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ARAŞTIRMA / RESEARCH

Karbon tetraklorür ile oluşturulan deneysel karaciğer hasarında benfotiamin'in karaciğer dokusu üzerine koruyucu etkilerinin incelenmesi

Investigation of benfotiamine's protective effects on liver tissue in experimental carbon tetrachloride induced liver injury

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Abstract

Purpose: In this study, we aimed to investigate the protective effects of benfotiamine on experimental liver injury caused by carbon tetrachloride (CCl4).
Materials and Methods: In this study, 30 male Wistar albino rats were used. Rats were equally divided into 5 groups. No application was made to control group. The CCl4 group was injected i.p with1ml/kg CCl4:olive oil (1:2) mixture on the 1st and 8th days, and the CCl4+benfotiamine group was treated i.p with 1 ml/kg CCl4: olive oil (1:2) mixture twice on the 1st and 8th days and orally with 70 mg/kg/day benfotiamine. To the benfotiamine group, 70 mg/kg/day benfotiamine was given orally for 14 days. To the olive oil group, 2 ml/kg olive oil was given i.p. on 1st and 8th days. Finally, rats were decapitated. Liver tissues were removed and paraffin blocks were prepared. Tissues were stored at –80 oC for malonaldeyhde (MDA) assay.
Results: There were no significant differences between the control, benfotiamine and olive oil groups. Compared with the control group, there was a significant increase in MDA, apoptosis and bax immunoreactivity in CCl4 group. Compared with the CCl4 group, there was a significant decrease in MDA, apoptosis and bax immunoreactivity in the CCl4+benfotiamine group.
Conclusion: CCl4 increases MDA, apoptosis and bax immunoreactivity, and benfotiamine, given as treatment, reduces these parameters.

Keywords:. Rat, carbon tetrachloride, benfotiamine, apoptosis

Öz

Amaç: Bu çalışmada karbon tetraklorür (CCl4) ile oluşturulan deneysel karaciğer hasarında benfotiaminin karaciğer dokusu üzerine koruyucu etkilerinin incelenmesi amaçlanmıştır.
Gereç ve Yöntem: Çalışmada, 30 adet Wistar albino cinsi erkek sıçanlar kullanıldı. Deney hayvanları 5 eşit gruba ayrıldı. Kontrol grubuna 14 günlük deney süresince herhangi bir uygulama yapılmadı. CCl4 grubuna ise 1. ve 8. günlerde 1ml/kg CCl4: zeytinyağı (1:2) intraperitoneal (i.p) verildi. CCl4 +benfotiamin grubuna 1. ve 8. günlerde iki defa 1 ml/kg CCl4: zeytinyağı (1:2) karışımı ve 70 mg/kg/gün oral benfotiamin verildi. Benfotiamin grubuna 70 mg/kg/gün oral benfotiamin verildi. Zeytinyağı grubuna 1. ve 8. günlerde 2 ml/kg zeytinyağı i.p. olarak verildi. Deney sonunda sıçanlar anestezi altında dekapite edildi. Karaciğer dokuları çıkarılıp incelemeler için parafin bloklar hazırlandı. Ayrıca malondialdehid (MDA) çalışması için dokular – 80 0 C de saklandı.
Bulgular: Kontrol, benfotiamin ve zeytinyağı grupları arasında bakılan parametrelerde anlamlı bir farklılık yoktu. Kontrol grubu ile karşılaştırıldığında CCl4 grubunda, MDA, apoptozis ve bax immünreaktivitesinde anlamlı artış izlendi. CCl4 grubu ile karşılaştırıldığında CCl4 + benfotiamin grubunda ise MDA, apoptozis ve bax immünreaktivitesinde anlamlı azalma görüldü.
Sonuç: CCl4'ün MDA, apoptozis ve bax immünreaktivitesini arttırdığı, tedavi olarak verilen benfotiaminin bu parametreleri azalttığı görüldü.

Anahtar kelimeler: Sıçan, karbon tetraklorür, benfotiamin, apoptozis

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INTRODUCTION

The liver, which accounts for nearly 2% of the body weight, is the largest gland in the organism and fulfills very important metabolic functions[1]. It is exposed to various toxic, harmful substances and medications due to its anatomical localization, and physiological and biochemical duties. Liver damage is a pathological condition with a difficult treatment due to the late manifestation of the symptoms[2-3]. It can arise from various factors and constitutes an important health problem. Therefore, the use of hepatoprotective agents, i.e agents that have a protective effect on the liver, is important as they significantly reduce mortality and morbidity, have a favourable effect on survival rates, and lower the high treatment costs. Various pharmacological agents are currently being investigated in order to prevent acute and chronic liver damage. Liver damage can be prevented by free radical scavengers and agents that inhibit lipid peroxidation[4]. Different forms of liver damage can arise from oxidative stress and the subsequent release of free radicals[5-8]. Toxic oxy-, and hydroxy radicals were shown to create damage in the cell membranes of hepatocytes through lipid peroxidation or other processes and also to cause damage to carbohydrates, proteins, lipids, and DNA (deoxyribonucleic acid) in vivo and in vitro[6-12].

Liver function tests (LFT) are needed to make a diagnosis, guide diagnostic efforts, predict the severity of the disease, determine the prognosis, ad evaluate the treatment. There exists no test that can address all of these problems. The available tests reflect distinct metabolic reactions. Although laboratory tests are essential in the follow-up and treatment of patients with liver disease, these have certain limitations[13].

Carbon tetrachloride (CCl_4)- induced hepatotoxicity is a model that has common use in the investigation of the hepatoprotective effects of certain medications and herbal extracts (14, 15). CCl_4 is a substance that creates liver injury by generating free radicals. This effect is realized through the following stages: The molecule is dehalogenized to the free radical trichloromethyl (CCI3) by the action of the cytochrome-P450 enzyme system. CCI3 forms the trichloromethyl peroxyl (CCI3OO) radical with the addition of oxygen molecules. This resulting reactive molecule initiates lipid peroxidation after a few more stages. Toxic peroxidation products that arise from lipid peroxidation damage the cell membrane. If such membrane damage is not prevented, it will lead to cell death[5,12,16,17] . CCl4 also causes hydropic degeneration and zone-3 hepatocellular necrosis in the liver[18, 19].

Benfotiamine is a fat-soluble form of Vitamin B1[20]. Some studies have shown benfotiamine to suppress reactive oxygen products[21, 22]. Reduced glutathione attenuates the effects of oxidizing agents within the cell and serves as an antioxidant by protecting the proteins that have an active role within the cell from the oxidation reaction. Consequently, the glutathione molecule is oxidized. It must be reduced again for it to fulfill its functions. This is achieved by the use of NADPH (Nicotinamide adenine dinucleotide phosphate). The pentose phosphate pathway is important for NADPH production. As thiamine plays an effective role in this pathway, it is considered an antioxidant[23, 24].

This study aims to investigate the protective effects of benfotiamine on the liver tissue in a CCl_4-induced experimental liver injury model. Our study is the first study in this field. The use of benfotiamine in the treatment phase of rats with liver damage shows the importance of our study to improve liver function.

MATERIALS AND METHODS

This study was granted an ethics approval by Firat University Animal Research Ethics Committee (Approval number: 15, Date: 02.02.2012) and conducted in Firat University Experimental Research Centre.

Procedure

This study included 200-250g male Wistar albino rats obtained from Firat University Experimental Research Centre. The experimental animals were assigned to 5 groups: control (group I), CCl_4 (group II), CCl_4 +benfotiamine (group III), benfotiamine (group IV), and olive oil (group V).

Five groups were formed by assigning 6 rats (n=6) to each group.

Group I (n=6): The control group did not receive any treatments for 14 days

Group II (n=6): Experimental animals in this group were applied i.p with 1ml/kg CCl_4/olive oil (1:2), twice on days 1 and 8.

Group III (n=6): Experimental animals in this group were applied i.p with 1ml/kg CCl_4/olive oil (1:2)

twice on days 1 and 8 and orally with 70 mg/kg/day benfotiamine.

Group IV (n=6): Experimental animals in this group were treated only with 70 mg/kg/day oral benfotiamine for 14 days.

Group V (n=6): Experimental animals in this group were applied i.p with 2 ml/kg olive oil, twice on days 1 and 8.

In the end of the experiment, all rats were treated i.p with ketamine (75 mg/kg) + xylazine (10 mg/kg) and decapitated under anesthesia. After decapitation, liver tissues of the rats were promptly extracted and fixed in 10% formaldehyde, and paraffin blocks were prepared for histological and histochemical evaluations. The tissues were stored at -80 C until the end of the study for MDA measurement in liver tissue. The Avidin-Biotin-Peroxidase complex method was used to assess bax immunoreactivity in the liver tissue.

Immunohistochemical evaluation

5-6 mm sections obtained from paraffin blocks were mounted onto polylysine slides. These were incubated with the primary antibody (Bax mouse monoclonal IgG, Santa Cruz Biotechnology, sc–7480, California, USA) for 60 minutes. After the primary antibody, the secondary antibody (biotinylated anti-mouse /rabbit IgG, Diagnostic BioSystems, KP 50A, Pleasanton, USA), streptavidin alkaline phosphatase and the Fast Red chromogen were applied. Then, counter-staining with Mayer's hematoxylin was performed. The negative control tissues were treated with phosphate buffered saline (PBS) instead of the primary antibody. The preparations were evaluated and photographed using a Novel N-800M microscope.

Immunohistochemical staining was evaluated based on the extent of staining. The extent of cytoplasmic immune staining was scored semi-quantitatively between 0 and +3 (0: none, +1: mild, +2: moderate, +3: strong) and statistical analyses were conducted.

TUNEL Method

5-6 mm sections obtained from paraffin blocks were mounted onto polylysine slides. An ApopTag Plus Peroxidase in Situ Apoptosis Detection Kit (Chemicon, cat no: S7101, USA) was used according to the manufacturer's instructions and the cells undergoing apoptosis were determined. Negative control tissues were treated with Reaction Buffer instead of the Tdt enzyme. The preparations were evaluated and photographed using a Novel N-800M microscope. In the evaluation of TUNEL staining, nuclei stained blue with Harris hematoxylin were considered normal and cells that showed brown nuclear staining were considered apoptotic. At least 500 normal and apoptotic cells were counted in randomly selected areas of the sections at x10 magnification. The Apoptotic Index (AI) was computed by dividing the number of apoptotic cells by the total number of cells (normal + apoptotic) and statistical analyses were conducted.

Malondialdehyde (MDA) Assay

0.42 gr Tris-Base + 1.43 gr Tris-HCI + 3 gr KCI, and 0.5 ml Tween 20 were prepared in 250 ml distilled water. This buffer was used in the homogenate of the samples. X g tissue was supplemented with and separated by 5 ml buffer. The homogenate was centrifuged at 5000 rpm for 5 minutes and 1ml from the supernatant was transferred to another tube. 1 ml 10% TCA (10 gr TCA prepared in 100 ml distilled water) was added to the obtained 1 ml sample. 1 ml 0.6% TBA (0.6 gr TBA is prepared in 100 ml distilled water, the prepared TBA can be stored at +4^{o}C for up to one day) was added. Then, 1 ml distilled water was added, and lastly, 0.5 ml 4% HCl was added (4ml HCl is prepared in 100 ml distilled water, the acid must be added in drops as there will be a strong reaction between the acid and water). The resulting mixture was left to incubate for 120 min at 90-95^{o}C. After incubation, the tubes were cooled at room temperature and vortexed after the addition of 3 ml butanol. Then, the red-pink component of the supernatant obtained by centrifuging the tubes at 5000 rpm for 5 min (butanol phase) was transferred to the spectrophotometer cuvette using a pipette and absorbance was read at 532 nm against butanol. The absorbance reading was calculated using the formula x: (reading$_{ABS}$ +0.0344)/0.0492. The obtained value was multiplied by 5 (as the tissue homogenate was prepared using 5 ml buffer), and the result was equal to the weight of tissue used in the homogenate.

Statistical analysis

Statistical analysis of the data was performed using IBM SPSS 22.0 statistical package program. Normal distribution of data was confirmed by the Shapiro-Wilk test before further analyses. Normally distributed data are expressed as mean±SD in the text and table. One-way analysis of variance was used for group comparisons of TAS, TOS and immunity

variables. Levene's statistic was used for the homogeneity test of variances. Tukey's pair-wise multiple comparison test was used to determine the differences between the groups of significant variables. The level of significance was accepted to be at least $p < 0.05$.

RESULTS

Spectrophotometrically measured tissue MDA levels were comparable across the control, benfotiamine, and olive oil groups. Compared to the control group, the CCl_4 group showed significantly higher MDA levels (p: 0,016). Compared to the CCl_4 group, the CCl_4 + benfotiamine group showed significantly reduced MDA levels in liver tissues (p: 0,042) (Table 1).

Table 1. Tissue MDA Levels

Group	MDA (nmol/g protein)
Control	111.83 ± 1.72
CCl4	165.83 ± 8.86a
CCl4+ Benfotiamine	104.33 ± 2.33b
Benfotiamine	121.50 ± 2.07c
Olive Oil	110.16 ± 9.28d

Values were presented in the form of mean ± standard deviation.
[a]Compared with the control group (p: 0,016),
[b]Compared with the CCI4 group (p: 0,042),
[c]Compared with the control group (p: 0,024),
[d]Compared with the control group (p: 0,516).

Table 2. Bax immunoreactivity.

Group	Bax Immunoreactivity
Control	1.16 ± 0.40
CCl4	2.83 ± 0.40a
CCl4+ Benfotiamine	1.83 ± 0.40b
Benfotiamine	1.33 ± 0.51c
Olive Oil	1.16 ± 0.40d

Values were presented in the form of mean ± standard deviation.
[a] Compared with the control group (p: 0,002),
[b] Compared with the CCI4 group (p:0.035),
[c] Compared with the control group (p: 0,002),
[d] Compared with the control group (p: 0,516),

The immunohistochemical staining performed to determine Bax immunoreactivity was evaluated using a light microscope and the bax immunoreactivity findings were comparable across the control, benfotiamine, and olive oil groups. Compared to the control group, the CCl_4 group showed significantly higher bax immunoreactivity (p: 0,002). The CCl_4 + benfotiamine group that received benfotiamine as treatment showed a significant decrease in bax immunoreactivity compared to the CCl_4 group, approaching the levels seen in the control group (p:0.035) (Table 2). The negative control did not manifest any staining (Figure 1).

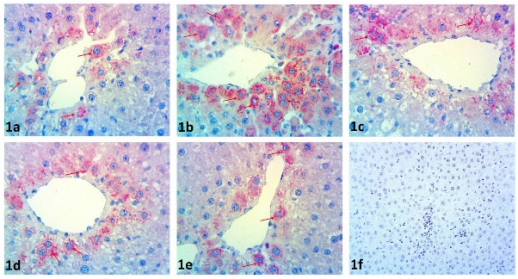

Figure 1. Bax immunoreactivity in liver tissue

1a: Bax immunoreactivity in liver tissue obtained from the control group (→), *1b:* Bax immunoreactivity in liver tissue obtained from the CCl_4 group (→), *1c:* Bax immunoreactivity in liver tissue obtained from the CCl_4+benfotiamine group (→), *1d:* Bax immunoreactivity in liver tissue obtained from the benfotiamine group (→), *1e:* Bax immunoreactivity in liver tissue obtained from the olive oil group (→), *1f:* Negative control.

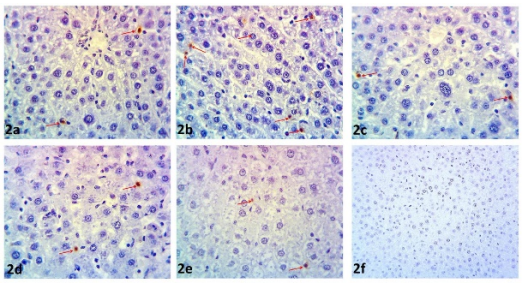

Figure 2. TUNEL positive cells in liver tissue

2a: TUNEL positive cells in liver tissue obtained from the control group (→), 2b: TUNEL positive cells in liver tissue obtained from the CCl4 group (→), 2c: TUNEL positive cells in liver tissue obtained from the CCl4+benfotiamine group (→), 2d: TUNEL positive cells in liver tissue obtained from the benfotiamine group (→), 2e: TUNEL positive cells in liver tissue obtained from the olive oil group (→), 2f: TUNEL negative control.

The evaluation of TUNEL staining conducted to identify apoptotic cells under a light microscope found TUNEL positivity in sinusoidal cells (red arrow) in the liver tissue. TUNEL positivity was comparable across the control, benfotiamine and

olive oil groups. Compared to the control group, the CCl_4 group showed a significant increase. Compared to the CCl_4 group, the CCl_4 + benfotiamine group showed a significant decrease (p:0.038) (Table 3). The negative control did not manifest TUNEL positivity (Figure 2).

Table 3. Apoptotic index (%)

Group	Apoptotic Index (%)
Control	2.16 ± 0.40
CCl4	6.66 ± 1.21a
CCl4+ Benfotiamine	3.16 ± 0.40b
Benfotiamine	1.83 ± 0.40c
Olive Oil	1.66 ± 0.51d

Values were presented in the form of mean ± standard deviation.
[a] Compared with the control group (p:0.026).
[b] Compared with the CCI4 group, (p:0.038).
[c] Compared with the control group, (p:0.065).
[d]Compared with the control group, (p:0.058).

DISCUSSION

The liver is exposed to the harmful effects of almost all oral and intravenous medications, toxic substances, and microbic agents; and it either detoxifies these effects or protects itself by responding to the damage created by these agents with regeneration[25]. The most recent studies have proposed that reactive oxygen metabolites have an active role in hepatotoxicity[28]. Numerous studies have shown that the elevated oxidative stress in liver diseases is linked to liver damage and fibrosis[29,30]. Free radicals with one or more unpaired electrons in their outer orbitals are short-lived, unstable, and highly active molecules that are closely linked to oxidative stress[37].

Metabolic reactions occurring in the tissues result in the formation of superoxide anions, hydroxyl radicals, hydrogen peroxide, and free oxygen radicals with very high reactivity[38,39]. The disruption of the intracellular electron balance, oxidative stress, mitochondrial defects, and the inadequacy of the antioxidant system may trigger apoptosis[40]. Oxidative stress can increase lipid peroxidation and cause fibrosis in the liver due to its unfavourable effects on the antioxidant mechanism[41]. CCl_4, which is hepatotoxic, results in the production of free radicals, and as a result, tissue damage[42]. Carbon tetrachloride has been one of the most widely used chemical agents in experimental rodent studies as the resulting liver degeneration closely resembles the development of cirrhosis in humans. Oxidative stress is known to play an important role in the appearance of CCl_4-induced liver toxicity[43]. The steps in the development of carbon tetrachloride-induced liver injury can be summarized as follows: reductive dehalogenation, covalent bonding of the radicals, inhibition of protein synthesis, lipid accumulation, loss of calcium sequestration, apoptosis, and lastly, fibrosis[44]. Experimental studies have reported CCl_4 to increase the synthesis of lipid peroxidation in liver and kidney tissues depending on the different administration methods and dosages; and also, to decrease levels of GSH (reduced glutathione)[45]. The body has developed certain defence mechanisms in order to prevent reactive oxygen species and their damage. These are known as antioxidant defence systems. Antioxidant molecules can be structures of endogenous or exogenous origin and they neutralize the damage inflicted by the produced oxidant molecules through intracellular and extracellular defence. Intracellular free radical scavenger enzymes are the main actors of antioxidant defence. These enzymes are superoxide dismutase, glutathione-S-transferase, glutathione peroxidase, glutathione reductase, catalase, and cytochrome oxidase[46]. In this study, it was aimed to investigate the protective effects of benfotiamine on liver tissue in experimental liver damage caused by CCl4.

Some studies have shown benfotiamine to have a suppressive effect on reactive oxygen products[21,22]. Highly significant changes such as steatosis, hydropic dystrophy, destructive changes in the hepatocyte cytoplasm, and severe hepatocyte necrosis have been described in the morphological evaluation of rat livers exposed to carbon tetrachloride[49].

In our study, the CCl_4 group was determined to have significantly higher MDA levels, bax immunoreactivity, which is a preapoptotic protein, and the number of apoptotic cells; and when administered as a treatment, benfotiamine was found to significantly reduce these parameters. In oxidative damage, calcium homeostasis and mitochondrial membrane potentials are altered. These changes damage the DNA and lead the cell to apoptosis, which is described as programmed death[47]. It is known that certain changes appear as the cell is damaged and undergoing apoptosis. As a result of these changes, cytochrome c is released to the cytosol. The release of cytochrome c to the cytosol can be stopped by the antiapoptotic members (Bcl-2, Bcl-XL). Meanwhile, the pro-apoptotic members (Bax, Bak, Bad) of the Bcl-2 family work to increase cytochrome c release. The survival and death of the

cell depends on this balance[50-52]. Benfotiamine, which is a fat-soluble form of the vitamin thiamine[20], shows antioxidant properties as it protects the cell from oxidizing agents and protein oxidation via reduced glutathione[23]. The use of antioxidants may be beneficial in the protection of the liver from CCl_4-induced damage and its treatment. In a study conducted by Vales and his team, administering NAS (N-Acetyl cysteine), which is an antioxidant substance, after CCl_4 was reported to prevent necrosis in the liver tissue[53]. Wang et al. reported that using melatonin caused liver fibrosis to regress and the levels of antioxidant enzymes SOD and GSH-Px to increase in rats treated with CCl_4[30]. At the same time, studies have explored various isoflavone variants with regard to their reparative effects in CCl_4-induced liver injury. In a study by Ustundag and colleagues, soy isoflavones were shown to be effective in preventing CCl_4 damage, to decrease the elevated levels of lipid peroxide products, and stimulate the paraoxonase enzyme, which has antioxidant properties[55]. Our study is the first study in this field. The use of benfotiamine in the treatment phase of rats with liver damage shows the importance of our study to improve liver function.

In conclusion, the present study suggests that CCl_4 significantly increases apoptosis, bax immunoreactivity, and MDA levels, that administering benfotiamine as treatment decreases these parameters, and that further and more detailed studies to be conducted in the future can investigate treatment approaches that involve benfotiamine in conditions associated with liver damage, primarily cirrhosis. Small number of rats in the study group can be seen as the most important limitation. Therefore, our findings must be supported by a larger rats groups.

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REFERENCES

1. Burroughs AK, Westaby D. Liver, biliary tract and pancreatic disease. In Clinical Medicine (6th ed) (Eds P Kumar, M Clark):347-417. Philadelphia, PA, Elsevier Saunders, 2005.
2. Dhanabal SP, Syamala G, Satish Kumar MN, Suresh B. Hepatoprotective activity of the Indian medicinal plant Polygala arvensis on D-galactosamine-induced hepatic injury in rats. Fitoterapia.2006;77:472-4.
3. Naaz F, Javed S, Abdin MZ. Hepatoprotective effect of ethanolic extract of phyllanthus amarus schum et thonn on aflatoxin B1-induced liver damage in mice. J Ethnopharmacol. 2007;113:503-9.
4. Seven A, Candan G. Antioksidan savunma sistemleri. Cerrahpaşa Journal of Medicine. 1996;27:41- 50.
5. Bacon BR, Tavill AS, Brittenham GM, Park CH, Recknagel RO. Hepatic lipid peroxidation in vivo in rats with chronic iron overload. J Clin Invest. 1983;71:429-39.
6. Brattin WJ, Glende EA, Recknagel RO. Pathological mechanisms in carbon tetrachloride hepatotoxicity. J Free Radio Biol Med. 1985;1:27-38.
7. Comporti M. Lipid peroxidation and cellular damage in toxic liver injury. Lab Invest. 1985;53:599-623.
8. Kaplowitz N, Fernandez-Checa J, Ookhtens M. Glutathione, alcohol and hepatotoxicity. In Nutrition and the Origins of Disease (Eds CH Halsted, RB Rucker):267-82. San Diego, Academic Press, 1989.
9. Halliwell B. The antioxidant paradox. Lancet. 2000;355:1179-80.
10. Halliwell B, Gutteridge JM. Oxygen toxicity, oxygen radicals, transition metals and Disease. Biochem J. 1984;219:1-14.
11. Hooper C. Free radicals: research on biochemical bad boys comes of age. J Natl Inst Health Res. 1989;1:101-6.
12. Slater TF. Free-radical mechanisms in tissue injury. Biochem J. 1984;222:1-15.
13. Musaoğlu A. Karaciğer hastaliklarinda laboratuvar testleri. Temel İç Hastalıkları. 1. Baskı (Eds G İliçin, S Ünal, K Biberoğlu, S Akalın, G Süleymanlar):1069-109. Ankara, Güneş Kitabevi, 1997.
14. Recknagel RO, Glende EA, Dolak JA, Waller RL. Mechanisms of carbon tetrachloride toxicity. Pharmacol Ther. 1989;43:139-54.
15. Weber LW, Boll M, Stampfl A. Hepatotoxicity and mechanism of action of haloalkanes: Carbon tetrachloride as a toxicological model. Crit Rev Toxicol. 2003;33:105-36.
16. Yao T, Delgi Esposti S, Huang L, Arnon R, Spangenberger A, Zern MA. Inhibition of carbon

tetrachloride-induced liver injury by liposomes containing vitamin E. Am J Physiol. 1994;267:476-84.
17. Zimmerman HJ. Hepatotoxicity: The Adverse Effects of Drugs and Other Chemicals in the Liver. 2nd Ed, Philadelphia, Lippincott Williams & Wilkins, 1999.
18. Poli G, Cottalasso D, Pronzato MA, Chiarpotto E, Marinari UM. Lipid peroxidation and covalent binding in the early functional impairment of golgi apparatus by carbon tetrachloride. Cell Biochem Funct. 1990; 8:1–10.
19. Poli G, Albano E, Dianzano MU. The role of lipid peroxidation in liver damage. Chem Phys Lipids. 1987;45:117–42.
20. Woelk H, Lehrl S, Bitsch R, Kopcke W. Benfotiamine in treatment of alcoholic polineuropathy; an 8-week randomized controlled study (BAPI Study). Alcohol. 1998;33:318-631.
21. Gadau S, Emanueli C, Linthout SV, Grainai G, Todaro M, Meloni M et al. Benfothiamine accelerate the healing of ischaemic diabetic limbs in mice through protein kinase B/Akt-mediated potentiation of angiogenesis and inhibition of apoptosis. Diabetologia. 2006;49:405-20.
22. Marchetti V, Menghinni R, Rizza S, ve ark. Benfothiamine counteracts glucose toxicity effects on endothelial progenitor cell differentiation via Akt/ FOXOsignaling. Diabetes. 2006;55:2231-37.
23. Bakker SJ, Heine RJ, Gans RO. Thiamine may indirectly act as an antioxidant. Diabetologia. 1997;40:741-2.
24. Pomero F, Molinar Min A, La Selva M, Allione A, Molinetti GM, Porta M. Benfotiamine is similar to thiamine in correcting endothelial cell defects induced by high glucose. Acta Diabetol. 2001;38:135-8.
25. Orrego H, Blake JE, Blendis LM, Medline A. Prognosis of alcoholic cirrhosis in the presence and absence of alcoholic hepatitis. Gastroenterology. 1987;92:208-14.
26. Ning QJ, Qin SW, Xu CS. Expression patterns and action analysis of genes associated with drug-induced liver diseases during rat liver regeneration. World J Gastroenterol. 2006;21:6966-72.
27. Cabre M, Comps J, Paternain JL, Ferre N, Joven J. Time course of changes in hepatic lipid peroxidation and glutathione metabolism in ratswith carbon tetrachloride-induced cirrhosis. Clin Exp Pharmacol Physiol. 2000;27:694-9.
28. Evans P, Halliwell B. Micronutrients: oxidant/antioxidant status. Br J Nutr. 2001;85:67-74.
29. Ökten A. Türkiye'de karaciger sirozunun etyolojisi. Hepatolojide Güncel Gelişmeler Sempozyum Kitabı. 1998:67.
30. Wang H, Wei W, Wang NP. Melatonin ameliorates carbontetrachloride-induced hepatic fibrogenesis in rats via inhibition of oxidative stres. Life Sci. 2005;77:1902-15.
31. Parola M, Robino G. Oxidative stress-related molecules and liver fibrozis. J Hepatol. 2001;35**:**297-306.
32. Poli G. Pathogenesis of liver fibrszis, role of oxidative stress. Mol Aspects Med. 2000;21:49-98.
33. Lee KS, Buck M, Houglum K. Activation of hepatic stellate cells by TGF alpha and collagen type I is mediated by oxidative stres through Cyb expression. J Clin Invest. 1995;96:2461-8.
34. Parola M, Pinzani M, Casini A. Stimulation of lipid peroxidation or 4- hdroxynonenal treatment increase procollagen alpha 1 (I) gene expression in human liver fat- storing cells. Biochem Biophysical Res Comm. 1993;194**:**1044-50.
35. Kumar V, Cotran RS, Robbins SL. Temel Patoloji. 2. Baskı, İstanbul, Nobel Tıp Kitapevleri, 1994.
36. Gochee PA, Jonsson JR, Clouston AD, Pandeya N, Purdie DM, Powell EE. Steatosis in chronic hepatitis C: association with increased messenger RNA expression of collagen I, tumor necrosis factor-alpha and cytochrome P450 2E1. J Gastroenterol Hepatol. 2003;18:386-92.
37. Evans P, Halliwell B. Micronutrients: oxidant/antioxidant status. Br J Nutr. 2001;2:67-74.
38. Castillo T, Koop DR, Kamimura S, Triadafilopoulos G, Tsukamodo H. Role of cytochrome P-450 2E in ethanol-carbon tetrachloride-and iron-dependent microsomal lipidperoxidation. Hepatology. 1992;16:992–6.
39. Hartley DP, Kolaja KL, Reinchord J, Peterson DR. 4-Hydroxynonenal and malondialdehyde hepatic protein adducts in rats treated with carbon tetrachloride: immunochemical detectionand lobular localization. Toxicol Appl Pharmacol. 1999;161:23–33.
40. Oren M, Rotter V. Introduction: p53 the first twenty years. Cell Mol Life Sci. 1999;55:9-11.
41. Bandyopadhyay U, Das D, Banerjee R. Reactive oxygen species: oxidative damage and pathogenesis. Curr Sci. 1999;77:658-65.
42. Pietta P G. Flavonoids as antioxidants. J Nat Prod. 2000;63:1035-42.
43. Mc Gee SA, Wiggins SA, Pierce JD. What advanced practise nurses to know about free radicals. Int J Adv Nurs Prac. 2003;6:1-10.
44. Hong RT, Xu JM, Mei Q. Melatonin ameliorates experimental hepatic fibrosis induced by carbon tetrachloride in rats. World J Gastroenterol. 2009;15:1452-8.
45. Guven A, Gulmez M. The effect of kefir on the activities of GSH-Px, GST, CAT, GSH and LPO levels in carbon tetrachloride-induced mice tissues. J Vet Med B. 2003;50:412–6.
46. Halliwell B Antioxidant characterization. Methodology and mechanism. Biochem Pharmacol. 1995;49:1341-8.
47. Burçak G, Andrican G. Oksidatif DNA hasarı ve yaşlanma. Cerrahpaşa Tıp Dergisi 2004;12:159-69.
48. Ellis RE, Yuan J, Horvitz HR. Mechanisms and

functions of cell death. Annu Rev Cell Biol. 1991;7:663-98.
49. Chin PL, Momand J, Pfeifer GP. In vivo evidence for binding of p53 to consensus binding sites in the p21 and GADD45 genes in response to ionizing radiation. Oncogene 1997;15:87-100.
50. Nagata S. Apoptosis by death factor. Cell. 1997;88:355-65.
51. Lee JI, Lee KS, Paik YH, Park JN, Han KH, Chon CY et al. Apoptosis of hepatic stellate cells in carbon tetrachloride induced acute liver injury of the rat: analysis of isolated hepatic stellate cells. J Hepatol. 2003;39:960-6.
52. Hoijman E, Rocha Viegas L, Keller Sarmiento MI, Rosenstein RE, Pecci A. Involvement of bax protein in the prevention of glucocorticoid-induced thymocytes apoptosis by melatonin. Endocrinology. 2004;145:418-25.
53. Valles EG, de Castro CR, Castro JA. N-acetlylcysteine is an early but also a late preventive agent aganist carbon tetrachloride induced liver necrosis Toxicol Lett. 1994;71:87-95.
54. Bayram İ. Askorbik asit ve alfa-tokoferol'ün karbon tetraklorürle oluşturulmuş akut karaciğer toksisitesi modelinde karaciğeri koruyucu etkisi. Van Tıp Dergisi 2004;11:32-8.
55. Üstündağ B, Bahçecioğlu İH, Şahin K, Gülcü F, Düzgün S, Özercan İH et al. Soy izoflavonların karbon tetraklorüre (CCl4) bağlı karaciğer hasarı ve plazma paraoksonaz ile arilesteraz aktivite düzeylerine olan etkileri. Fırat Üniversitesi Sağlık Bilimleri Dergisi. 2005;19:263-71.