Ultrastructural Pathology

ISSN: 0191-3123 (Print) 1521-0758 (Online) Journal homepage: https://www.tandfonline.com/loi/iusp20

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To cite this article: Neriman Colakoglu, Ilter Kus, Aysel Kukner, Hidir Pekmez, Enver Ozan & Mustafa Sarsilmaz (2011) Protective Effects of CAPE on Liver Injury Induced by CCL_4: An Electron Microscopy Study, Ultrastructural Pathology, 35:1, 26-30, DOI: 10.3109/01913123.2010.527036

To link to this article: https://doi.org/10.3109/01913123.2010.527036

Published online: 25 Jan 2011.

Ultrastruct Pathol, 35(1), 26–30, 2011

ISSN: 0191-3123 print/ 1521-0758 online
DOI: 10.3109/01913123.2010.527036

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ORIGINAL ARTICLE

Protective Effects of CAPE on Liver Injury Induced by CCL_4: An Electron Microscopy Study

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ABSTRACT

This study was designed to investigate the protective effects of caffeic acid phenethyl ester on carbon tetrachloride-induced liver damage in rats. Twenty-four male Wistar rats were divided in three groups. Group I was used as control. Rats in group II were injected with carbon tetrachloride every other day for 1 month, whereas rats in group III were injected with carbon tetrachloride and caffeic acid phenethyl ester every other day for 1 month. At the end of the experiment, all animals were killed by decapitation and their livers were removed. Liver tissues were processed for electron microscopy. Histopathologically, hepatocytes of rats treated with carbon tetrachloride had damage in the cytoplasmic organelles and nuclei membranes as well as an excessive lipid accumulation in the hepatocytes. However, those histopathological changes were reduced with the coadministration of carbon tetrachloride and caffeic acid phenethyl ester. We conclude that caffeic acid phenethyl ester treatment has the capability to prevent carbon tetrachloride-induced liver damage in rats.

Keywords: Caffeic acid phenethyl ester (CAPE), Carbon tetrachloride, Electron microscopy, Hepatotoxicity, Rat

Carbon tetrachloride (CCl_4) is a well-known hepatotoxic agent used to induce experimental liver injury [1]. It is suggested that CCl_4 is not toxic by itself [2] but causes oxidative stress and lipid peroxidation by producing trichloromethyl (CCl_3) [3–5]. CCl_3 free radicals derived from CCl_4 react with sulfoethyl groups like glutathione (GSH), catalase, and superoxide dismutase and protein thiols. Furthermore, covalent binding of CCl_3 to cell membrane induces lipid peroxidation [2,6], protein oxidation leading to hepatocellular membrane damage [3,5], and, finally, cell necrosis [2]. After hepatocellular damage, the altered permability of cell membrane leads to release of hepatospecific enzymes into blood circulation [5]. CCl_4 stimulates Kupffer cells that leads to a production of proinflammatory mediators [7]. Madro et al. determined that CCl_4 does not cause cirrhotic changes but it activates Ito cells, causes focal retraction of the stroma and fibrosis. An increased number of Ito cells is a sign of the activation of liver fibrosis due to CCl_4 administration [8]. Fibrosis caused by CCl_4 was reduced in mice genetically lacking B and T lymphocytes. On the other hand, mice lacking B and T lymphocytes as well as natural killer (NK) cells had a significant increase in hepatic fibrosis, which emphasizes the anti-fibrotic capacity of the NK cells [9].

Caffeic acid phenethyl ester (CAPE) is an active component of propolis that has antioxidant, immunomodulatory, antiinflammatory, anticarcinogenic [10–12], antiviral, antiatherosclerotic, antiproliferative, and neuroprotective properties [13]. Antiinflammatory properties of CAPE are thought to be due to the supression of eicosanoid synthesis, inhibition of arachidonic acid release from cell membrane, expression of cyclooxygenase-2 (cox-2) gene, and the inhibition of COX-1 and COX-2 activity [14]. CAPE also has free-radical scavenging activities [13,14]. CAPE reduces bioactivation of carcinogens like benzo(*a*) pyrene [15]. It was proven that CAPE suppresses lipid peroxidation and stimulates the activitiy of

Received 17 August 2010; accepted 22 September 2010

We would like to thank Mehmet Sertac Ozcan from the University of Illinois at Chicago for providing language help.
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antioxidant enzymes [16]. In fact, CAPE has been used in folk medicine for many years [10].

Liver disease is a widespread health problem throughout the world. That's why it is necessary to find alternative protection or therapy against liver disorders. Our objectives were to confirm the liver injury induced by CCl_4 and to detect whether CAPE may provide protection against the CCl_4-induced liver injury.

MATERIALS AND METHODS

Animals and Treatment

Ethical approval for this study has been obtained from Firat University Faculty of Medicine Ethics Board and all procedures conformed to the "Guide for the Care and Use of Laboratory Animals." Twenty-four adult male Wistar albino rats (weighing 170–220 g) were used in this study. Rats were randomly divided into 3 groups with 8 animals per group. The rats were kept in Plexiglas cages (4 animals per cage) and received standard chow and water ad libitum in an air-conditioned room with automatically regulated temperature (22 ± 1°C) and light cycle (light: 07.00–19.00). All rats were allowed to acclimatize for 1 week prior to experimentation. Control rats (group I) received pure olive oil (1 mL subcutaneously (sc)) alone. Rats in group II were injected with CCl_4 (0.5 mL/kg body weight per 1 mL olive oil sc; EM Science, Cherry Hill, NJ, USA) every other day for 1 month. Rats in group III received CAPE (10 µmol/kg body weight intraperitoneally (ip)) and a subcutaneous injection of CCl_4 every other day for 1 month. CAPE was synthesized in the Physico-Chemistry Laboratory using the technique described by Grunberger et al. [17]

Histopathological Analysis of Live

All animals were killed by decapitation at the end of the experiment. A midsaggital incision was made and livers of all rats were removed and fixed in 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.2) within 24 h of removal. After a rinsing with phosphate buffer, tissues were postfixed with 2% osmium tetraoxide in sodium phosphate buffer. Dehydration was accomplished by gradual ethanol series, and tissues were embedded in epoxy resin. Semithin sections were stained with toluidine blue and examined with a BH2 light microscope (Olympus, Tokyo, Japan). Ultrathin sections (800 nm) were stained with uranyl acetate and lead citrate. Sections were then viewed and photographed with a Zeiss 9EM TEM.

RESULTS

Histopathological Findings

Hepatocytes, sinusoids, and sinusoidal cells structures in group I (control group) were normal (Figure 1). In group II, there was excessive lipid accumulation and vacuolization in the cytoplasm of hepatocytes, invagination of nuclear membranes of hepatocytes, and as well as nuclei of different sizes (Figures 2, 3). Moreover, karyorectic hepatocytes were also detected (Figure 4). In the electron microscopic examination, lipid accumulation in the hepatocytes was evident (Figure 5). In addition, microvilli of hepatocytes disappeared, cytoplasmic organelles of hepatocytes were damaged, and the intercellular boundaries were obscured (Figure 6). In group III, CAPE provided protection for hepatocytes, sinusoids, and sinusoidal cells (Figure 7). Different from group II, microvilli of hepatocytes were not damaged. Cytoplasmic organelles had a more organized appearance (Figure 8).

DISCUSSION

CCl_4 is widely used to induce experimental liver injury. Peroxidation of membrane lipids and formation of free

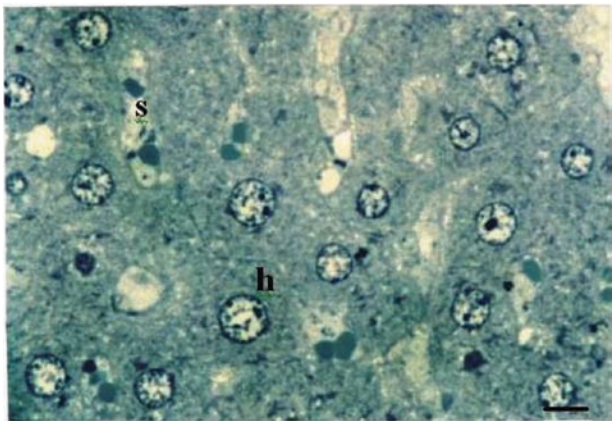

FIGURE 1 Group I: hepatocytes (h), sinusoids (s), and sinusoidal cells are normal in appearance. Toluidine blue; bar, 4 mµ.

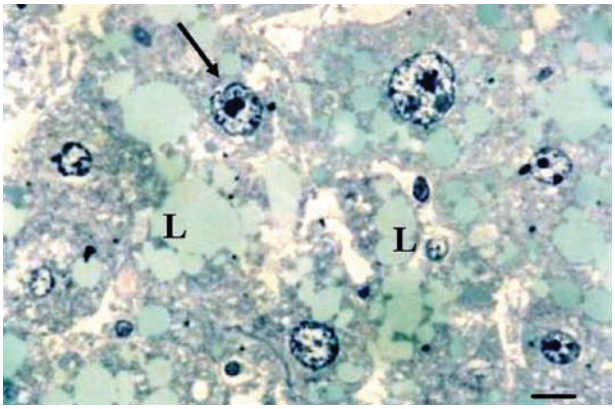

FIGURE 2 Group II: excessive lipid (L) accumulation, nuclear membrane invagination (arrow) and different sized nuclei are evident in the hepatocytes. Toluidine blue; bar, 4 mµ.

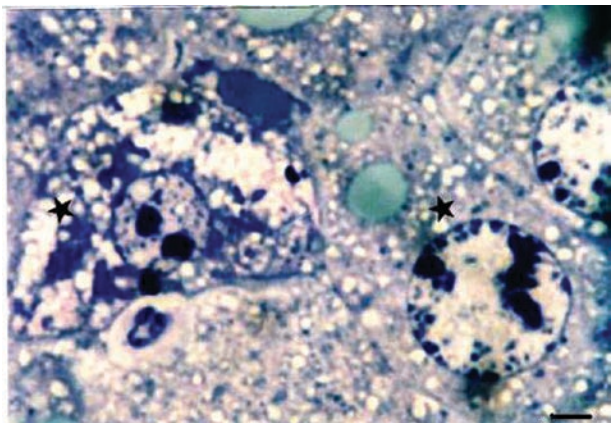

FIGURE 3 Group II: serious vacuolization (*) in the hepatocytes. Toluidine blue; bar, 10 µm.

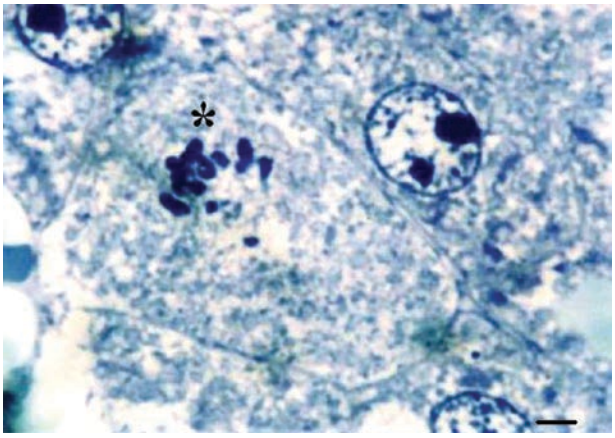

FIGURE 4 Group II: One of the karyorectic hepatocytes (*). Toluidine blue; bar, 10 mµ.

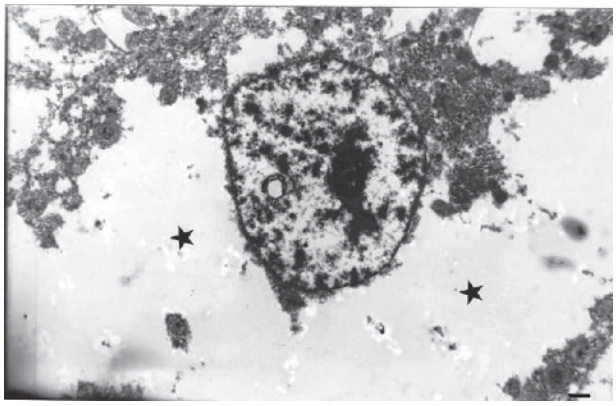

FIGURE 5 Group II: excessive lipid accumulation (*) and disorganized cytoplasmic organelles. Lead citrate–uranyl acetate; bar, 2.5 mµ.

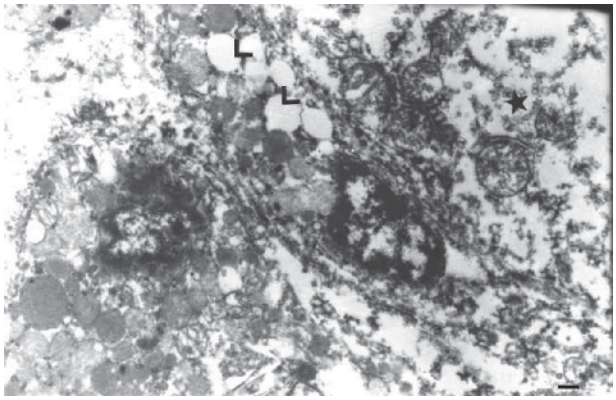

FIGURE 6 Group II: lipid (L) loaded Ito cell in the Disse space. Microvilli of hepatocytes disappear and cytoplasmic organelles of hepatocytes disorganize (*). Lead citrate–uranyl acetate; bar, 1.1 mµ.

radicals are responsible for the toxic effects of CCl_4. Lipid peroxidation, particularly those containing polyunsaturated fatty acids, can significantly change the properties of biological membranes. This can lead to severe cell damage and play an important role in the pathogenesis of diseases [18]. Under normal conditions, the free radical levels in the body are low and healthy organisms can neutralize, metabolize, or decrease their toxic effects by free radical scavengers, such as superoxide dismutase (SOD) and catalase. Hepatic injury caused by CCl_4 is thought to be due to an increased production of reactive oxygen species (ROS) [19]. Excessive levels of ROS damage lipids, proteins, and nucleic acids. Following this, cell death occurs by necrosis or apoptosis [20].

Cell death is one of the important steps in the development of liver injury, fibrosis, alcoholic liver disease, and hepatitis [21,22]. Mitochondria are notable among the hepatocytic organelles affected by CCl_4 administration. It was found that even a small amount of CCl_4 causes ultrastructural changes of hepatic mitochondria [23,24]. This situation plays a key role in controlling cell death [25].

Chronic administration of CCl_4 induces fibrosis, as indicated by an increase in the serum levels of AST and ALT [26]. The increased serum levels of ALT are an indicator of the degree of cell membrane damage, while AST is an indicator of mitochondrial damage [25]. We also determined damage in the biological membranes and organelles, including mitochondria.

Hepatic fibrosis is triggered by hepatocyte damage, which recruits inflammatory cells and platelets, activates Kupffer cells, along with a release of cytokines and growth factors. These factors activate hepatic stellate cells (HSC). Activated HSC proliferate and transform into myofibroblast-like cells that deposit large amounts of connective tissue components [26].

Xu et al. observed that CCl_4 administration causes inflammation, necrosis, and collagen deposition [26,27]. It was found that administration of estradiol decreases the serum enzyme and subsequently protects the structural integrity of the hepatocellular membrane against CCl_4 [26].

Tang et al. noticed that CCl_4 caused fatty changes, necrosis, and loss of cellular boundary in liver as well as infiltration of lymphocytes and Kupffer cells [25]. Mas et al. observed a breakdown of organization of the rough endoplasmic reticulum (RER), a vacuolization of the

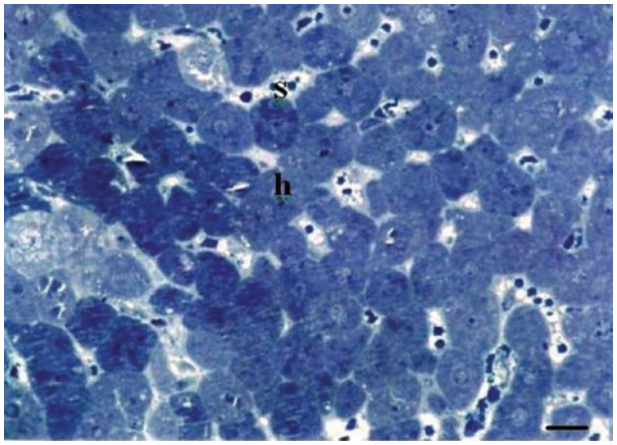

FIGURE 7 Group III: hepatocytes (h), sinusoids (s), and sinusoidal cells seem like group I through CAPE protection. Toluidine blue; bar, 2 mμ.

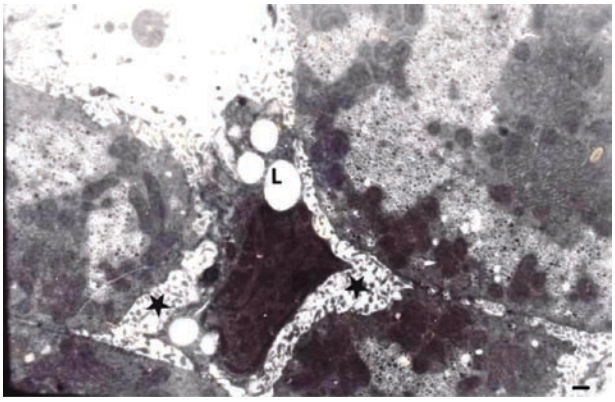

FIGURE 8 Group III: cytoplasmic organelles and microvilli (*) of hepatocytes and intercellular boundaries are well preserved thanks to CAPE protection. Lipid (L) loaded normal appearanced Ito cell in the Disse space. Lead citrate–uranyl acetate; bar, 2.5 mμ.

smooth endoplasmic reticulum (SER), and an irregularity of nuclear content after CCl_4 injection [28]. Yao et al. found that hepatocytes enlarged with shrunken nuclei, along with different sizes of lipid droplets in the nuclei and cytoplasm. Moreover, CCl_4 decreased the number of organelles [29]. Another effect of CCl_4 was a swelling of endoplasmic reticulums [30].

Junnila et al. revealed that CCl_4 caused centrilobular steatosis and reduced the number of mitochondria. Fatty infiltration of the liver is thought to develop as a result of the action of free alkyl radicals on biomembranes, which in turn causes haloalkylation-dependent blocking of the exit of lipoprotein micelles from the Golgi apparatus [31].

Histhopathologically, we detected many structural damages, including an excessive lipid accumulation in the hepatocytes as well as damage to microvilli, cytoplasmic organelles, and nuclei of the hepatocytes in this study. Damage of microvilli results in difficult exchange of substance between the bloodstream and the hepatocytes. Widespread of microvilli damage can lead to functional failure in the tissue.

Exogenous antioxidant molecules have the capability to detoxify ROS even if the endogen antioxidant system fails. One of them, CAPE, can enhance endogenous antioxidant enzyme activities and prevent lipid peroxidation in intestinal tissue caused by intestinal ischemia–reperfusion injury [32]. Aladag et al. also reported that CAPE has a powerful antioxidant effect by suppressing the formation of ROS and MDA [33]. CAPE can protect the brain by its antioxidant and antiinflammatory effects, which was shown in rabbits with focal permanent middle cerebral artery occlusion [34].

We also detected protective effects of CAPE against the CCl_4 in this study. Administration of CAPE preserved liver structure. We suggest that CAPE provided a reconstitution of the antioxidant defense system. In short, CAPE helped to maintain the integrity of membranes in both the organelles and cells.

As a result, CCl_4-induced hepatic damage is frequently used as a model for studying hepatoprotective drugs. Lipid peroxidation, ROS, and damage of endogenous antioxidant defense caused by CCl_4 are important factors for liver pathogenesis. CAPE, due to its antioxidant properties, can reduce lipid peroxidation and play key role for radical scavenging. Moreover, CAPE can support the endogenous antioxidant system. CAPE may also block the biotransformation of CCl_4 to CCl_3, which is the main toxic substance. Due to these different useful qualities of CAPE, it can be considered a protective drug for liver.

ACKNOWLEDGMENT

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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