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Protective Mechanism of *Urtica Dioica* on Carbon Tetrachloride-Induced Hepatic Encephalopathy in Rats

Gülşah YILDIZ DENİZ

Atatürk University, Health Services Vocational School, Department of Medical Lab Techniques Erzurum, Turkey

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ABSTRACT Around the world, species from the genus *Urtica* are commonly used because of their peripheral and central medicinal effects; this is prepared as teas. In recent years, it has become increasingly important to study the beneficial properties of derivatives of *Urtica dioica* (UD). The aim of the present study was to evaluate the effects of UD against carbon tetrachloride (CCl_4) induced hepatic encephalopathy (HE). Forty-nine adult (2-months-old) male *Sprague dawley* rats were used in this study. The models were established by CCl_4 (1 mL/kg body weight; twice a week) given intraperitoneally for 8 weeks. The animals were euthanized by decapitation and rat brains were removed to assess histopathologic changes. Biochemical parameters were assessed in serum samples from the CCl_4-treated rats. UD extracts provided significant protection against CCl_4-induced brain damage by increasing the preventing alterations in biochemical serum parameters, such as the levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), glutamyl transferase (GGT) and ammonia relative to the control group. Histopathological and immunohistopathological changes of the brain tissue was observed using Hematoxylin-eosin (H&E) staining and c-Fos expression method. In the present study, the protective effect of UD on CCl_4 toxicity was demonstrated through studies of biochemistry and immunohistopathology. Administration of UD may have potential protective effects against CCl_4-induced brain toxicity.

Keywords: *CCl_4, Hepatic encephalopathy (HE), Rat, Urtica dioica*

ÖZ

Urtica dioica'nın Sıçanlarda Karbon Tetraklorür ile İndüklenen Hepatik Ensefalopati Üzerine Koruyucu Mekanizması

Dünya çapında, Urtica cinsinden türler periferik ve merkezi tıbbi etkileri nedeniyle yaygın olarak kullanılmaktadır; bunlar çay olarak hazırlanır. Son yıllarda, *Urtica dioica* (UD) türevlerinin yararlı özelliklerini incelemek gittikçe önem kazanmıştır. Bu çalışmanın amacı, UD'nin karbon tetraklorür (CCl4) ile indüklenen hepatik ensefalopatiye (HE) karşı etkilerini değerlendirmektir. Bu çalışmada kırk dokuz yetişkin (2 aylık) erkek Sprague dawley sıçanı kullanıldı. Modeller 8 hafta boyunca intraperitoneal olarak verilen CCI_4 (1 mL / kg vücut ağırlığı; haftada iki kez) ile oluşturuldu. Hayvanlar başları kesilerek ötanazi edildi ve sıçan beyinleri histopatolojik değişiklikleri değerlendirmek için çıkarıldı. Biyokimyasal parametreler CCI_4 ile tedavi edilen sıçanlardan alınan serum örneklerinde değerlendirildi. UD ekstraktları, kontrol grubuna göre aspartat aminotransferaz (AST), alanin aminotransferaz (ALT), glutamil transferaz (GGT) ve amonyak seviyeleri gibi biyokimyasal serum parametrelerindeki önleyici değişiklikleri arttırarak CCI_4'ün neden olduğu beyin hasarına karşı önemli bir koruma sağlamıştır. Hematoksilin-eozin (H & E) boyama ve c-fos ekspresyon metodu kullanılarak beyin dokusunun histopatolojik ve immünohistolojik değişiklikleri gözlendi. Bu çalışmada, UD'nin CCI_4 toksisitesi üzerindeki koruyucu etkisi, biyokimya ve immünohistoloji çalışmaları ile gösterilmiştir. UD'nin uygulanması CCI_4'ün neden olduğu beyin toksisitesine karşı potansiyel koruyucu etkilere sahip olabilir.

Anahtar Kelimeler: *Hepatik ensefalopati (HE), CCI_4, Sıçan, Urtica dioica*

INTRODUCTION

Hepatic encephalopathy (HE) or portosystemic encephalopathy (PSE) is a reversible syndrome of impaired brain function occurring in patients with advanced liver failure. Most of the episodes of HE occur in cirrhotic patients (Ferenci et al., 1984). HE is frequent: overt HE is found in some 30–40% of patients with cirrhosis (Vilstrup et al., 2014). HE is characterized by increased blood ammonia level and is one of the major

Corresponding author: Gülşah YILDIZ DENIZ
Atatürk Univ. Sağlık Hiz. MYO, Tıbbi Lab Tekn Böl. Erzurum, Turkey *e-mail: gulsah.deniz@atauni.edu.tr*

complications of cirrhosis. But efforts to halt disease progression by reducing ammonemia have failed considerably (Vaquero et al., 2003; Cichoż et al., 2013). However, HE is not a single clinical entity. It may reflect either a reversible metabolic encephalopathy, brain atrophy, brain edema or any combination of these conditions (Kaplan and Rossetti, 2011). HE is associated with confusion, altered levels of consciousness, or coma as a result of liver failure (Losowsky and Scott 1973; Vaquero et al., 2003). Moreover, recent reports postulate that pro-inflammatory and oxidative stress pathways may be crucial mechanisms involved in the pathogenesis of this disease (Bémeur et al., 2013). CCl_4 is a potent hepatotoxic agent used extensively to induce in vivo liver degeneration by oxidative stress. The lipid solubility of CCl_4 renders it readily available to cells. Hence, it is deposited and mediates injury not only in the liver but also in the CNS, kidneys and several other organs. Liver and other tissue damage were manifested by elevation of the ALT and AST activities in blood (Sanzgiri et al., 1997; Basu, 2003). It is well established that liver metabolism of CCl_4 causes the formation of reactive oxygen and nitrogen species, making it suitable to evaluate the role of pro-oxidant and antioxidant mechanisms, as well as the impact of different types of interventions on these mechanisms (Tirkey et al., 2005).

UD is a common green plant that grows all over the world. From the past to the present day, it has been used in many different applications, such as alternative medicine, food, paint, fiber, manure and cosmetics (Ak et al., 2006). The extract of this plant contains different chemical compounds including neophytadiene (25.21%), sinapic acid (25%), phthalic acid (8.15%), dibutyl phthalate (7.37%), bis (2-ethyl hexyl) maleate (6.32%) and 1,2-benzene di carboxylic acid (7.62%) (Lahighi et al., 2011). Nettle is nutritionally high in vitamins A, C and D, also minerals iron, manganese, potassium and calcium (Bisht et al., 2012).

Even though UD has many therapeutic effects, data no literatures have pointed out its neuro protective effect in CCl_4 induced cirrhotic *Sprague dawley* rats. In the present study, the effect of UD extract in CCl_4 induced HE was evaluated in rats.

MATERIALS and METHODS

Animals

Forty-nine adult (2-months-old) male *Sprague dawley* rats were used in this study. The animals had an average body weight of 250-300 g and were housed in polypropylene cages, at 22–24 ^{o}C with a controlled 12 h light. They were fed and watered ad libitum and treated. All procedures were performed in conformity with the Institutional Ethical Committee for Animal Care and Use at Ataturk University (protocol number: 54826478-483/13) and the Guide for the Care and Use of Laboratory Animals.

Preparation of herbal extract

The UD plant was obtained from the wildlife in the mountains of Ağrı. After rinsing, the leaves of the plant were dried in a controlled temperature and humidity setting. Next, the dried materials were powdered using a blender and transferred to the 10-L extraction reactor equipped with a rotation and temperature sensor. The extraction process was run using dichloromethane solvent for 24 hours. To reach the greatest purity percentage, the extract was passed through Whatman filter paper no. 40. The resultant filtrate was removed under reduced pressure using a rotary vacuum evaporator.

Experimental design

The rats were randomized into seven groups of seven each. The rats from the different groups received the administration of medications and toxicants as below:

Group 1: Rats were intraperitoneally injected with physiological saline solution (twice a week) and served as a control.

Group 2: Rats were intraperitoneally injected with soybean oil without CCl_4 (1 mg/kg body weight, twice a week for eight weeks),

Group 3: Rats were intraperitoneally injected with 30% CCl_4 mixed with soybean oil (1 mL/kg body weight; twice a week).

Group 4: Rats treated with UD (200 mg/kg bw; twice a week)

Group 5: Rats treated with UD (400 mg/kg bw; twice a week)

Group 6: Rats treated with (CCI_4 + UD 200 mg/kg bw; twice a week)

Group 7: Rats treated with (CCI_4 + UD 400 mg/kg bw; twice a week)

Biochemical analysis

Blood samples which were received from animals were collected in gel-activated tubes for the assessment of specific liver markers. The gel-activated tubes were allowed to clot, then centrifuged at 4000 × g for 10 min at 4°C. The serum samples were collected for measuring liver markers, alanine aminotransferase (ALT), aspartate aminotransferase (AST), glutamyl transferase (GGT) and blood ammonia (Commercial kits on a Beckman Coulter AU5811 device, Japan).

Liver histology

At the end of the 8th week, the rats were anesthetized with ether. The brain tissues were immediately removed, washed by cold ice saline, dried by filter paper, and weighed in the wet state. The tissue specimens fixed in 10% phosphate-buffered formaldehyde, routinely processed and blocked into paraffin for detecting image analysis, while others were snap-frozen in liquid nitrogen and stored at −70 °C.

Hemotoxylin–eosin (H&E) stain: Formalin-fixed brain tissue was processed and 5 µm thick paraffin sections were stained with Hemotoxylin–eosin for 10 min, rinsed with water, then putted in 75% HCl–ethanol for 30 s, rinsed with water and putted in eosin–ethanol for 1–2 min, dehydrated and mounted.

Immunohistochemistry

Immunohistochemistry was performed as described previously (Mamiya et al., 2009; Inaba et al., 2015). We used a polyclonal rabbit primary antibody for c-fos and biotinylated goat anti-rabbit IgG. Structures were defined anatomically according to the atlas of Franklin and Paxinos (2012).

Statistical analysis

Data recording and analysis was performed on "SPSS 20.0 for Windows" (SPSS Inc., IL, USA) software. Descriptive data were expressed as mean±standard deviation. Serum AST, ALT, GGT and T-BIL results was assessed using the Kolmogorov-Smirnov test. Since all results were normally distributed, comparisons of them among the groups was performed using parametric one-way ANOVA, while

degree of significance of differences between groups was determined using the post hoc LSD test. Correlations between results were assessed using Pearson correlation analysis. P<0.05 was regarded as significant.

RESULTS

Effect of CCl_4 and UD on biochemical parameters of hepatic function

In order to evaluate if the experimental model is mimicking hepatic encephalopathy in the rats, hepatic toxicity markers (ALT and AST) were evaluated. As shown in Table 1, i.p. injection of CCl_4 significantly increased the serum levels of ALT, AST and GGT in toxic group, compared to the control group (p<0.001 for all) *UD* extract at doses of 200 and 400 mg/kg inhibited the CCl_4-induced hepatic encephalopathy according AST (p<0.001 for both), ALT (p<0.001 for both) and GGT (p<0.001 for both). The extract also decreased the serum levels of ammonia at doses of 200 (p<0.001) and 400 mg/kg (p<0.001) compared to the toxic group.

Pathological changes of the brain tissues

Sections from the cortex of control group exhibited normal neuronal structure. Neurons retained their shape and normal cellularity with obvious nuclei (Figure 1A). Sections from the CCI_4-treated rats showed marked neuronal degeneration; neurons decreased in number and had indistinct boundaries. The sections also exhibited irregular damaged cells and cytoplasmic shrinkage. There was evidence of pyknotic nuclei and chromatin condensation. Necrosis and perineuronal vacuolation were observed (Figure 1B). The cortex of CCI_4+UD-200 treated rats and CCI_4+UD-400 treated rats showed few pyknotic nuclei (Figure 1C and Figure 1D).

Induction of c-Fos expression in hippocampal region of brain

We analyzed expression of c-fos-positive areas in the hippocampal region (Fig. 2). Importantly, the CCI_4 group showed significantly more c-fos-positive areas than the other groups (Fig. 2B). In the CCI_4+low dose UD and CCI_4+high dose UD groups (Figure 2C and 2D) showed fewer c-Fos-positive cells compared to only CCI_4 group (Figure 2B). (original magnification ×10).

Table 1. Effect of UD on serum biochemical parameters after treated with CCl4

Treatment	AST U/L	ALT U/L	GGT U/L	Ammonia µmol/L
Control	44.64± 0.86	15.96 ± 0.34	6.33±0.36	12.7±0.24
SOC	41.36± 0.57^{a3}	14.03± 0.45^{a3}	5.4±0.56^{a2}	12.63±0.32^{a3}
UD-200	37.21± 0.79^{a3}	11.12± 0.75^{a3}	5.08±0.32^{a2}	12.55±0.14^{a3}
UD-400	39.82± 0.54^{a3}	13.19± 0.54^{a3}	5.25±0.82^{a3}	12.51±0.13^{a3}
CCI_4	138.48± 0.43^{a3,b3,c3,d3}	55.38± 0.86^{a3,b3,c3,d3}	40.23±0.56 ^{a3,b3,c3,d3}	59.83±0.45 ^{a3,b3,c3,d3}
CCI_4+UD-200	57.49± 0.58^{a3,b3,c3,d3,e3}	18.29± 0.75^{a3,b3,c3,d3,e3}	27.26±0.73 ^{a3,b3,c3,d3,e3}	38.30±0.11^{a3,b3,c3,d3,e3}
CCI_4+UD-400	65.14± 0.56^{a3,b3,c3,d3,e3,f3}	20.09± 0.34 ^{a3,b3,c3,d3,e3,f3}	32.53±0.90 ^{a3,b3,c3,d3,e3,f3}	35,77±0.22 ^{a3,b3,c3,d3,e3,f3}

Results of the study expressed as means ± SD (n=7). ^{a2} and ^{a3} significant differences with respect to the control at p<0.01 and p<0.001. ^{b3} significant differences with respect to the SOC group at p<0.001. ^{c3} significant differences with respect to the UD-200 group at p<0.001. ^{d3} significant differences with respect to the UD-400 group at p<0.001. ^{e3} significant differences with respect to the Cirrhotic group at p<0.001. ^{f3} significant differences with respect to the Cirrhotic+UD-200 group at p<0.001. Significant P ≤ 0.05

DISCUSSION

A HE is a neuropsychiatric disorder resulting from acute or chronic liver failure. HE results from impaired ability of the liver to metabolize neurotoxins particularly ammonia leading to spectrum of psychiatric/neurological deficits ranging from shortened attention span to coma (Vilstrup et al., 2014).

Hyperammonemia is a well-known complication of acute and chronic liver diseases and plays a central role in the pathogenesis of HE leading to neurological dysfunction (Drotman et al., 1978). The data obtained shows an increase in ammonia content in CCI_4 group was accompanied by elevated serum activities of liver enzymes as compared to control group. AST and ALT have been used as useful hallmarks of CCI_4 hepatotoxicity (Bhondave et al., 2014). In our experimental model, it was observed an increased activity of ALT and AST serum levels in rats treated with CCI_4 compared to the control. Therefore, our experimental model could be considered adequate to mimic the effects of hepatic encephalopathy, since it provoked hepatic failure. Moreover, in this work it is also verified that UD prevented the increase in ALT and AST in serum of rats. In the previous studies, it has been shown that UD decreased the lipid peroxidation and liver enzymes and increased the antioxidant defense system activity in the CCI_4 treated rats (Kanter et al., 2005). It is well established that hepatic encephalopathy is due to hyperammonemia which results from hepatic failure (Webster et al., 1957).

The present study showed that UD treatment appeared to be beneficial in decreasing liver injury and improving liver function. These protective effects can be ascribed, at least partly, to the decreased levels of AST, ALT and blood ammonia.

Studies have already demonstrated that antioxidants may have a protective effect against the oxidative damage caused by CCl_4 in a hepatic encephalopathy model (Sikander et al., 2013; Yang et al., 2013). Enhanced oxidative stress leads to deposition of senile plaque and synaptic loss that result in neurodegeneration. Therefore, one of the most important factors for preventing or treatment of this process is controlling of oxidative stresses. UD is known to possess antioxidative and anti-inflammatory properties. Recently, studies on novel preparation of ethanolic herbal extract from UD with an immune system modulator effect, have shown positive effects on reduction of oxidative stresses and pro-inflammatory status and they seem to act as anti-aging drugs (Mohraz et al., 2009; Ghanbari et al., 2012).

The expression and accumulation of c-fos gene, a protooncogen that is a member of IEG (Immediate Early Gene) group, and Fos proteins, a member of AP-1 (Activator Protein-1) family oncoproteins, upsurge along with cellular activation under the affect of certain stimuli. This gene and proteins play a very important role during cellular proliferation, differentiation and programmed cell death (apoptosis) (Roche et al., 1999). To understand the molecular mechanisms involved in behavioral alterations of cirrhotic rats, we analyzed the expression of neural markers. In this study, it was examined that the pattern of c-Fos expression in the brains of rat. We explored alternations in c-Fos expression, using immunohistochemical techniques. In many of the regions that it was investigated, CCl_4 treatment enhanced the c-Fos response. The present study demonstrated that UD extract prevented the CCl_4-induced increase of the apoptotic cell and loss of neurons in cerebral cortex of rats. According to our results rats with hepatic failure showed increased expression of C-fos. Thus, our results were similar to those obtained in previous studies examining c-Fos expression in the mouse brain and rat brain (Yanagida et al., 2016; Matsuda et al., 2017). Nevertheless, the beneficial effect of UD on the brain confirmed the previous investigations, which demonstrated that the polyphenolic natural product is responsible for its neuroprotective effects (Otterbein., 2011).

Based on the results obtained from the current study it can concluded that UD may be a useful candidate to minimize the hyperammonemia occurring by acute or chronic liver damage. However, the underlying mechanisms need to be elucidated in further studies.

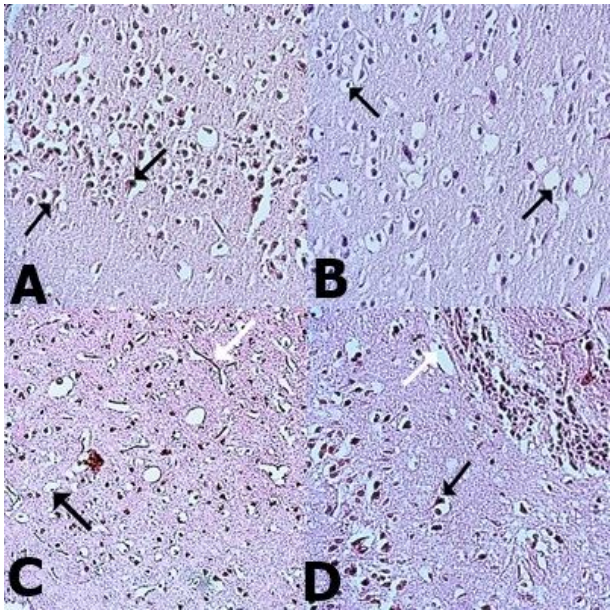

Figure 1. Representative light microphotographs of H&E stained sections from rats brain treated with physiological saline solution, CCI_4, or CCI_4 + UD. (A) Control group: Normal neurons (black arrows). (B) CCI_4 : Neurons appear smaller and shrunken with slight vacuolation of neuropil. Pyknotic darkly stained nuclei, apoptotic cells and cytoplasmic vacuolations (black arrows). (C) CCI_4+UD-200: Apoptotic cells and cytoplasmic vacuolations (black arrows), vein dilatation (white arrow). (D): CCI_4+UD-400: Normal neurons (black arrows), apoptotic cell (white arrow) (magnification at 40×).

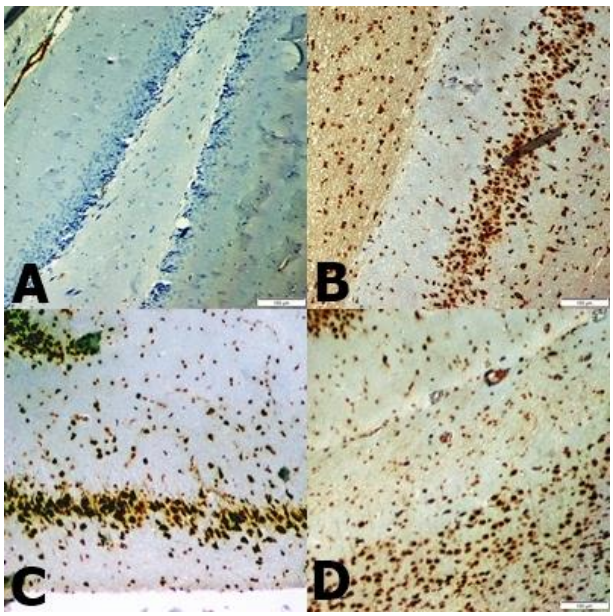

Figure 2. C-Fos staining showing the protective effect of UD against CCI4-induced neurodegenetive disease. The immunohistochemical localization of c-Fos appears as brown staining. (**A**) Control group: No c-Fos positive reaction (Normal saline), (**B**) CCI_4 treated group: Increased c-Fos positive area, (**C**) Low dose UD + CCI4: Decrease of c-Fos staining compared to CCI4 treated group and (**D**) High dose UD: Decrease of c-Fos staining similar to low dose UD (magnification at 40×).

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