Kafkas Univ Vet Fak Derg
22 (3): 403-408, 2016
DOI: 10.9775/kvfd.2015.14825

Kafkas Universitesi Veteriner Fakultesi Dergisi
Journal Home-Page: http://vetdergi.kafkas.edu.tr
Online Submission: http://vetdergikafkas.org

Research Article

The Protective Effect of Kefir on Carbon Tetrachloride-induced Histopathological Changes in the Livers of Rats

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Article Code: KVFD-2015-14825 Received: 07.12.2015 Accepted: 12.01.2016 Published Online: 11.01.2016

Abstract

The aim of the study was to investigate the protective effects of kefir in the liver damage of rats, at experimental carbon tetrachloride (CCl_4) intoxication by histologically and immunohistochemically. During the 45 days trial period, 18, female, Wistar albino rats were used. One of them was control, three experimental group was created. Twice a week 0.5 cc carbon tetrachloride (CCl_4) + olive-oil (1:1) suspension was injected subcutaneously to the second and third group. At third group additionally to this administration 30 ml kefir was given daily by oral gavage. CCl_4-induced hepatocellular damage and apoptosis was observed but these adverse findings reduced with kefir administration. These findings indicate that kefir may have a protective role at liver damage.

Keywords: *Apoptosis, Carbon tetrachloride, Histopathology, Liver, Rat*

Karbon Tetraklorüre Bağlı Ratların Karaciğerinde Oluşan Histopatolojik Değişikliklere Karşı Kefirin Koruyucu Etkisi

Özet

Çalışmada deneysel karbon tetraklorür (CCl_4) toksikasyonu oluşturulan ratlarda kefirin karaciğer hasarına karşı koruyucu etkilerinin histolojik ve immunohistokimyasal olarak araştırılması amaçlandı. Kırkbeş günlük deneme boyunca, 18 adet, dişi, Wistar albino rat kullanıldı. Biri kontrol olmak üzere, üç deneme grubu oluşturuldu. Grup 2 ve 3'e haftada 2 kez 0.5 cc karbon tetraklorür (CCl_4) + zeytinyağı (1:1) süspansiyonu subkutan yolla verildi. Grup 3'e buna ek olarak günlük 30 ml kefir oral gavaj yoluyla verildi. CCl_4'e bağlı hepatoselüler dejenerasyon ve apoptosis gözlendi, ancak kefir eklenmesi ile bu olumsuz değişiklikler azaldı. Bu bulgular kefirin karaciğer hasarında koruyucu rolü olabileceğini gösterdi.

Anahtar sözcükler: *Apoptozis, Karbon tetraklorür, Histopatoloji, Karaciğer, Rat*

INTRODUCTION

Carbon tetrachloride is obtained by the chlorination of carbondisulfide or reacting of the same compound with sulfur monochloride. This material absorbed by respiration, skin and gastrointestinal tract. They are used as anthelminthic, against parasites in veterinary medicine [1]. When carbon tetrachloride used in high doses the accumulation of it causes damage in liver even cirrhosis can be created. It also makes degeneration in many other organs in the body [1-3].

In regard to FAO/WHO; probiotics means 'for life' organisms, are useful for humans and animals [4]. The probiotics include some yeast such as; *Lactobacillus bulgaricus, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus helveticus, Lactobacillus lactis, Lactobacillus salivarius, Lactobacillus plantarum, Streptococcus thermophilus, Enterococcus faecium, Enterococcus faecalis, Bifidobacterium spp., Escherichia coli* and *Saccharomyces*. Some of them also content *Bacillus subtilis* [5,6]. It has been reported that probiotic applications might be protective against to the urogenital, gastrointestinal and surgical infections [7,8].

A fermented milk product kefir drink, obtained from kefir grains, is Caucasian origin and ethyl alcohol and lactic acid fermentation are shaped together in it [9,10]. The polysaccharide structure, white-yellowish color kefir grains contains microorganisms such as, lactobacilli, lactococci, leuconostocs, acetobacteri and fungi (*Kluyveromyces marxianus, Torulaspora delbrueckii, Saccharomyces cerevisiae, Candida kefir*) [11-13]. It was reported that kefir has antioxidant,

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antifungal [14], antibacterial, antitumor, immunological [15,16], cholesterol-lowering [17] and anti- the apoptotic effect [18].

Both physiological and pathological inducible cell death mechanism apoptosis known as programmed cell death or cell suicide. Organism refers to apoptosis during organogenesis of multicellular organisms or completed the development in living, damaged or the removal of cells that potentially tumor predisposed [19,20]. Apoptosis occurs by one of two pathways: (1) a death receptor pathway, and (2) the mitochondrial pathway [21]. It was reported that in cultured rat hepatocytes, the hydrophobic bile acid glycochenodeoxycholate, GCDC, at pathophysiologically relevant concentrations (20-100 μM) induces apoptosis, as documented by cell shrinkage, nuclear condensation and lobulation, caspase activation, DNA fragmentation, and phosphatidylserine externalization [22].

In the study, it was aimed that detection the protective effect of kefir at histologic and apoptotic changes with TUNEL method, induced by carbon tetrachloride in the livers of rats.

MATERIAL and METHODS

A total of 18 female Wistar albino rats were used in the study. Three experimental group (one of them was control) formed and each group consisting 6 animals. All groups were fed with pellet (standart commercial rat chow) and drinking water was given *ad libitum*. The research project and animal housing conditions were approved by the Mustafa Kemal University Ethical Committee for Animal Studies (approval 2014-01/11). Rats were obtained from the Mustafa Kemal University Laboratory Animal Breeding Unit. The rats were assigned randomly to three groups. The first group was the control group, were fed only rat chow and drinking water. Twice a week 0.5 cc carbon tetrachloride (CCl_4) + olive-oil (1:1) suspension was injected subcutaneously to the second and third group. A total of 12 injections applied for 45 days. At third group additionally to this administration 30 ml kefir was given daily by oral gavage. Kefir drink was prepared as; kefir grain to sterile milk, 3% (w/v) and fermenting at 30°C for 24 h. After fermentation kefir was diluted 1:3, before given to rats.

At the end of the 45-day experimental period animals were sacrificed by decapitation under anesthesia [intra-muscular injection of ketamine (50 mg/kg) and xylazine (20 mg/kg)]. Necrospy was performed and liver tissues took out and routine process was done. Initially tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin then were cut in 5-6 μm for hematoxylin and eosin (H & E) staining [23] and for in situ detection of apoptotic cells.

In-situ Detection of Apoptotic Cells by TUNEL Assay: DNA fragmentation was assessed in situ in liver sections using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin nick endlabelling (TUNEL) method used as catalog procedure *(In Situ Cell Death Detection Kit, POD, Roche, Mannheim, Germany)*. In summary sections were de-waxed and rehydrated using routine methods. Firstly sections were held in 3% H_2O_2 for 20 min later in proteinase K (20 mg/ml; Roche, *Mannheim, Germany)* for 15 min at room temperature. As following step; sections initially washed with Phosphate buffer solution (PBS, pH 7.4) for 3 times for 5 min, later 50 μl TUNEL reaction mixture (including TdT & dUTP) was dropped and incubated in 37°C humid camera for 1 h. Again washed with PBS. Later sections were incubated with conjugate anti-fluorescein-POD for 40 min at room tempereture and washed three times with PBS. To visiualize reaction products, samples were incubated with AEC *(3-Amino 9-Ethyl Carbasole, Dako, Glostrup, Denmark)* for 5 min, and counterstained with Mayer's hematoxyline stain. As a control, samples were treated with labeling solution instead of TdT.

Finally all sections were examined by light microscopy *(Olympus CX31)* and microphotographed *(Olympus DP12)*.

RESULTS

At control group liver was normal in colour and consistency, any macroscopical change was observed. Liver sections of the control group showed a normal histological appearance of the sinusoids and hepatic central vein, any fatty degeneration was observed *(Fig. 1)*.

At CCl_4 treated group livers yellowish pigmentation and crumbly-fatty consistency noticed at macroscopical examination. Liver histopathology revealed centrolobular lipid accumulation with necrosis in the hepatocytes *(Fig. 2)*. Also sinusoidal congestion, locally yellowish-green gall pigmentation, increase in the number of kupffer cells and inflammatory cell infiltration around the necrotic tissue was noted. With TUNEL staining DNA fragmentation was observed at some liver epithelium cells. TUNEL reaction in cell cytoplasms was demonstrated as granular staining *(Fig. 3)*.

At kefir added group macroscopical appearance of the liver was resembled to control group, normally in colour and pigmentation. The parenchymal structure of the liver was preserved via kefir administration. Kefir significantly reduced fatty degeneration, hepatocytes necrosis, sinusoidal congestion and inflammatory cell infiltration *(Fig. 4)*. Compatible with this histopathological results any staining was observed with TUNEL reaction test.

At positive and negative control sections dropping terminal transferase-free solution instead of TUNEL reaction mixture, test gave negative result in all sections.

DISCUSSION

Due to chemicals or infectious agents the liver

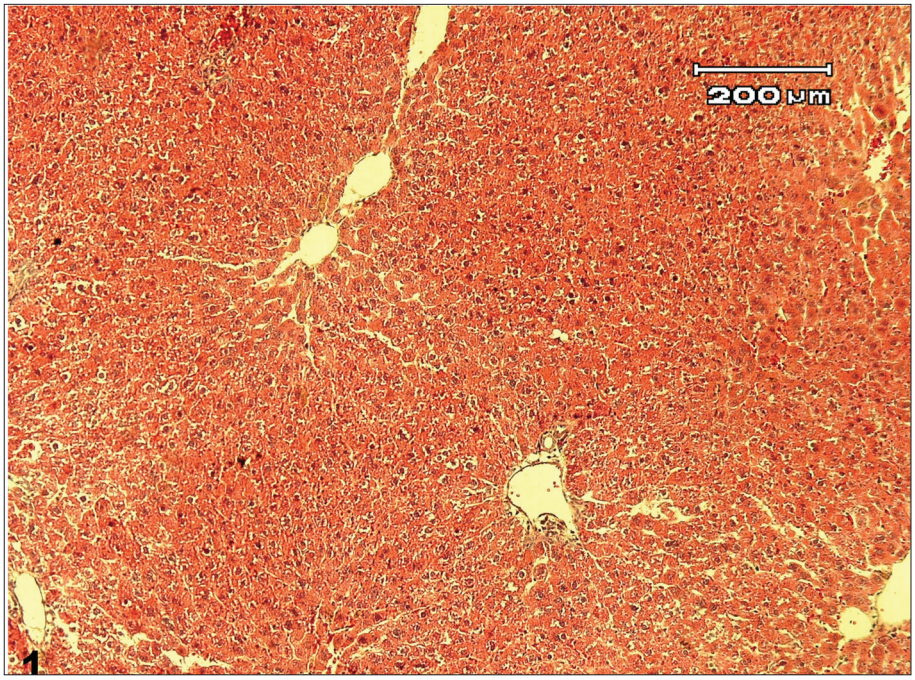


Fig 1. Control group, liver, normal histological appearance of the sinusoids and hepatic central vein, H&E

Şekil 1. Kontrol grup, karaciğer, sinuzoidler ve hepatik sentral damarın normal histolojik görüntüsü, H&E

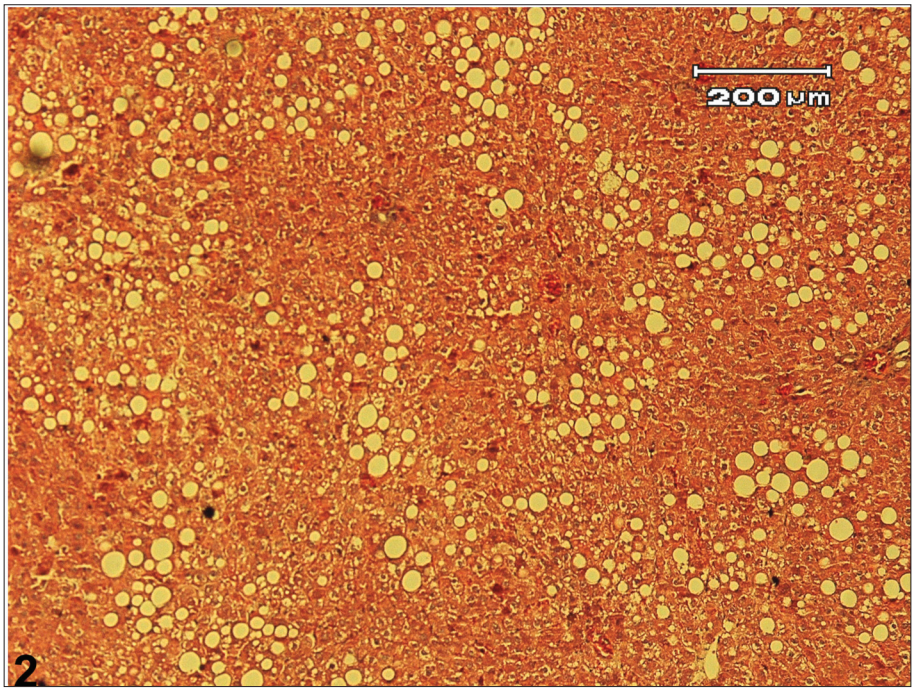


Fig 2. CCl_4 group, liver, large, diffuse, lipid vacuoles with necrosis in the hepatocytes, H&E,

Şekil 2. CCl_4 grubu, karaciğer, geniş, diffuz, yağ vakuolleri ve hepatositlerde nekroz, H&E

disorders are one of the world problems and unfortunately no effective treatment that prevent disease damage, progression and complications has yet been found [24,25]. But each passing day investigators studied with new agents to prevent liver damage. Many hepatotoxin agent was found in the environment [26,27], carbon tetrachloride is one of them [28]. Carbon tetrachloride and other halogenated hydrocarbons are used as liquid cleaner (detergents) and oil-repelling substances immemorial. In veterinary medicine they are used against the parasitic anthelminthic [2]. Low doses of CCl_4 caused fatty degeneration of the liver cells, while high doses caused the necrosis of liver cells has been reported [1,29]. In the our study due to 45 day, low doses CCl_4 administration; hepatocellular degeneration, necrosis and lipid accumulation was observed as described before.

Human and animal beings can encounter with many hepatotoxic agents during their life beacuse of this each passing day both humans, pet owners and of course investigators use a lot of functional foods such as kefir. In the developing world kefir drink increasingly become as popular. Its known that Caucasian origin acidic and midly alcolic fermented milk kefir drink contain benefical microorganisms and can treat some diseases [9] and has

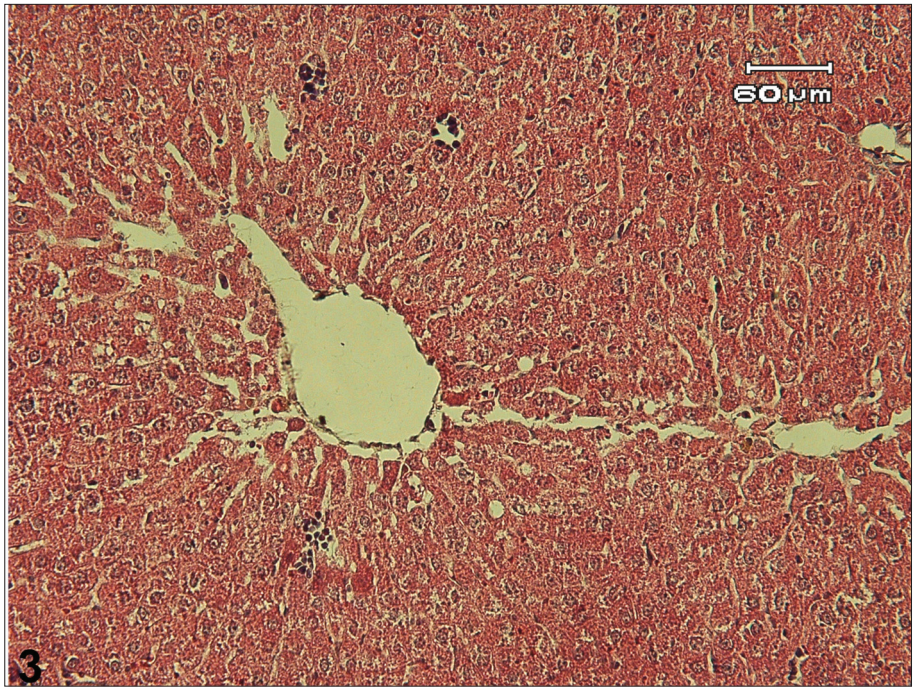
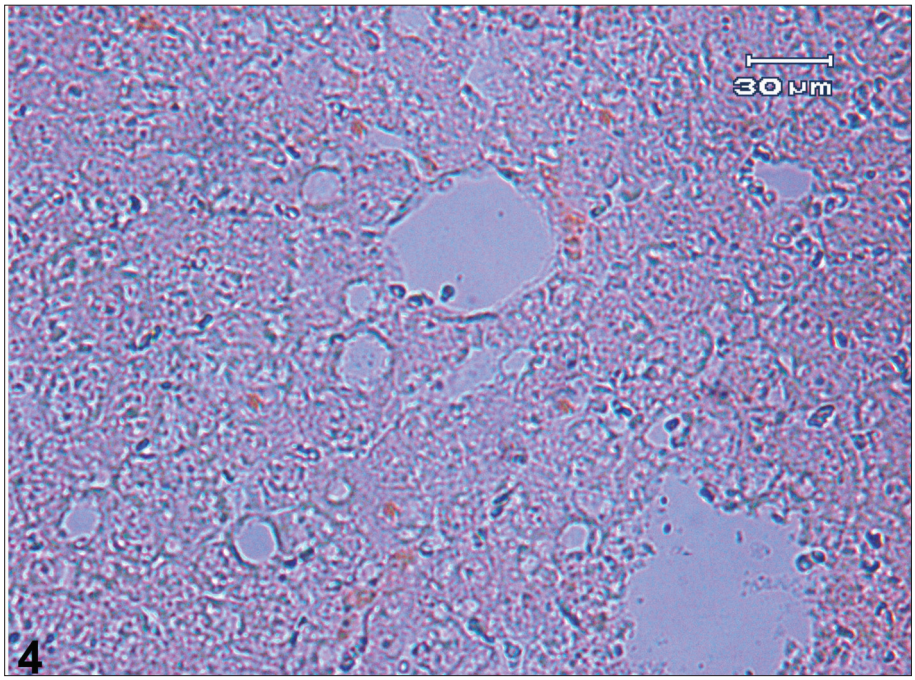


Fig 3. Kefir group, liver, only few lipid vacuoles, H&E

Şekil 3. Kefir grubu, karaciğer, birkaç yağ vakuolü, H&E

Fig 4. CCl_4 treated group, liver, apoptotic hepatocytes, TUNEL stain

Şekil 4. CCl_4 grubu, karaciğer, apoptotik hepatositler, TUNEL boyama



an effect on natural immune system it modulates the immune system [30] and also previous studies reported that kefir including many benefical general health properties such as antioxidant features [14,31]. In the study its clearly observed that affiliated to CCl_4 administration severe liver damage was formed but kefir drink significantly reduced pathological changes. Initial positive change observed at macroscopical appearance of the liver. While in CCl_4 tretaed group yellowish pigmentation and crumbly-fatty consistency noticed, with kefir administration nearly to normally colour and consitency was observed. Due to CCl_4 toxication histopathologically diffuse, big lipid vacuoles were replaced with few and small lipid vacuoles with kefir addition. Also kefir protect the paranchymal structure of the liver. This situation was explained at previous studies by this; kefir can able to inhibite the adipocyte differentiation due to its ability to eliminate lipid accumulation at mature adiposites. GPDH (gliserol 3 fosfat dehidrogenaz) is an enzyme that converse glycerole to triglyceride and regular kefir consumption also can be significantly reduced GPDH activity [32]. Kefir has other benefical effect on decreasing chlosterol levels [33-35]. By apoptosis dying cells are promptly removed by phagocytosis and replaced by new cells generated by mitosis, also apoptosis is an essential feature

of a wide variety of acute and chronic diseases, including liver diseases [36]. In the study cytoplasmic reactions that were detected with TUNEL test, were associated with apoptotic bodies that including nucleous residuals. We absorved apoptosis in some hepatocytes depending on CCl_4 exposed. But although severe lipid accumulation, hepatocellular degeneration and inflammatory reaction; apoptotic changes were very mild. Based on kefir supplementation microscopically nearly to control group liver paranchymal structure was observed. Otherwise any reaction that was related to apoptosis was determinated.

In conclusion, our results indicate that CCl_4 induce histopathological changes and apoptosis at hepatocytes. Kefiran intake decreased these adverse alterations and did not show any negative effects in the liver of rats. As a results the study shows that kefir is a healthy food that protect liver from CCl_4 toxication and inhibits hepatocellular degeneration, lipid accumulation and apoptosis.

Acknowledgment

We are thankful to *Antgen Medical* for chemical material supply.

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