Protective Role of Vitamin C on Histopathological and Enzymatic Changes in Experimental Liver Cirrhosis of Rats

Deneysel Karaciğer Sirozlu Sıçanlarda Enzimatik ve Histopatolojik Değişikliklerde C Vitamininin Koruyucu Rolü

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ABSTRACT Objective: Our aim in this study was to investigate the possible protective role of vitamin C on experimental liver necrosis, fibrosis and cirrhosis generated by carbon tetrachloride (CCl_4) in rats. **Material and Methods:** This present study was carried out on sixty Wistar albino rats divided equally into three groups. The first group (control group) received only saline (0.9% NaCl), the second group was given intraperitoneal 0.15 mL/kg CCI_4 in olive oil and the third group was administered CCl_4 (0.15 mL/kg, i.p.) plus vitamin C (100 mg/kg, by gastric gavage). After the treatments, blood was collected; aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP) and gamma glutamyle transferase (GGT) enzymes were measured as the biochemical markers of hepatotoxicity and liver tissue samples were taken for histopathological examination. **Results:** While the activities of AST, ALT and ALP were increased with CCl_4 injection, the activities of these enzymes after the administration of vitamin C were significantly decreased ($p< 0.01$, $p< 0.01$ and $p< 0.05$, respectively). The level of AST, ALT and ALP in the control group was lower ($p< 0.01$, $p< 0.01$, $p< 0.05$, respectively) than those in the CCl_4 group. Histopathological examination showed that CCl_4 induced necrotic, fibrotic and cirrhotic changes in the liver tissue, which decreased with vitamin C administration in the CCl_4 plus vitamin C group. **Conclusion:** Our results showed that CCl_4 caused an increase in the activities of liver enzymes in plasma and fibrotic, necrotic and cirrhotic changes in the liver. Vitamin C has an important role in the reduction of hepatic cellular injury produced by CCl_4.

Key Words: Vitamin C; carbon tetrachloride; liver function tests; fibrosis; rats

ÖZET Amaç: Bu çalışmada amacımız, karbon tetraklorür (CCl_4) verilerek sıçanlarda oluşturulan karaciğer nekrozu, fibrozu ve sirozunun gelişmesinde C vitamininin muhtemel koruyucu rolünü araştırmaktır. **Gereç ve Yöntemler:** Bu çalışmaya, 60 adet Wistar albino sıçan dâhil edildi ve bu sıçanlar eşit olarak üç gruba ayrıldı. Birinci gruba (kontrol) %0.9'luk NaCl verildi. İkinci gruba 0.15 mL/kg zeytin yağında hazırlanmış CCI_4 periton içine uygulandı. Üçüncü gruba CCl_4 (0.15 mL/kg) ile eş zamanlı olarak C vitamini (100 mg/kg, gastrik gavaj ile) verildi. Tedavi sonrasında kan alınarak, hepatotoksik biyokimyasal göstergeler olarak kabul edilen aspartat aminotransferaz (AST), alanin aminotransferaz (ALT), alkalen fosfataz (ALP) ve gama glutamil transferaz (GGT) enzimleri ölçüldü ve karaciğer doku örnekleri alınarak histopatolojik inceleme yapıldı. **Bulgular:** CCl_4 enjeksiyonu ile AST, ALT ve ALP artarken, C vitamini verilen grupta bu enzimlerin aktiviteleri azaldı ($p< 0.01$, $p< 0.01$ ve $p< 0.05$). Kontrol grubunda AST, ALT ve ALP düzeyleri, zeytin yağında hazırlanmış CCI_4 verilen gruba göre daha düşük idi ($p< 0.01$, $p< 0.01$, $p< 0.05$). Histopatolojik incelemede, CCl_4'ün karaciğerde nekrotik, fibrotik ve sirotik değişikliklere neden olduğu ve C vitamininin eklenmesiyle bu değişikliklerin azaldığı görüldü. **Sonuç:** CCl_4 karaciğerde, nekrotik, fibrotik ve sirotik değişiklikler oluşturur ve aynı zamanda karaciğer enzim aktivitelerini artırır. C vitamini, karaciğerde CCl_4 ile oluşturulan hücre hasarının önlenmesinde önemli bir role sahiptir.

Anahtar Kelimeler: Vitamin C; karbon tetraklorür; karaciğer fonksiyon testleri; fibrozis; sıçanlar

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Oxidative stress in the cells is identified as a common factor in the ethiopathogenesis of many degenerative human diseases. An imbalance between oxidative and antioxidative systems leads to oxidative damage in the body. Several substances have been defined as antioxidants which include free radical scavengers, exogenous agents such as vitamins A, C, E and endogenous substances like superoxide dismutase (SOD), catalase (CAT), glutathione (GSH), glutathione peroxidase (GSH-Px), uric acid, bilirubin and albumin.[1-3]

CCl_4 can cause damages in the liver, the kidney and the central nervous system. The liver is especially sensitive to CCl_4 and thus is commonly used as an experimental model for the liver damage in animals.[4-6] While the hepatocytes, acutely affected by CCl_4, are damaged or destroyed, the chronic parenchymal exposure leads to fatty degeneration, necrosis, fibrosis and cirrhosis.[7-9] This well-known hepatotoxic agent produces haloalkane radicals during its biotransformation in the liver microsomes. These free radicals produce cell damage through covalent binding to cellular macromolecules, particularly, to the cellular lipid and lipoproteins.[10,11]

The oxidative damage caused by free radicals may be avoided or reduced with antioxidants in the biological environment.[12] Vitamin C is known as the most important antioxidant in extracellular fluids and may scavenge many toxic oxygen-derived products such as superoxide radical, hydrogen peroxide, hypochloride, and hydroxyl and peroxyl radicals before they damage cellular unsaturated lipids in the body. It is also known to have many biological functions such as collagen biosynthesis.[1,2,13] Indeed, its antioxidant function is related to reversible oxidation and reduction characteristics thereby protecting lipids and lipoproteins in cellular membranes against oxidative damage caused by toxic free radicals at early stages.[14,15] Therefore, the present study was designed to investigate the effects of vitamin C on experimental liver cirrhosis generated by CCl_4 in rats using biochemical measurements of AST, ALT, ALP and GGT in plasma and histopathological examination of the liver tissues.

MATERIAL AND METHODS

ANIMALS AND TREATMENTS

This study was carried out on sixty Wistar albino rats weighing 200-250 g. Rats were supplied from the Center of Experimental Animal Research (İnönü University, Malatya, Turkey). The experimental protocol was designed according to the Principles of Laboratory Animal Care and ethical standards for animal use and was approved by the local ethical committee. All rats were fed with a rodent pellet and drinking water ad-libitum. The animals were randomly divided into three equal groups each containing twenty rats, housed in cages at room temperature throughout the study.

The first group was used as control and was injected intraperitoneal (i.p.) placebo (0.9% NaCl). The second group was injected i.p. CCl_4 (0.15 ml/kg) dissolved in olive oil (1/4, v/v). The third group was administered i.p. CCl_4 0.15 mL/kg plus vitamin C (L-ascorbic acid, 100 mg/kg-body weight, gastric gavage). All treatments were administered three times in a week for seven weeks.

Mortality rate was 5-20%, which was similar to those reported by investigators using this model.[14] During the study, one rat from the first group (5% mortality), four rats from the second group (20% mortality), and three rats from the third group (15% mortality) died.

The blood samples were taken 24 hours after the last dose under ether anesthesia. Subsequently, the animals were sacrificed and their abdomens were immediately opened. The livers of all animals were removed by dissection and were fixed in formaldehyde (10%, v/v) for histopathological examination. Vitamin C (L-ascorbic acid) and olive oil were provided from Sigma Chemical Co. (St. Louis, MO, USA). CCl_4 was purchased from Merck AG (Darmstadt, Germany).

ENZYME ANALYSIS IN PLASMA

Whole blood samples were collected in heparinized tubes, were subsequently centrifuged at 1500 x g for 15 min (Heraus® Inst, Mega Fuge 1.0 centrifuge), and their plasma was separated. In plasma samples, the activities of AST, ALT, ALP and GGT

were determined by an autoanalyzer (Technicon® RA-XT, New York, USA).

HISTOPATHOLOGICAL EXAMINATION OF LIVER TISSUES

Liver specimens were embedded in paraffin for an ordinary histological examination and were sectioned as 3 to 5 μm serial sections using a rotary microtome. The sections were stained with hematoxylin and eosin (H&E) for histological examinations and with Masson trichrome stain for evaluation of the fibrosis. Microscopic studies were reviewed by an experienced pathologist blinded to the study. The degree of fibrosis and necrosis within the periportal tracts and lobules were assessed according to Zhang et al.[14] Histological grading was made according to four severity grades: 0 (none), no fibrosis and normal liver architecture; I (mild), fatty degenerations around portal areas and central veins, fibrosis increased in portal areas and sinusoidal space and regular liver architecture; II (moderate), thin fibrous septae present connecting portal areas and pseudolobules seen frequently; and III (severe), thick fibrous septa and collagen bands accompanied by pseudolobules.

STATISTICAL ANALYSIS

For statistical analyses, nonparametric independent group comparisons were made. For multiple comparisons the Kruskal-Wallis test was used. Comparisons between the groups were made by the Mann-Whitney U test. A level of 5% ($p< 0.05$) was considered statistically significant. Data were expressed as median and range.

RESULTS

Plasma AST, ALT and ALP levels were significantly higher in the CCl_4-treated group than those in the CCl_4 + Vitamin C treated and control groups ($p< 0.01$ for AST and ALT, $p< 0.05$ for ALP) (Table 1). While the activities of AST, ALT and ALP were increased by CCl_4 injection, they were significantly decreased by the administration of vitamin C. There was no significant difference between the GGT values of the groups. The results were summarized in Table 1.

Histopathological examinations of the liver tissues revealed that CCl_4 induced necrotic, fibrotic and cirrhotic changes. In addition, animals treated with vitamin C showed significant attenuation compared with the CCl_4 group (Table 2). Histological analysis of the liver specimens of control animals showed no evidence of histopathological alterations (Figure 1A). The chronic effect of CCl_4 on the rat liver specimens usually occurred as typical fibrotic and cirrhotic changes. Some areas showed varying degrees of hepatic degenerations.

TABLE 1: Clinical parameters in the plasma of control, CCl_4-treated and CCl_4+Vitamin C-treated groups.

	Control (n= 19)	CCl_4 (n= 16)	CCl_4 + vitamin C (n= 17)
AST (U/L)	112 ± 80	369 ± 154 ^{a**}	131 ± 34 ^{b*,c**}
	(79-159)	(273-427)	(126-160)
ALT (U/L)	79 ± 57	115 ± 46 ^{a**}	79 ± 5 ^{b*,c**}
	(30-87)	(105-151)	(76-81)
ALP (U/L)	242 ± 120	263 ± 358 ^{a*}	227 ± 121 ^{b*,c*}
	(163-283)	(32-390)	(172-293)
GGT (U/L)	2 ± 2	3 ± 3	2 ± 2
	(2-4)	(2-5)	(1-3)

AST: Aspartate aminotransferase, ALT: Alanine aminotransferase, ALP: Alkaline phosphatase and GGT: Gamma glutamyle transferase (GGT).

Values are Median ± SD (Min -Max)

a: Difference between control and CCl_4-treated groups

b: Difference between control and CCl_4+Vitamin C treated groups

c: Difference between CCl_4-treated and CCl_4+Vitamin C treated groups

** = $p< 0.01$

* = $p< 0.05$.

TABLE 2: The histopathological grading for fibrotic and necrotic changes in liver of control, CCl_4-treated and CCl_4 + Vitamin C-treated groups.

		Histopathological grading			
Groups	**n**	**0**	**I**	**II**	**III**
Control	19	19	0	0	0
CCl_4	16	0	0	5	11
CCl_4 + vitamin C	17	0	8	5	4

Collagen bands bridged between the portal areas or extended from central regions to portal areas. Broad fibrous septae was often seen as surrounding pseudolobules. Collagen bands were observed with Masson trichrome stain. Portal areas were accompanied by a variable degree of hyperplasia of the biliary epithelium and biliary duct proliferations. These biopsies were considered strong suggestion of cirrhosis (Figure 1B).

The liver sections obtained from animals treated with vitamin C showed consistent reduction in necrotic, fibrotic, and cirrhotic processes of the liver. Collagen bands connecting central regions with portal areas and pseudolobules were not seen. In addition, cell injury, coagulative necrosis, inflammatory infiltration and centrilobular fatty metamorphosis were always observed. Intralobular necrosis, comprising all single cells and cell groups, including piece-meal and bridge necrosis, was seen. Fatty degeneration within hepatocytes was present especially in the centrilobular zone of the liver. Variable numbers of inflammatory cells consisting of granulocytes, round cells were observed in the periportal tract and intralobular areas. These specimens showed more regular liver architecture, in which only thin fibrous bands were seen to connect portal areas (Figures 1C and D).

DISCUSSION

Vitamin C is known to have antioxidant ability to inhibit oxidative processes of lipids and lipoproteins in cell membranes.[1-3] Antioxidant function of vitamin C is related to its reversible oxidation and reduction characteristics. Its effects on the toxicity of CCl_4 may also be attributed to its antioxidant feature by which it scavenges superoxide radicals, hydrogen peroxide, hypochloride, and hydroxyl and peroxyl radicals at early stages of oxidative damage.[1,2] Thus, vitamin C may partially prevent certain types of hepatic cellular degeneration.[4]

In this study, we used vitamin C as an exogenous antioxidant against oxidative damage caused by CCl_4 in the liver. Liver is an important target organ for CCl_4 with hepatocytes damaged by haloalkane free radicals produced during its biotransformation.[4,5,8-10,12] The haloalkane free radicals such as trichloromethyl free radical (CCl_3) and trichloromethyleperoxy radical (CCl_3O_2) are in mixed function oxidase system utilizing the nicotinamide adenine dinucleotide phosphate (NADPH)-cytocrome P-450 electron transport chain. They may bind to subcellular macromolecules and can react with free amino groups on proteins.[8-10] Hence, the macromolecules may lose their physiological functions. Haloalkane free radicals were held responsible for CCl_4-hepatotoxicity and were reported to cause the oxidative damage of unsaturated lipids in some cellular components of hepatic tissues. Damage of these components may be an important factor for liver cell injury.[8,9,11,12] Thus, AST, ALT and ALP may be mobilized into the blood and their plasma levels increase, which is usually indicative for liver degeneration in animals.[9,10,16-18] Therefore, in this investigation, using these enzymes as biochemical markers of the oxidative hepatic damage, which was developed by CCl_4 injection, we tried to find out the protective effects of vitamin C. Our results suggested that CCl_4 led to increased activities of AST, ALT and ALP, but the activities of these enzymes were significantly decreased by the administration of vitamin C. The levels of AST, ALT and ALP in the control group were lower than the levels in both the CCl_4 and the CCl_4 plus vitamin C groups. These results were consistent with the findings in previous investigations.[4,10,16,17,19-23] Vitamin C decreased these values near to the control levels and provided a strong evidence for the presence of oxidative injury caused by CCl_4 and for the protective effects of antioxidant vitamins like vitamin C. Although our results were similar to and in agreement with those in the study by Halim et al, in which they used 50% smaller amount (50 mg/kg) of vitamin C, they are discordant

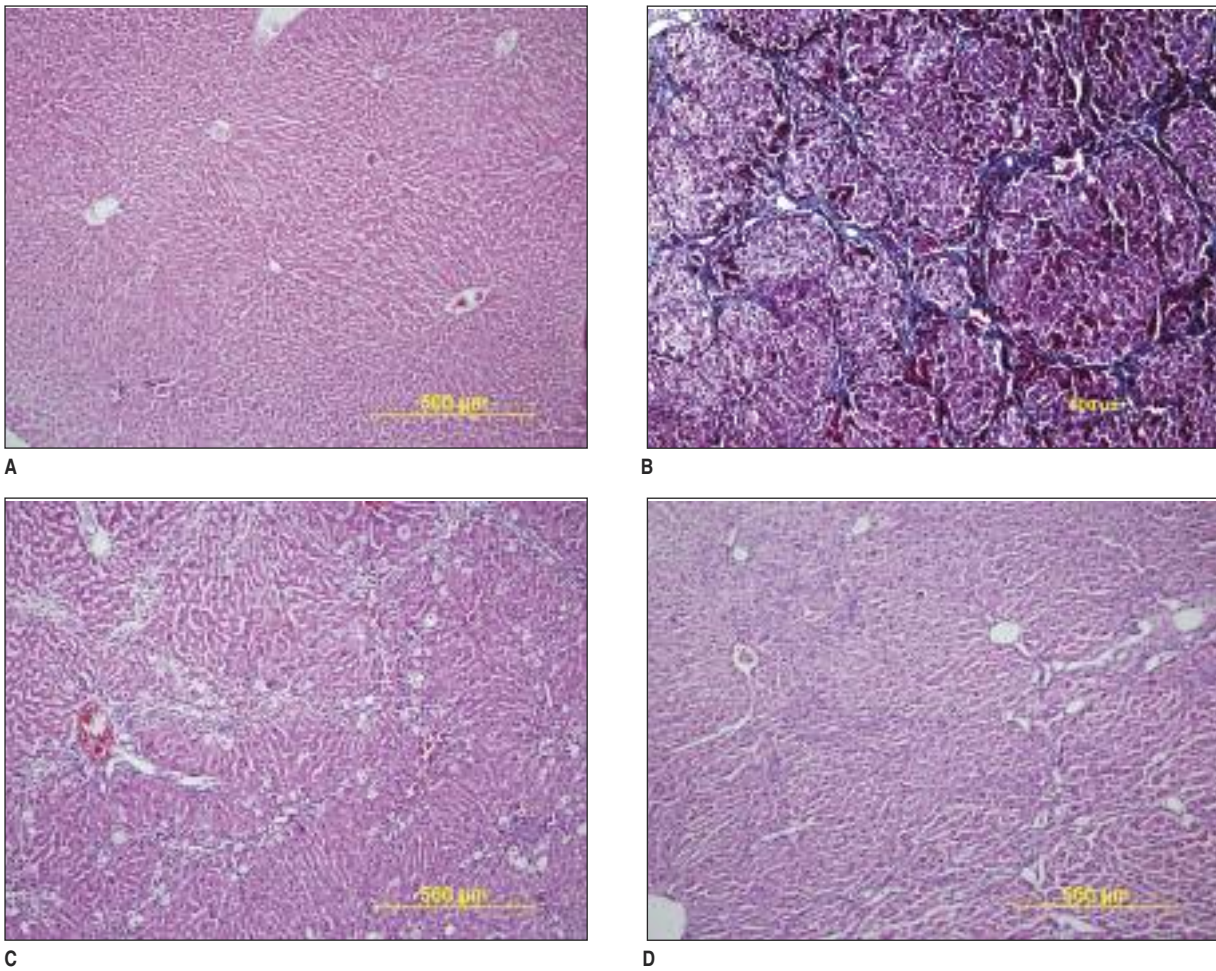

FIGURE 1: Slides of H&E-stained liver sections. 1A: Histological appearance of a liver specimen from the control group, with no histopathological evidence of hepatic injury (H&E stain, x100); 1B: CCl4-injured rats group shows a typical cirrhotic appearance, with thick septae often surrounding pseudolobules (Masson trichrome stain, x100); 1C: CCl4 plus vitamin C-group, prominent intralobular inflammatory reaction consisting of granulocytes, round cells and fatty metamorphosis in hepatocytes (H&E stain, x100); 1D: periportal and intralobular delicate fibrous bands are seen (H&E stain, x100).

with the data of another previous work undertaken by Türkdoğan et al in which they found vitamin C to be ineffective in oxidative liver injury caused by CCl_4.[4,5]

Histopathological results of our study suggested that vitamin C could reduce liver injury, fibrosis and cirrhosis induced by CCl_4. The protective effects of vitamin C against CCl_4-induced chronic liver damage and cirrhosis were confirmed by conventional histological examination.[7-9,12] Indeed, vitamin C efficiently traps free radicals, which damage the lipids and lipoproteins in the cell membranes before they can initiate lipid peroxidation. Thus, it can protect the biomembranes.[1,2,14,15]

In the light of these findings, it is possible to conclude that vitamin C, a well-known antioxidant, is quite effective to avoid hepatic cellular damage, and for the prevention and corrective treatment of CCl_4-toxicity. However, more precise comparative work is required to verify this conclusion employing other antioxidants like vitamins A, E and selenium.

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