Protective Effect of the Coffee Diterpenes Kahweol and Cafestol on *tert*-Butyl Hydroperoxide-induced Oxidative Hepatotoxicity

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Kahweol and cafestol significantly reduced *t*-BHP-induced oxidative injuries in cultured rat hepatocytes, as determined by cell cytotoxicity, intracellular glutathione (GSH) content and lipid peroxidation in a dose-dependent manner. In addition, kahweol and cafestol provided good protection from the *t*-BHP-induced production of intracellular reactive oxygen species and DNA damage. The *in vivo* study showed that pretreatment with kahweol and cafestol prior to the administration of *t*-BHP significantly prevented the increase in serum levels of hepatic enzyme markers (alanine aminotransferase and aspartate aminotransferase) and reduced oxidative stress, such as GSH content and lipid peroxidation, in the liver in a dose-dependent manner. The histopathological evaluation of the livers also revealed that kahweol and cafestol reduced the incidence of liver lesions induced by *t*-BHP. Taken together, these results support the anti-oxidative role of kahweol and cafestol and demonstrate that kahweol and cafestol can protect hepatocytes from oxidative stress.

Key Words : Kahweol, Cafestol, *tert*-Butyl hydroperoxide, Hepatotoxicity

Introduction

The liver produces large amounts of oxygen free radicals in the course of detoxifying xenobiotic and toxic substances. Oxidative stress caused by reactive oxygen species (ROS) has been shown to be linked to liver diseases, such as hepatotoxicity, and other liver pathological conditions.[1,2] Furthermore, oxidative stress is considered to be associated with many diseases, such as inflammation, cardiovascular diseases, aging and cancer.[3] Normally, the ROS produced are scavenged by endogenous antioxidants which are abundant in the liver tissue.[4] However, liver injury can occur when large acute doses of, or chronic exposure to toxic substances overpower the hepatic antioxidant defense system. It is well known that these cellular changes induced by ROS lead to DNA damage in a variety of cell types.[5]

A number of prooxidant drugs and other chemicals have been implicated in producing oxidative stress and cell injury resulting from the intracellular production of ROS.[6] *tert*-Butyl hydroperoxide (*t*-BHP), a short-chain analog of lipid hydroperoxide, has often been used as a model to investigate the mechanism of cell injury initiated by acute oxidative stress.[7-10] *t*-BHP can be metabolized to free radical intermediates by cytochrome P450 in hepatocytes or by hemoglobin in erythrocytes, which in turn can initiate lipid peroxidation and glutathione (GSH) depletion, affect cell integrity, and result in cell injury in hepatocyte cultures and in livers.[10,11] It also causes a mitochondrial depolarization within intact hepatocytes and mediates DNA base damage in cultured mammalian cells.[12]

Kahweol and its dehydro derivative, cafestol, are two diterpenes (Fig. 1) found in considerable quantities in coffee beans, as well as in final, unfiltered coffee beverages, *e.g.* in Turkish or Scandinavian style coffees.[13] They have been shown to have both adverse and chemoprotective properties.[14,15] Both kahweol and cafestol increase the blood cholesterol level in both humans and animals.[14] However, animal studies have shown that kahweol and cafestol can offer protection against the action of well-known carcinogens.[16,17] In line with these observations, there is epidemiological evidence suggesting that the consumption of coffee with a high amount of kahweol and cafestol is associated with a lower rate of colon cancer in humans, which is one of the most common cancers in the western world.[18] The chemoprotective effects of kahweol and cafestol are related to the beneficial modifications of the xenobiotic metabolism.[19] Oxidative stress has been implicated in the processes of inflammation.[20,21] Recently, we reported that kahweol and cafestol have *in-vitro* and *in-vivo* anti-inflammatory activity and reduce the COX-2 and iNOS expression levels by inhibiting the transcription factor, nuclear factor-κB, by targeting the inhibitor κB kinase complex as well as prostaglandin E_2 and nitric oxide production in lipopolysaccharide-stimulated mouse macrophages.[22-24] Although these studies suggest that at least some of these effects are due to the anti-oxidative potential of kahweol and cafestol, unequivocal proof of this assumption is still lacking. However, it is not known whether kahweol and cafestol can prevent or

[a]The first two authors contributed equally to this work.

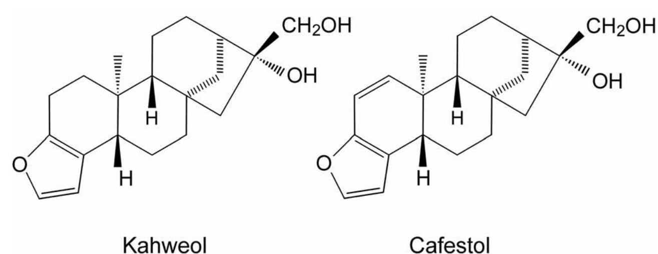

Figure 1. Chemical structures of kahweol and cafestol.

alleviate liver injury induced by oxidative stress. In our present work, we examined the protective potential of kahweol and cafestol against *t*-BHP-induced oxidative hepatocyte injury in primary hepatocytes cultures and in the liver. Various parameters, such as cell viability, lipid peroxidation, thiol status, intracellular ROS, and DNA damage, were measured as an index of oxidative stresses.

Experimental Section

Reagents. Cafestol acetate, kahweol acetate, *tert*-butylhydroperoxide (*t*-BHP), collagenase, thiobarbituric acid, reduced glutathione (GSH), *o*-phthalaldehyde, and aspartate aminotransferase (AST) and alanine aminotransferase (ALT) diagnostic kits from Sigma Co.; lactate dehydrogenase (LDH)- and MTT-based colorimetric assay kit from Roche Co.; Williams' E medium, penicillin, streptomycin neomycin, glutamine, and fetal bovine serum (FBS) from Life Technologies, Inc.; other chemicals were of the highest commercial grade available.

Isolation and culture of hepatocytes. Hepatocytes were isolated from male Sprague-Dawley rats (200 ± 10 g, Dae Han Laboratory Animal Research and Co., Daejeon, Korea) according to the method of two-stage collagenase perfusion,[25] were transferred to collagen-precoated culture plates and first cultured for 4 h at 37 °C (95% humidity, 5% CO_2) in Williams' E medium supplemented with an antibiotic mixture of penicillin, streptomycin and neomycin (1%), glutamine (1%) and FBS (10%), and then the medium was changed to Williams' E medium. Culture medium containing kahweol and cafestol and/or *t*-BHP was added to cultures 24 h after seeding, in order to ensure the uniform attachment of the cells at the onset of the experiments. Kahweol and cafestol were dissolved in dimethylsulfoxide (DMSO) and *t*-BHP was dissolved in the culture media. At the concentration of 30 μM kahweol or cafestol no cell toxicity, DNA damage and lipid peroxidation by kahweol or cafestol alone were detected.

Cell viability assay. The cell viability was assessed by measuring the release of LDH and by means of the MTT assay, according to the manufacturer's instructions. The cells were incubated in culture medium for 6 h containing *t*-BHP (250 *μ*M) and kahweol or cafestol. The level of LDH release was measured in the supernatants. After the supernatant was removed for LDH determination, the cells were used for the MTT assay. The release of LDH into the supernatant was then analyzed following a procedure involving the colorimetric test based on NADH synthesis. The controls included untreated cells and cells lysed with 2% Triton X-100 (100% mortality). The viability of the cells was calculated according to this scale. For the MTT assay, cells were rinsed with PBS and then MTT was added to the wells. After 4 h incubation, the medium was removed, and the blue formazan crystals, which was formed, were dissolved in DMSO. Relative cell viability was quantified by absorption measurements at 570 nm using a microtiter plate reader (Molecular Devices, Menlo Park, CA). This wavelength was found not to interfere with cafestol or kahweol.

GSH and lipid peroxidation assay. The non-protein GSH content of hepatocytes was determined in cell homogenates by a fluorometric assay using *o*-phthalaldehyde as previously described.[26] Malondialdehyde (MDA), the lipid peroxidation product in the cells, was assayed according to a thiobarbituric acid fluorometric method using 1,1,3,3-*tetra*-methoxypropane as the standard.[27] The protein concentration was determined by the method of Bradford, using bovine serum albumin as the standard.

Measurement of intracellular ROS production. The fluorescent probe, dichlorodihydrofluorescein diacetate, was used to monitor the intracellular generation of reactive oxygen species by *t*-BHP, as described previously.[28]

Comet assay. Oxidative DNA damage was evaluated using the Comet assay. Culture medium was aspirated from the cell monolayer and the cells were then exposed to different concentrations of kahweol or cafestol and *t*-BHP in PBS for 20 min on ice. Following the exposure to the oxidant, the cells were washed twice with ice-cold PBS. The cells were then detached from the culture dishes and processed for the Comet assay as previously described.[29] To quantitate the Comet assay, ethidium bromide-stained nucleoids were examined under a Nikon fluorescence microscope using Image-Pro 4.5 software (Media Cybernetics). One hundred comets per slide were visually scored according to the amount of DNA present in the tail. Under these conditions, the tail moment, Tm = [(fluorescence intensity of the tail)/(fluorescence intensity of the head) × tail length], was used as a measure of the DNA damage.

Animal treatment and hepatotoxicity assessment. Male ICR mice (25 ± 3 g, Dae Han Laboratory Animal Research and Co., Daejeon, Korea) were used for the experiments. The mice were allowed free access to Purina Rodent Chow and tap water, maintained in a controlled environment at 21 ± 2 °C and 50 ± 5% relatively humidity with a 12 h dark/light cycle, and acclimatized for at least 1 week before use. To study its protective effect against *t*-BHP-induced hepatotoxicity, kahweol or cafestol dissolved in corn oil were administered intragastrically (ig) at 10-100 mg/kg once daily for 3 consecutive days. Three hours after the final treatment, the mice were treated with *t*-BHP (20 mg/kg, intraperitoneally (ip), dissolved in saline). Twenty-four hours after the administration of *t*-BHP, the mice were anesthetized with CO_2, blood was removed by cardiac puncture to determine the serum ALT and AST activities, and the animals were

sacrificed by cervical dislocation. After bleeding, the livers were weighed and a thin slice preserved in a buffered formalin solution for the purpose of obtaining histological sections. The remaining livers were frozen quickly in dry ice/methanol and stored at −70 °C for GSH content and lipid peroxidation analysis. The hepatotoxicities were assessed by quantifying the serum activities of ALT and AST, the hepatic lipid peroxidation, and GSH content. The serum ALT and AST activities were measured with a spectrophotometric diagnostic kit obtained from the Sigma Co. The hepatic lipid peroxidation level and GSH content were measured by the fluorometric methods described above.[26,27]

Histological examinations. Fresh liver tissues, previously trimmed to approximately 2 mm thickness, were placed in plastic cassettes and immersed in neutral buffered formalin for 24 h. Fixed tissues were processed routinely, and then embedded in paraffin, sectioned, deparaffinized, and rehydrated using standard techniques. The extent of *t*-BHP-induced necrosis was evaluated by assessing the morphological changes in the liver sections stained with hematoxylin and eosin (H&E), using standard techniques.

Statistical analysis. All experiments were repeated at least three times. Results are reported as means ± SD. ANOVA was used to evaluate the difference between multiple groups. If significance was observed between groups, a Duncan's 't' test was used to compare the means of two specific groups, with $P < 0.01$ considered as significant.

Results

Effect of kahweol and cafestol on the *t*-BHP-induced cytotoxicity. The protective effects of kahweol and cafestol against the *t*-BHP-induced cytotoxic injury of the cultured hepatocytes were quantified by LDH and MTT assay. To assess whether kahweol and cafestol would protect the hepatocytes from *t*-BHP-induced damage, the cells were simultaneously incubated with kahweol or cafestol and *t*-BHP. Kahweol and cafestol, which are nontoxic even at a high concentration (10 μM), afforded full protection from cell injury (Fig. 2). To more precisely quantify the protective effects of kahweol and cafestol toward *t*-BHP, we also performed LDH assays, and those results were consistent with those obtained from the MTT assay. Adding kahweol and cafestol to the primary cultured hepatocytes effectively protected the cells from the cytotoxicity induced by *t*-BHP as expressed by the leakage of LDH (Fig. 2).

Effects of kahweol and cafestol on *t*-BHP-induced depletion of GSH and lipid peroxidation on hepatocytes. GSH is one of the most abundant intracellular antioxidants and the determination of changes in its concentration provides an alternative method of monitoring oxidative stress within cells. GSH is known to have a protective role in *t*-BHP-induced toxicity.[8] Therefore, the influences of kahweol and cafestol on the *t*-BHP-induced depletion of GSH in cultured rat hepatocytes were investigated. As expected, exposure to 250 μM *t*-BHP resulted in a dramatic depletion of GSH, which coincided with the onset of cytotoxicity. In the presence of kahweol and cafestol, the loss of intracellular GSH was largely prevented in a dose-dependent manner (Fig. 3). These results suggest that kahweol and cafestol reduced *t*-BHP-induced oxidative stress in the cells. Lipid peroxidation has been recognized as a potential mechanism of cell injury. Therefore, to explore the consequences of *t*-BHP-induced oxidative damage to cellular macromolecules

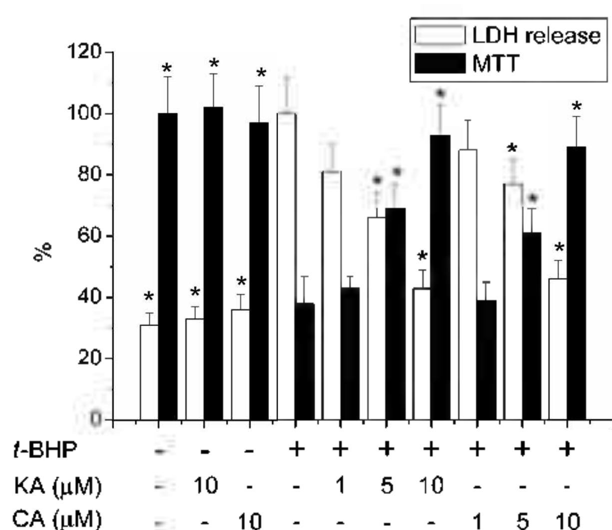

Figure 2. Cytoprotective effects of kahweol and cafestol on *t*-BHP-induced oxidative hepatotoxicity in cultured hepatocytes. The cultured hepatocytes were treated with *t*-BHP (250 μM) and kahweol (KA) or cafestol (CA) (1, 5 or 10 μM) for 6 h and oxidative hepatotoxicities were evaluated using the LDH and MTT assays as described in the Materials and Methods section. Each value represents the mean ± SD of three independent experiments, performed in triplicate. $^{*}P < 0.01$, significantly different from the *t*-BHP-treated cells.

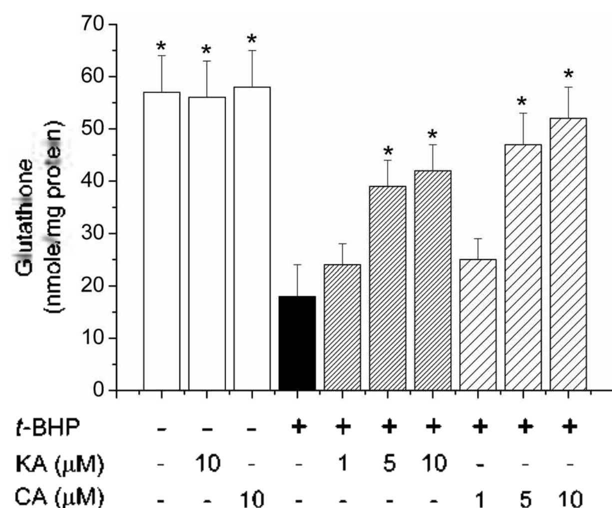

Figure 3. Effect of kahweol and cafestol on the *t*-BHP-induced depletion of cellular glutathione in cultured hepatocytes. The cultured hepatocytes were treated with *t*-BHP (250 μM) and kahweol (KA) or cafestol (CA) (1, 5 or 10 μM) for 6 h and cellular glutathione contents were measured as described in the Materials and Methods section. Each value represents the mean ± SD of three independent experiments, performed in triplicate. $^{*}P < 0.01$, significantly different from the *t*-BHP-treated cells.

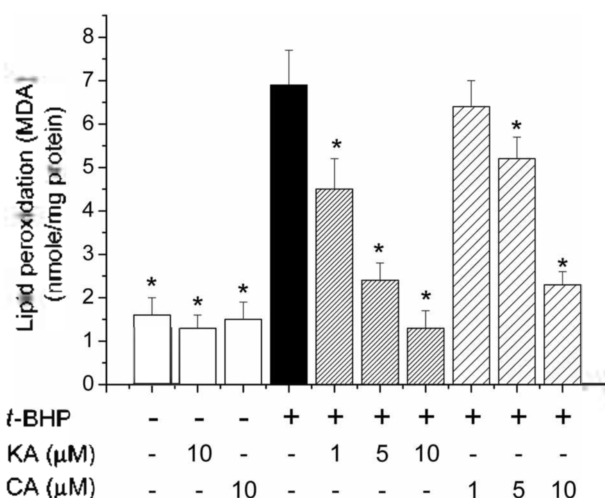

Figure 4. Effect of kahweol and cafestol on the lipid peroxidation induced by *t*-BHP in cultured hepatocytes. The cultured hepatocytes were treated with *t*-BHP (250 μM) and kahweol (KA) or cafestol (CA) (1, 5 or 10 μM) for 6 h and lipid peroxidation was evaluated by malondialdehyde (MDA) formation as described in the Materials and Methods section. Each value represents the mean ± SD of three independent experiments, performed in triplicate. $^*P < 0.01$, significantly different from the *t*-BHP-treated cells.

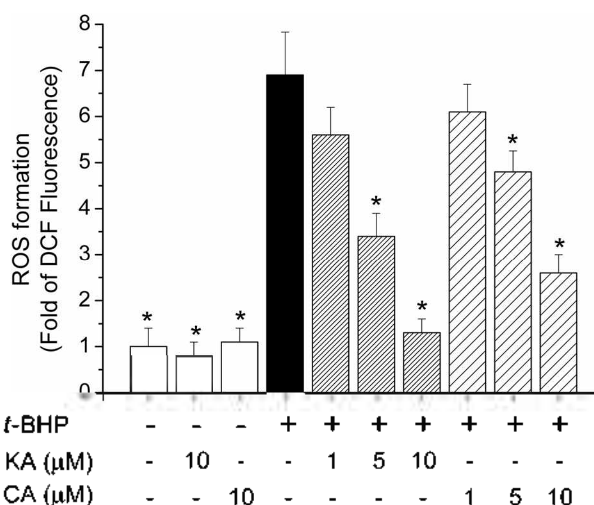

Figure 5. Effect of kahweol and cafestol on the intracellular ROS formation induced by *t*-BHP in cultured hepatocytes. The cultured hepatocytes were treated with 25 mM of dichlorodihydrofluorescein diacetate for 20 min and the medium was replaced by fresh medium containing *t*-BHP (250 mM) and kahweol (KA) or cafestol (CA) (1, 5 or 10 μM). After 30 min of treatment, the intracellular reactive oxygen species were measured by monitoring the fluorescence increases. Each value represents the mean ± SD of three independent experiments, performed in triplicate. $^*P < 0.01$, significantly different from the *t*-BHP-treated cells.

cafestol, we analyzed the formation of malondialdehyde (MDA) as a marker for membrane lipid peroxidation. Exposure to *t*-BHP alone for 6 h increased the amount of cell-associated MDA in the hepatocytes (Fig. 4). The presence of kahweol and cafestol significantly prevented MDA production induced by *t*-BHP and these inhibitions were strongly dependent upon the concentrations of kahweol and cafestol, indicating that kahweol and cafestol were able to inhibit the membrane lipid peroxidation triggered by the injurious peroxy radicals generated from *t*-BHP.

Effects of kahweol and cafestol on *t*-BHP-induced intracellular ROS production in hepatocytes. To confirm that kahweol and cafestol reduce *t*-BHP-induced oxidative stress in hepatocytes, the intracellular ROS production was assessed by monitoring dichlorodihydrofluorescein (DCF) fluorescence. Rapid increases in intracellular oxidant levels were noted in the cells after *t*-BHP treatment, as assessed by the increased DCF fluorescence, but the oxidant burden after *t*-BHP exposure decreased in the presence of kahweol and cafestol in a dose-dependent manner (Fig. 5). These results demonstrate that the kahweol and cafestol exhibited significantly antioxidant activity.

Effects of kahweol and cafestol on *t*-BHP-induced oxidative DNA damage on hepatocytes. Based on the hypothesis that kahweol and cafestol have an impact on the defense against oxidative stress, and to examine the relationship between the observed intracellular ROS-inducing action of *t*-BHP and oxidative-induced DNA damage, we further assessed the effects of kahweol and cafestol on oxidative DNA damage caused by *t*-BHP using the Comet assay. The Comet assay detects single and double-stranded DNA breaks in naked supercoiled DNA. Strand breaks cause supercoiled DNA to relax, allowing loops of DNA to migrate toward the anode upon electrophoresis, forming a 'Comet tail'. *t*-BHP (250 mM) treatment for 20 min increased about 3.5 fold the tail moments in the cells versus the control cells, indicating a 3.5 fold increase in oxidative DNA damage (Fig. 6). As shown in Figure 5, *t*-BHP rapidly increased DCF fluorescence, indicating that *t*-BHP induced a rapid increase in intracellular ROS levels. These results

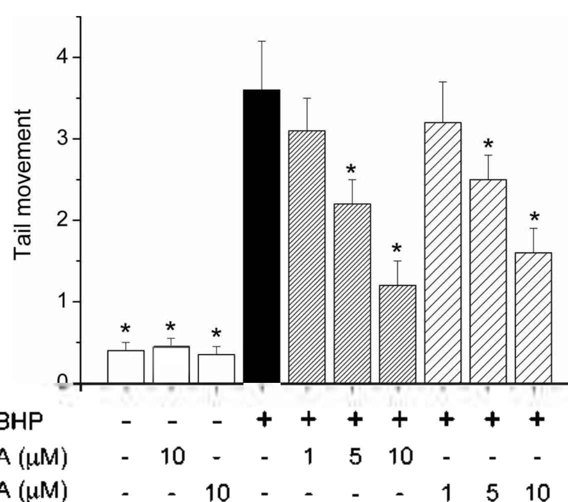

Figure 6. Effects of kahweol and cafestol on *t*-BHP-induced oxidative DNA damage to hepatocytes. The cultured hepatocytes were treated with *t*-BHP (250 mM) and kahweol (KA) or cafestol (CA) (1, 5 or 10 μM) for 20 min and the oxidative DNA damage was evaluated using the Comet assay, as described in the Materials and Methods section. Each value represents the mean ± SD of three independent experiments, performed in triplicate. $^*P < 0.01$, significantly different from the *t*-BHP-treated cells.

Table 1. Effects of the pretreatment of mice with kahweol and cafestol on *t*-BHP-induced oxidative hepatotoxicity

Treatment	Serum ALT (U/L)	Serum AST (U/L)	Lipid Peroxidation (nmole/g liver)	Glutathione (μmole/g liver)
Control	72 ± 8^{b}	96 ± 11^{b}	2.71 ± 0.51^{b}	9.22 ± 1.41^{b}
KA 100	82 ± 9^{b}	116 ± 17^{b}	2.46 ± 0.49^{b}	$12.62 \pm 1.61^{a,b}$
CA 100	87 ± 9^{b}	113 ± 18^{b}	2.62 ± 0.53^{b}	$12.25 \pm 1.53^{a,b}$
t-BHP	$2{,}772 \pm 341^{a}$	$1{,}627 \pm 215^{a}$	6.92 ± 1.02^{a}	4.63 ± 0.87^{a}
KA 10 + *t*-BHP	$1{,}923 \pm 244^{a,b}$	$751 \pm 104^{a,b}$	6.56 ± 0.97^{a}	$7.31 \pm 0.82^{a,b}$
KA 50 + *t*-BHP	$753 \pm 95^{a,b}$	$484 \pm 61^{a,b}$	$5.42 \pm 0.81^{a,b}$	$9.25 \pm 1.23^{a,b}$
KA 100 + *t*-BHP	$236 \pm 36^{a,b}$	$187 \pm 32^{a,b}$	$3.44 \pm 0.52^{a,b}$	$11.23 \pm 1.38^{a,b}$
CA 10 + *t*-BHP	$2{,}241 \pm 321^{a,b}$	$1{,}314 \pm 196^{a}$	6.68 ± 1.03^{a}	$6.78 \pm 0.84^{a,b}$
CA 50 + *t*-BHP	$824 \pm 102^{a,b}$	$624 \pm 95^{a,b}$	$5.61 \pm 0.82^{a,b}$	$9.52 \pm 1.42^{a,b}$
CA 100 + *t*-BHP	$512 \pm 76^{a,b}$	$358 \pm 52^{a,b}$	$4.47 \pm 0.65^{a,b}$	$12.42 \pm 1.36^{a,b}$

The mice were pretreated with kahweol (KA) or cafestol (CA) (10, 50 or 100 mg/kg, ig) once daily for 3 consecutive days. The control mice were given corn oil. Three hours after the final treatment, the mice were treated with *t*-BHP (20 mg/kg, ip). Hepatotoxicity was determined 24 h later by quantifying the serum activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), hepatic lipid peroxidation, and the glutathione level. Each value represents the mean ± SD of five mice. $^{a}P < 0.01$, significantly different from the control. $^{b}P < 0.01$, significantly different from the *t*-BHP.

further suggest that *t*-BHP increased DNA damage through a mechanism involving intracellular ROS production. However, the increased tail moments caused by *t*-BHP-induced oxidative DNA damage were decreased in the presence of kahweol and cafestol in a dose-dependent manner (Fig. 6). These results demonstrate that the increase of intracellular ROS production may mediate the *t*-BHP-induced DNA damage of the cells and that kahweol and cafestol significantly prevent *t*-BHP-induced oxidative DNA damage through its anti-oxidative effects.

Effect of kahweol and cafestol on *t*-BHP-induced hepatotoxicity in mice. The hepatic enzymes, AST and ALT, were used as biochemical markers for early acute hepatic damage. A single dose of *t*-BHP (20 mg/kg) caused hepatotoxicity in the mice, as indicated by the increase in their ALT and AST serum levels after *t*-BHP administration (Table 1). Kahweol and cafestol pretreatment prevented the *t*-BHP-induced elevation of the ALT and AST serum levels in a dose-dependent manner. In consistent with the effect on the serum levels of ALT and AST, the pretreatment with kahweol and cafestol significantly decreased *t*-BHP-induced lipid peroxidation in the liver (Table 1). As the oxidative stress of tissue generally involves the GSH system, we therefore measured the level of GSH for each group of livers. Whereas the administration of *t*-BHP significantly depleted the GSH level, pretreatment with kahweol and cafestol significantly protected the GSH depletion produced by *t*-BHP in a dose-dependent manner (Table 1). Kahweol or cafestol treatment alone slightly increased the hepatic GSH levels.

Histopathology of the liver. Histopathological studies of liver showed that *t*-BHP, compared to normal, induces degeneration in hepatocytes and hepatic cords, focal necrosis, congestion in central vein and sinusoids, and infiltration of lymphocytes (Fig. 7). According to microscopic examinations, severe hepatic lesions induced by *t*-BHP were remarkably reduced by the administration of kahweol and cafestol, which were largely in agreement with the results of the serum aminotransferase activities and hepatic lipid peroxidation. Kahweol or cafestol (50 mg/kg) treatment alone did not cause any change in the liver histology (data not shown).

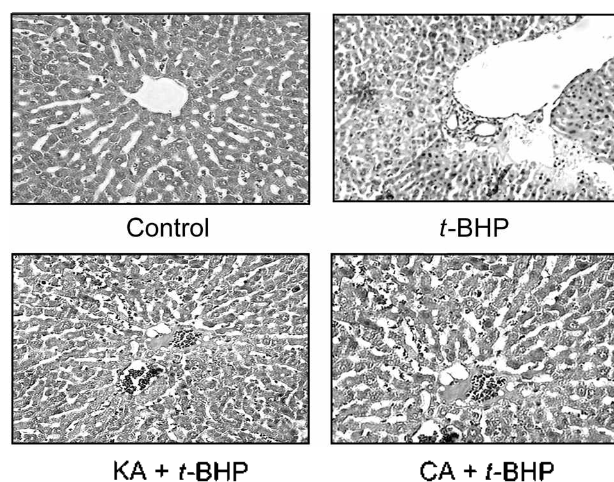

Figure 7. Effects of kahweol and cafestol pretreatment on *t*-BHP-induced liver damage in mice. The mice were pretreated with kahweol (KA) or cafestol (CA) (50 mg/kg, ig) once daily for 3 consecutive days. The control mice were given corn oil. Three hours after the final treatment, mice were treated with *t*-BHP (20 mg/kg, ip). The mice were sacrificed 24 h after the *t*-BHP administration and the liver was removed, fixed and embedded in paraffin. Sections were stained with hematoxylin-eosin. × 100.

Discussion

Liver disease, especially viral and alcoholic hepatitis, occurs predominantly in the developing world and has an enormous impact on both public health and economics. Due to their high metabolic function, high cytochrome P450 content, and their central role in the biotransformation of xenobiotics, hepatocytes are very much exposed to oxidative stress *in-vivo*, as indicated by the large number of liver pathologies which have been linked to oxidative stress.[1,2] Oxidants may generate lipid hydroperoxides, which sub-

sequently decompose into alkoxyl and peroxyl radicals, which are able to oxidize cellular constituents.[6] The antioxidant and free radical scavenging activities of many substances have often been assessed, since many substances that possess anti-hepatotoxic activity also show strong antioxidant activity.[8,30]

The aim of this study was to evaluate the ability of kahweol and cafestol, with their inherent antioxidant activity, to have an effect on the cellular and DNA damage in rat primary hepatocyte cultures, and hepatotoxicity in mice caused by *t*-BHP-induced oxidative stress. In this study, we used *t*-BHP, which has frequently been employed as an oxidative stress inducer[7,9,10,29] to induce oxidative hepatotoxicity *in vitro* and *in vivo*. Our present study showes that *t*-BHP produces loss of cell viability, as evidenced by the leakage of cytosolic LDH and by means of the MTT assay, the depletion of intracellular GSH, and the increase of lipid peroxidation in cultured hepatocytes. The incubation of the cultured hepatocytes with kahweol and cafestol resulted in the protection of the hepatocytes against *t*-BHP induced oxidative hepatotoxicities in a dose-dependent manner (Fig. 2, 3 and 4). Kahweol and cafestol also attenuated *t*-BHP induced cellular ROS production (Fig. 5) and DNA damage (Fig. 6) in the cultured hepatocytes.

An antioxidant action has been reported to play an important role in the hepatoprotective activity of many plants.[9] Silymarin, obtained from the plant *Silybum marianum* has been intensively studied, because of its hepatoprotective effects.[31] Silymarin is now used clinically in the treatment of many liver diseases.[32] In this study, we demonstrated that kahweol and cafestol, diterpenes found in considerable quantities in coffee beans, had the antioxidant activity in cultured hepatocytes and the liver. This provides biological evidence supporting the effectiveness of kahweol and cafestol for the liver disorders.

One of the potential mechanisms by which *t*-BHP may be cytotoxic is through the generation of free radical intermediates, such as the toxic peroxyl and alkoxyl radicals, which then have an effect on various components of the iron metabolism, such as on cytochrome P450 and hemoglobin, which are present in the hepatocytes and erythrocytes, respectively.[7,11] These radicals can then initiate and propagate lipid peroxidation in cells which are susceptible to oxidative stress, and may also form covalent bonds with cellular molecules resulting in DNA damage and cell injury.[12] In the hepatotoxicity and lipid peroxidation experiments, kahweol and cafestol protected the primary cultured hepatocytes effectively from the injury caused by *t*-BHP, as reflected in the increased cell viability (LDH release and the MTT assay) and the decreased formation of MDA (Fig. 4). LDH release and the MTT assay are known to be general indices of hepatic cytotoxicity. MDA, on the other hand, is the major oxidative degradation product of membrane unsaturated fatty acid, having toxic and genotoxic properties.[33] These data confirm the hepatoprotective and anti-lipid oxidation activities of kahweol and cafestol. However, we cannot rule out the possibility that kahweol and cafestol may interact directly with *t*-BHP in the medium, in such a way as to alleviate the cell damage caused by *t*-BHP. Furthermore, the protective effects of kahweol and cafestol on *t*-BHP-induced oxidative hepatotoxicity could be associated with the inhibition of cytochrome P450-mediated metabolism of this organic hydroperoxide to active alkoxyl radicals that subsequently initiate lipid peroxidation, thus leading to liver damage. Another potential mechanism of *t*-BHP cytotoxicity is possible interference with the detoxification process that causes depletion of GSH, which can indirectly lead to loss of cell integrity and DNA damage and result in cell injury.[6] GSH acts as an essential intracellular reducing agent both for the maintenance of thiol groups on intracellular proteins and for antioxidant molecules. It is well established that GSH, the most important biomolecule providing protection against oxidative stress and chemically induced cytotoxicity, is able to participate both in the elimination of reactive intermediates, by conjugation and hydroperoxide reduction, and that of free radicals by direct quenching. This study showed that *t*-BHP reduced the level of GSH, an index of oxidative stress, in cultured hepatocytes and in the mouse liver, and that treatment with kahweol and cafestol significantly prevented the *t*-BHP induced depletion of GSH levels (Fig. 3 and Table 1). In our *in-vivo* studies, we also showed that treatment with kahweol and cafestol increased the GSH content in the liver (Table 1). In line with these observations, the chemoprotective effects of kahweol and cafestol have been known to be related to be enhanced detoxification, *e.g. via* the induction of carcinogen-detoxifying enzyme systems such as γ-glutamylcysteine synthetase, the rate limiting enzyme of chemoprotective GSH synthesis, and glutathione S-transferase.[15-17] The formation of lipid peroxidation products and the depletion of the GSH level implicate oxidative stress. Therefore, the results showed that kahweol and cafestol attenuated the oxidative stress induced by *t*-BHP (Table 1).

Oxidative stress appears to be involved in the mechanism of various types of cell injury.[3] Liver cells have a particularly high probability of being subjected to ROS-induced toxicity, because hepatocytes produce large amounts of ROS during the detoxification of xenobiotics and toxic substances.[2] The DCF assay is widely used for measuring overall ROS formation in biological systems.[28] The method is based on the formation of fluorescent DCF from non-fluorescent DCF by oxidizing species. When hepatocytes were cultured with *t*-BHP, a pronounced increase in DCF fluorescence was observed, indicating the existence of an overproduction of ROS derived from the *t*-BHP metabolism inside the cell. In the presence of kahweol and cafestol, the generation of excess ROS by *t*-BHP was strongly reduced, and this resulted in the DCF fluorescence being significantly decreased. There is accumulating evidence that the increased intracellular ROS occurring in cells acts as a common mediator of DNA damage caused by physical or chemical stimuli, such as hydrogen peroxide or nitric oxide.[34] Thus, in this study we investigated the possible involvement of intracellular ROS production in the *t*-BHP-induced DNA

damage to the cells and the protective effects of kahweol and cafestol on this process. The results showed that *t*-BHP increased DNA damage through increased intracellular ROS production (Fig. 5 and 6). The significant blockade of the *t*-BHP-induced DNA damage brought about by the treatment with kahweol and cafestol (Fig. 6) indicates that the increase of ROS production mediates the observed DNA-damage caused by *t*-BHP. It was reported that, in isolated hepatocytes, *t*-BHP induced unscheduled DNA synthesis-a marker of DNA damage.[9] These results suggest that DNA damage may be a key step in the pathogenesis of various liver diseases related to oxidative stress, and it can be concluded that kahweol and cafestol exert an antioxidant action inside the cells, and are responsible for the observed modulation of the cellular response to oxidative challenge. This was confirmed by the markedly higher intracellular GSH levels and ROS reduction observed in response to *t*-BHP-induced oxidative stress in kahweol and cafestol treated hepatocytes. The *in-vivo* study also showed that oral pretreatment with kahweol and cafestol significantly lowered *t*-BHP-induced serum levels of hepatic enzyme markers (ALT and AST) and reduced oxidative stress of the liver, as determined by evaluating the lipid peroxidation and GSH levels (Table 1). Histopathological evaluation of the mouse livers also revealed that kahweol and cafestol reduced the incidence of liver lesions and inflammation induced by *t*-BHP (Fig. 7). It has been proposed that ROS generated in inflamed tissue can cause injury to target cells.[35] We therefore assumed that kahweol and cafestol could inhibit *t*-BHP-induced oxidative damage in the liver by blocking the cytotoxicity induced by *t*-BHP and by decreasing the progressive damage caused during inflammation.

In conclusion, this study demonstrated that kahweol and cafestol showed protective effects against *t*-BHP-induced oxidative hepatotoxicity, due to its antioxidant potential, in primary cultured hepatocytes and the liver. However, further studies are needed in order to clarify the exact mechanism. Recently, much attention has been focused on the protective biochemical function of naturally occurring antioxidants in biological systems, and on their mechanism of action. Many natural antioxidant products are capable of preventing or inhibiting the process of pathogenesis of various diseases, including liver injury. This study therefore provides biological evidence supporting the use of kahweol and cafestol for the treatment of liver disorders.

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