لوحظ زياده في معدل الدهون الكليه والكوليستيرول والجليسريدات الثلاثيه والدهون منخفضه الكثافه , صحب ذلك انخفاض في مستوي الدهون عاليه الكثافه.كذلك اظهرت الدراسه ارتفاع محتوي المصل من الصوديوم والحديد مع انخفاض في مستوي البوتاسيوم والزنك.

الجرذان التي تم حقنها بمركب الكيتوزان اوالكيتوزان مع النحاس بجرعه 200 مجم /كجم من وزن الجسم لمده احدي وعشرين يوما قبل الحقن برابع كلوريد الكربون احدث وقايه ملموسه لمعظم المعايير ورفع من قوه جهاز مضاد الاكسده الدفاعي وقام بتحسين وظائف الكبد . وكان لمركب الكيتوزان مع النحاس دورا اقوي من الكيتوزان بمفرده في الوقايه من اثار سميه رابع كلوريد الكربون.