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# Beneficial Effect of Caffeic Acid Phenethyl Ester (CAPE) on Hepatocyte Damage Induced by Bile Duct Ligation: An Electron Microscopic Examination

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## ABSTRACT

Recently the authors have reported the potent beneficial effect of caffeic acid phenethyl ester (CAPE) on cholestatic oxidative liver injury induced by acute bile ligation in Swiss albino rats. Herein, they report the ultrastructural hepatocellular alterations induced by acute bile duct ligation and the effect of CAPE administration on these alterations. Bile duct ligation resulted in many degenerative changes, such as vacuolization, mitochondrial degeneration, endoplasmic reticulum dilatation, and lysosome accumulation within the cytoplasm of hepatocytes. Mitochondrial degeneration was also observed within the cytoplasm of the cells of biliary ductular epithelium. CAPE potentially protected the hepatocytes from the cholestasis-induced cellular injury.

**Keywords:** bile duct ligation, caffeic acid phenethyl ester, cholestasis, ultrastructure

Cholestasis is a common pathophysiological process in many human diseases, leading to the accumulation of toxic bile salts within the liver [1,2]. Bile acids cause oxidative damage by stimulating the generation of oxygen free radicals from mitochondria, as well as promoting their release from neutrophils and macrophages [3]. It seems likely that the detergent action of bile salts is responsible for solubilization of plasma membranes and cell death, which in turn might lead to oxidative stress, oxidation of reduced glutathione (GSH), and lipid peroxidation [4]. Several studies have shown the role of oxygen free radicals and the protective effect of antioxidants in the cholestasis syndrome [5–9]. Since experimental cholestasis was found to be associated with increased lipid peroxidation in remote organs such as kidney, brain, and heart [10], it is concluded that oxidative stress in cholestatic

liver disease is a systemic phenomenon probably influencing all tissues and organs. This result shows the importance of the inhibition of oxidative liver injury, induced by cholestasis, by administration of antioxidant agents.

Caffeic acid phenethyl ester (CAPE), exhibiting a broad spectrum of biological activities, has antimicrobial [11], anti-inflammatory [12], antiatherosclerotic [13], antioxidative [14, 15], antiangiogenic [15], neuroprotective [14], and antitumoral actions [16]. The in vitro antioxidant activity of CAPE has also been shown, since it reduced the levels of intracellular H<sub>2</sub>O<sub>2</sub> and oxidized bases in DNA, probably by its selective scavenging activity [17]

Recently, we have reported the potent beneficial effect of CAPE on cholestatic oxidative liver injury induced by acute bile ligation in Swiss albino rats [5]. Herein,

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we report the ultrastructural hepatocellular alterations induced by acute bile duct ligation and the effect of CAPE administration on these alterations.

## MATERIALS AND METHODS

### Experimental Protocol

Three-month-old male Swiss albino rats, weighing 300–350 g were included in this study. The study was approved by the Inonu University Ethics Committee.

Rats were allowed access to food and water ad libitum. Food was withheld 8 h prior to surgery; free access to water was allowed. Rats were subjected to a controlled environment regarding temperature and humidity and a 12-h light–dark cycle. All surgical procedures were performed while rats were under intraperitoneal ketamine (50 mg/kg) and xylazine HCl (10 mg/kg) anesthesia.

A total of 21 rats were randomly divided into 3 groups as follows: sham operation group (SO), bile duct ligation group (BDL), and bile duct ligation and CAPE administration group (BDL+CAPE). All of the rats from all groups were subjected to a simple laparotomy. But the rats from the BDL and BDL+CAPE groups underwent ligation and division of the bile duct. Abdominal layers were closed with appropriate suture material.

CAPE was synthesized by the standard method of Grunberger [18]. Animals from the BDL+CAPE group were treated with CAPE (10 mg/kg/day/ip) (25  $\mu\text{mol}/\text{mL}$  solution in 1% ethanol) for 14 days. All rats were sacrificed under anesthesia at the end of day 14 and livers were rapidly removed.

### Electron Microscopic Procedure

Liver was sliced into small pieces. The samples were fixed in 2.5% gluteraldehyde buffered with 0.2M  $\text{NaH}_2\text{PO}_4 + \text{NaHPO}_4$  (pH=7.2–7.3) and postfixed in 1%  $\text{OsO}_4$ . After dehydration in acetone, they were embedded in Araldite CY 212. Ultrathin sections were stained with uranyl acetate and lead citrate and were examined in a Carl Zeiss Libra 120 electron microscope.

## RESULTS

All animals survived until the end of the experiment. Jaundice was observed in the visceral and parietal peritoneum of the rats from the BDL and BDL+CAPE groups. The livers of the animals from these groups were enlarged and their bile ducts above the obstruction point were dilated.

Examination of livers obtained from the SO group showed normal ultrastructural appearance. The rough

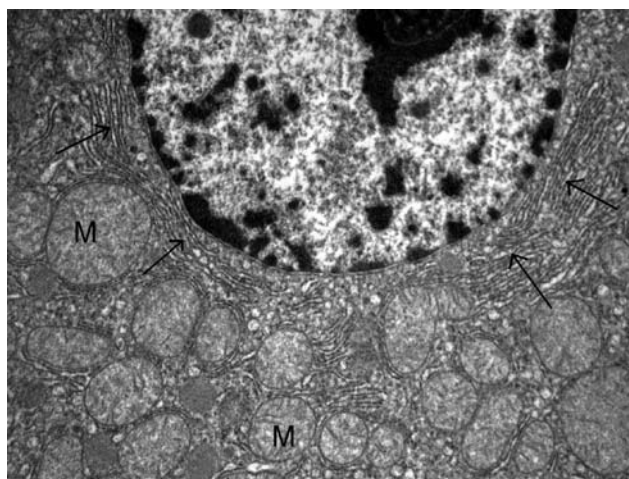


Figure 1. SO group. Extensive rough endoplasmic reticulum (arrows) and many mitochondria (M) are observed. Uranyl acetate and lead citrate  $\times 10,000$ .

endoplasmic reticulum was extensive, and numerous mitochondria and ribosomes were observed. Nuclei with dispersed chromatin and prominent nucleoli were round in shape (Figure 1).

In the thin sections from BDL group, the most prominent ultrastructural alterations were vacuolization (Figures 2A, 2B), mitochondrial degeneration, and endoplasmic reticulum dilatation (Figure 2B). Mitochondria were edematous; cristae decrease or loss, myelinic figure formation, and alteration in the density of mitochondrial matrix were observed (Figures 2C–E). Mitochondrial degeneration was also observed in the cytoplasm of the cells of biliary ductular epithelium (Figure 2F). Many huge lysosomes containing granular or membranous debris (sometimes onion-like membranous bodies) were present within the cytoplasm of hepatocytes (Figure 2G). Nuclei were irregular in shape (Figures 2C, 2E). Occasionally, plasma membranes were ruptured; degenerated organelles were spilled into intercellular space (Figure 2H). Cell infiltration including neutrophils, plasma cells, and lymphocytes was observed (Figure 2J).

CAPE administration resulted in a substantial normalization in the ultrastructural picture of hepatocytes (Figure 3A). However, hepatocyte edema (Figure 3B), vacuolization (Figure 3C), mitochondrial edema, cristae loss, and myelinic figure formation (Figure 3C), nuclear irregularity, and plasma membrane rupture were rarely observed. Biliary ductular epithelium was normal in ultrastructural appearance.

## DISCUSSION

There is growing evidence suggesting that considerable impairment of oxidative stress regulation may

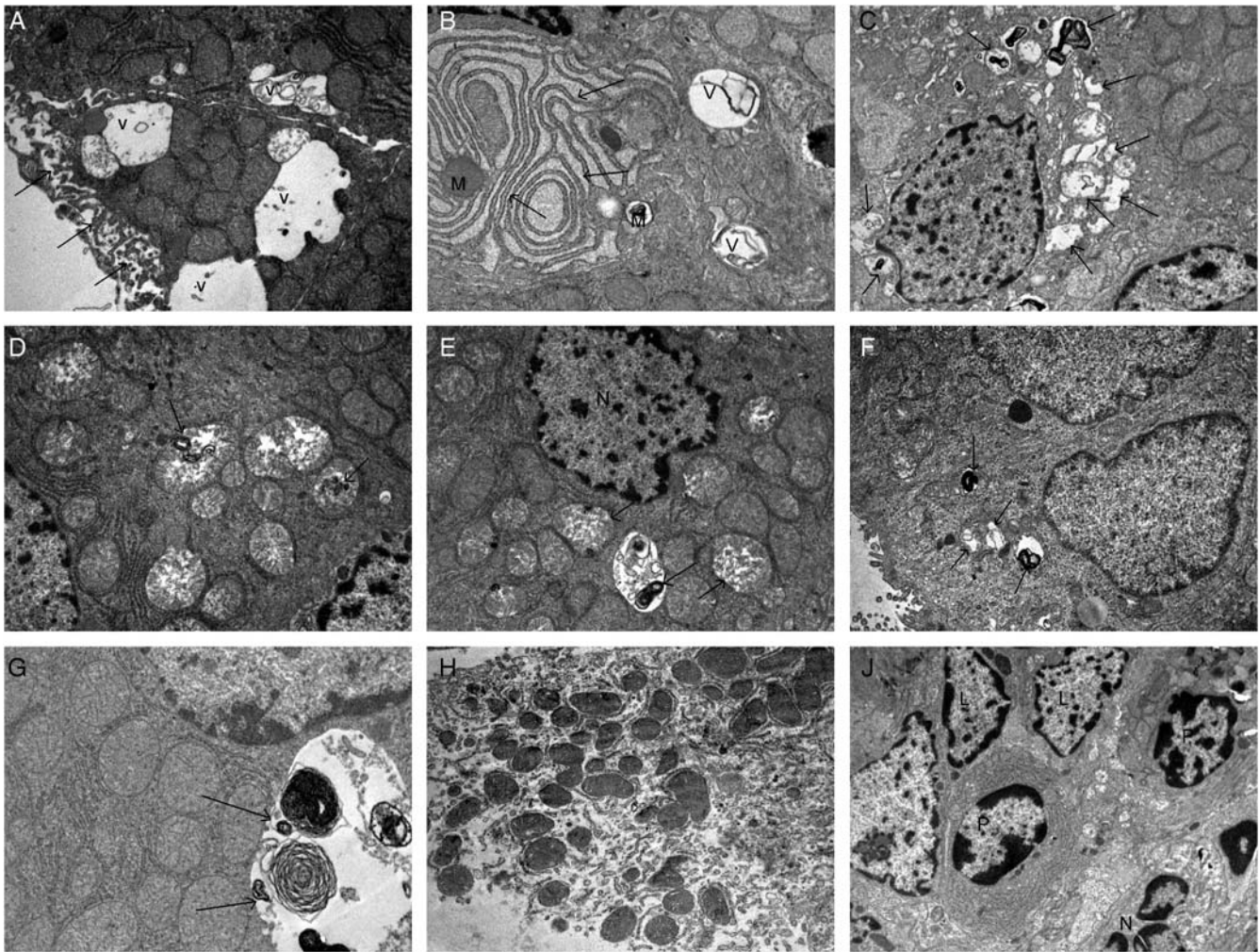


Figure 2. BDL group. (A) Large vacuoles (V) within the cytoplasm adjacent to Disse space (arrows) are observed. Uranyl acetate and lead citrate  $\times 10,000$ . (B) Vacuolization (V), mitochondrial degeneration (M) and endoplasmic reticulum dilatation (arrows) are seen. Uranyl acetate and lead citrate  $\times 12,500$ . (C) Mitochondrial edema, cristae loss and myelinic figure formation are obvious (arrows). Uranyl acetate and lead citrate  $\times 8,000$ . (D) Myelinic figure formation is observed (arrows). Uranyl acetate and lead citrate  $\times 10,000$ . (E) Nuclear irregularity (N), cristae loss and myelinic figure formation (arrows) are observed. Uranyl acetate and lead citrate  $\times 8,000$ . (F) Cristae loss and myelinic figure formation (arrows) are seen in the cytoplasm of the cells of biliary ductular epithelium. Uranyl acetate and lead citrate  $\times 10,000$ . (G) A huge lysosome containing granular and membranous debris (onion-like membranous bodies) are observed (arrows). Uranyl acetate and lead citrate  $\times 16,000$ . (H) Degenerated organelles are observed within the extracellular space. Uranyl acetate and lead citrate  $\times 10,000$ . (J) Neutrophils (N), plasma cells (P) and lymphocytes (L) are present among the adjacent hepatocytes. Uranyl acetate and lead citrate  $\times 6,300$ .

play an important role in cholestatic liver injury [10,19]. Acute bile duct obstruction is characterized by increased lipid peroxidation and by marked decline in reduced GSH, a major cellular antioxidant [20]. It is known that bile duct ligation (BDL) results in a shift in the oxidant/prooxidant balance in favor of increased free radical activity [21]. Enhanced production of reactive oxygen intermediates augments lipid peroxidation by disturbing oxidant-antioxidant balance in hepatic mitochondrial fraction [22]. Recently, we have reported the light microscopic and biomedical results of the present experimental study. Bile duct ligation resulted in an increased hepatic damage score and

hepatic MDA levels, but decreased GSH levels [5]. MDA is the breakdown product of the major chain reactions leading to oxidation of polyunsaturated fatty acids, and thus serves as a reliable marker of oxidative stress-mediated lipid peroxidation. An increased concentration of MDA is considered a marker of tissue injury.

In attempting to limit the oxidative damage, a number of antioxidants have been tested in experimental bile duct obstruction models [6, 8, 9, 20, 23]. It has been proposed that antioxidants, which maintain the concentration of reduced GSH, may restore the cellular

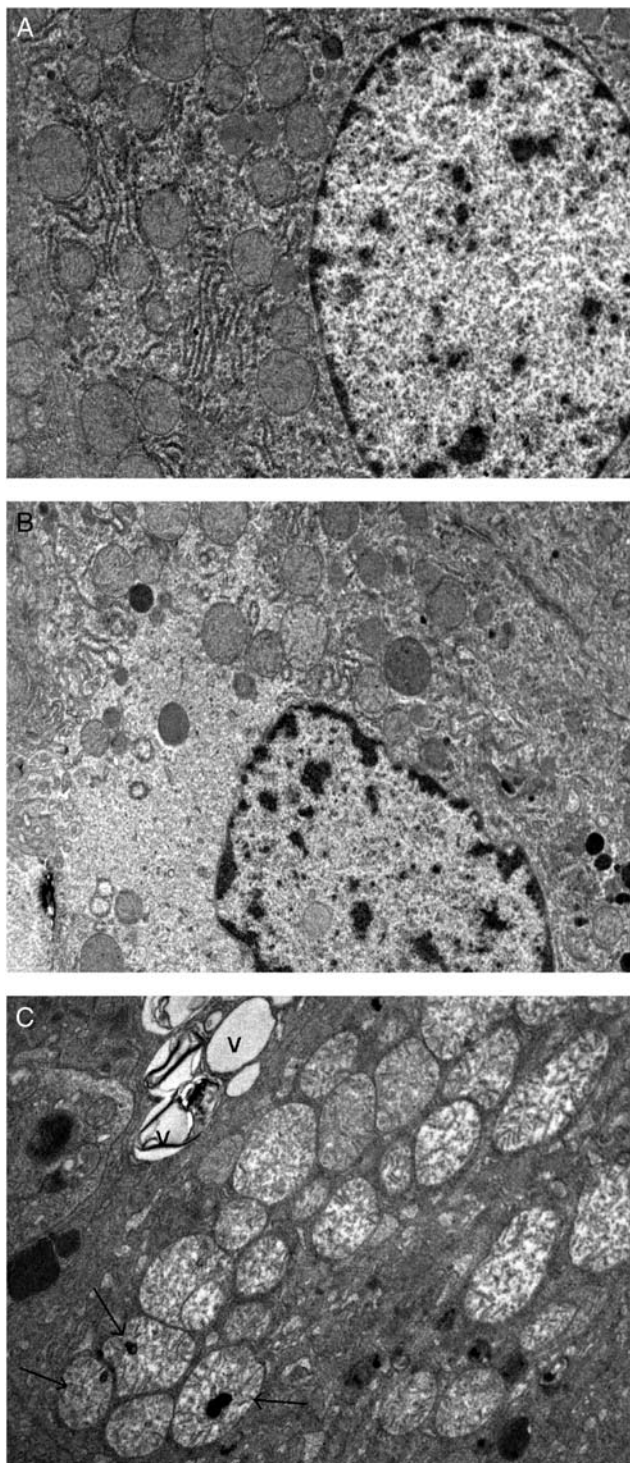


Figure 3. BDL+CAPE group. (A) A healthy hepatocyte is seen. Uranyl acetate and lead citrate X. 8,000. (B) Hepatocyte edema is observed. Uranyl acetate and lead citrate  $\times 8,000$ . (C) Vacuolization (V) and mitochondrial degeneration (arrows) are observed. Uranyl acetate and lead citrate  $\times 10,000$ .

defense mechanism and block lipid peroxidation. CAPE has been proved to have the greatest impact not only on oxidative stress, but also on systems of defense against free radicals, restoring the oxidative

balance in treated experimental animals. Recently, we have demonstrated the beneficial effect of CAPE on oxidative stress induced by cholestatic hepatic damage. CAPE administration resulted in decreases in hepatic damage score and hepatic MDA levels, but an increase in hepatic GSH content [5].

CAPE has been shown to inhibit lipo-oxygenase activities, to suppress lipid peroxidation [24, 25], but to increase cellular antioxidant enzyme levels or activities [5, 14, 26, 27]. It is rapidly absorbed and metabolized by plasmatic esterases [28]. It is nontoxic, and readily available as the active component of propolis of honeybee hives [8]. It has been shown to be one of the most potent lipophilic antioxidants [29]. As a natural lipophilic small phenolic component, it can penetrate cellular membranes and protects cellular components from oxidative damage induced by several factors.

We provided many clues about the cytoprotective effect of CAPE by examining hepatocytes by transmission electron microscope. We detected many degenerative cellular alterations, such as vacuolization, mitochondrial degeneration, endoplasmic reticulum dilatation, lysosome accumulation, and nuclear irregularity in the cytoplasm of hepatocytes of the rats from BDL group. Occasionally, plasma membranes were ruptured; degenerated organelles were spilled into intercellular space. Reactive oxygen species (ROS) are involved in a variety physiological and pathological processes. Excessive ROS can damage cellular components such as lipids, proteins, and nucleic acids (e.g., DNA), which leads to necrotic or apoptotic cell death [30].

Although any biologically important molecule in a cell can be the target of injury producing stress, the cell membrane, energy metabolism, protein synthesis, and gene systems are particularly vulnerable. The integrity and function of whole cell and the organelles depend on the integrity of their phospholipid membranes. By controlling the selective transport of molecules, the plasma membrane keeps the cell in osmotic equilibrium with extracellular fluid. Damage to plasma membrane increases the cell's permeability to sodium and water, leading to swelling and even disruption of the cell [31]. The cellular edema, mitochondrial swelling, and dilatation of endoplasmic reticulum we observed represent the damage to membranes of organelles and the cell itself. Kilicoglu et al. [23] reported cholestasis-induced hepatocyte edema and degeneration.

Mitochondria are central to the life of eukaryotic cells, and the role of mitochondria in cell death has been clarified. Mitochondria are the source of energy required for nearly all functions of the cell. It is the most important source of ROS, but also one of the main targets of ROS. ROS production is an effective inducer of a series of downstream cellular injuries. It primarily damages the components of mitochondria and subsequently impairs

the mitochondrial functions, leading to subsequential cell death. We detected mitochondria swelling, cristae decrease or loss, myelinic figure formation, and alteration in the density of mitochondrial matrix in both hepatocytes and also cells of biliary ductular epithelium. Degree of mitochondrial damage is an indicator of cellular injury. Therefore, the use of radical scavengers to prevent mitochondria from oxidative damage should be of critical importance to prevent and cure many diseases.

CAPE and its related phenolic components have been reported to be protective by limiting the mitochondrial membrane lipoperoxidation and membrane fluidity, which resulted in the maintenance of mitochondrial function. These compounds also diminish protein carbonylation levels. It is clear that proteins are another target of ROS and protein enzyme function can be destroyed by their carbonylation. CAPE can prevent the damage induced by ROS on both mitochondrial phospholipids and proteins. CAPE protects mitochondria mainly correlated to its antioxidative activities [32]. It also completely blocks the production of free radicals in human neutrophils [24]. So it may protect liver from oxidative damage by its anti-inflammatory effects. In the present study, mitochondrial alterations were rarely observed in CAPE-administered rats. This observation emphasizes the potent cytoprotective effect of CAPE.

Dufour et al. [33] have reported an increase in lysosomal enzyme activities 1 week after bile duct ligation. We did not evaluate lysosomal enzyme activities, but observed lysosome accumulation within the cytoplasm of hepatocytes. There were many large lysosomes containing granular or membranous debris. CAPE administration resulted in a prominent decrease in lysosomal content of the hepatocytes. Lysosomes constitute an intracellular digestive system capable of breaking down materials originating both outside and inside the cell. In damaged cells, lysosomes are the sites of elimination of damaged organelles and of inclusions. Lysosome increase is also an indicator of cell injury. We observed that CAPE administration resulted in a normalization of cellular lysosome content.

As a conclusion, we report here that CAPE potentially protects liver via protecting hepatocyte ultrastructure against cholestasis-induced oxidative injury.

**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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