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Protective effect of saponins derived from roots of *Platycodon grandiflorum* on *tert*-butyl hydroperoxide-induced oxidative hepatotoxicity

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Abstract

There is increasing evidence that oxidative stress is implicated in the pathogenesis of various diseases, including alcoholic liver injury. In the present work, we investigate the protective effects of the saponins isolated from the roots of *Platycodon grandiflorum* A. DC (Campanulaceae), Changkil saponins (CKS), on the *tert*-butyl hydroperoxide (*t*-BHP)-induced oxidative injury (hepatotoxicity) in cultured rat primary hepatocytes and in rat livers. CKS 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. CKS provided good protection from the *t*-BHP-induced production of intracellular reactive oxygen species and DNA damage. In addition, CKS was able to quench 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals and the superoxide radical. The in vivo study showed that the pretreatment with CKS prior to the administration of *t*-BHP significantly prevented the increase in the 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. These results support the anti-oxidative role of CKS, and demonstrate that CKS can scavenge oxygen free radicals and protect cells from oxidative stress.

Keywords: *Platycodon grandiflorum*; Saponin; Antioxidant; *tert*-Butyl hydroperoxide; Hepatotoxicity

1. Introduction

The liver produces large amounts of oxygen free radicals in the course of detoxifying xenobiotic and toxic substances, and oxidative stress caused by oxygen free radicals (reactive oxygen species (ROS)) has

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been shown to be linked to liver diseases, such as hepatotoxicity, and other liver pathological conditions (Mehendale et al., 1994; Stohs, 1995). Furthermore, oxidative stress is considered to be associated with many diseases, such as inflammation, cardiovascular diseases, aging, and cancer (Spector, 2000). Normally, the ROS produced are scavenged by endogenous antioxidants which are abundant in the liver tissue (Yu, 1994). However, liver injury can occur when large acute doses of or chronic exposure to toxic substances overpower the hepatic antioxidant defense system (Halliwell, 1996). ROS readily interact with cellular macromolecules and structures, resulting in changes in membrane permeability, the activation of proteases and nucleases, and altered gene expression (Yu, 1994; Halliwell, 1996). It is also well known that these cellular changes induced by ROS lead to DNA damage in a variety of cell types (Collins, 1999; Termini, 2000).

A number of prooxidant drugs and other chemicals have been implicated in the oxidative stress and cell injury resulting from the intracellular production of injurious ROS (Halliwell and Gutteridge, 1999). *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 (Rush et al., 1985; Joyeux et al., 1990; Hwang et al., 1996; Soszynski and Bartosz, 1997). *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 GSH depletion, affect cell integrity and result in cell injury in hepatocyte cultures and in rat livers (Thornalley et al., 1983; Rush et al., 1985; Lin et al., 2000). It also causes a mitochondrial depolarization within intact hepatocytes and mediates DNA base damage in cultured mammalian cells (Altman et al., 1994). Alternatively, *t*-BHP can be rapidly converted by glutathione peroxidase to *t*-butyl alcohol and glutathione disulfide (GSSG). GSSG is then converted to reduced glutathione (GSH) by GSSG reductase, resulting in pyridine nucleotide oxidation. The loss of GSH and the oxidation of pyridine nucleotides are associated with altered Ca^{2+} homeostasis, which is considered to be a critical event in *t*-BHP-induced toxicity (Shimizu et al., 1998). These phenomena are similar to the oxidative stress which occurs in cells and tissue.

Recently, herbs have begun to attract attention as health-beneficial foods (physiologically functional foods) and as a raw material for the development of drugs. Herbal medicines derived from plant extracts are increasingly being utilized to treat a wide variety of clinical diseases, even though relatively little is known about their modes of action. Platycodi radix, the root of *Platycodon grandiflorum* A. DC (Campanulaceae), commonly known as Doraji (Chinese drug, “Jiegeng”, and Japanese name, “Kikyo”) is used as a traditional oriental medicine and its biological significance has previously been reviewed (Lee, 1973). Extracts from the roots of *P. grandiflorum* have been reported to have wide ranging health benefits. In Korea, the root of *P. grandiflorum* (4 years old) is used both as a food and as a folk remedy for adult diseases, such as, bronchitis, asthma and pulmonary tuberculosis, hyperlipidemia, and inflammatory diseases, as well as being taken as a sedative (Lee, 1973). In our previous studies, it was observed that Changkil (CK), the aqueous extract made from the root of *P. grandiflorum* cultivated for more than 20 years (Lee, S.H., 1991, Patent on the method of cultivating the perennial balloonflower, Patent no. 045971, Korea), prevented hypercholesterolemia and hyperlipidemia (Kim et al., 1995) and enhanced the functions of macrophages (Choi et al., 2001). Recently, we showed that CK exhibited protective effects against the acetaminophen- and carbon tetrachloride-induced hepatotoxicity in rats and that these protective effects, at least in part, are associated with the inhibition of the cytochrome P450-mediated bioactivation of these hepatotoxicants (Lee et al., 2001; Lee and Jeong, 2002). In addition, we also found that CK exhibited antioxidant effects in $FeCl_2$-ascorbate induced lipid peroxidation and in relation to the superoxide radical scavenging activity (Lee and Jeong, 2002). Although these studies suggest that at least some of these effects are due to the anti-oxidative potential of CK extracts or their constituents, unequivocal proof of this assumption is still lacking. In a continuing study to discover the components responsible for the antioxidant activity derived from CK, we were able to determine that the saponin fraction (CKS) derived from CK has a potent antioxidant effect, such as a superoxide radical scavenging activity generated by the xanthine and xanthine oxidase system, and a reduction of ROS production by *t*-BHP in hepatocytes. Even though CKS showed

antioxidant activities in the preliminary examination, the biological effects of CKS have so far not been well elucidated, and it is not known whether CKS can prevent or alleviate liver injury induced by oxidative stress. In the present work, we examined the protective potential of CKS against *t*-BHP-induced oxidative hepatocyte injury and the mechanism(s) underlying these protective effects in rat primary hepatocytes cultures and in the rat liver. Various parameters, such as cell viability, lipid peroxidation, thiol status, intracellular ROS, DNA damage, and free radical scavenging activity, were measured as an index of oxidative stresses. Our results indicate that CKS significantly protected both rat primary hepatocytes cultures and the rat liver against *t*-BHP-induced oxidative hepatotoxicities.

2. Materials and methods

2.1. Chemicals

Chemicals and cell culture materials were obtained from the following sources: *tert*-butyl hydroperoxide, collagenase, thiobarbituric acid, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 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.

2.2. Preparation of CKS

The CK used in this study is the aqueous extract obtained from the roots of *P. grandiflorum* (22 years old), which was supplied by Jang Saeng Doraji Co., Jinju, South Korea. The composition of the roots of *P. grandiflorum* has been previously published (Kim et al., 1995). The CK was prepared as described previously (Lee et al., 2001; Lee and Jeong, 2002). CK was subjected to column chromatography over amberlite XAD-2, Diaion MCl Gel HP20 or Kogel BG4600. After removing the saccharides and amino acids with water, the column was eluted with methanol to obtain CKS, the saponin fraction of CK, as described previously (Tada et al., 1975).

2.3. Isolation and culture of hepatocytes

Hepatocytes were isolated from male Sprague–Dawley rats (200 ± 10 g, KRIBB, Korea) according to the method of two-stage collagenase perfusion (Bonney et al., 1974), 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 CKS 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. CKS and *t*-BHP were dissolved in the culture media. At the concentration of 5 μg/ml no cell toxicity, DNA damage and lipid peroxidation by CKS alone were detected.

2.4. 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 μM) and CKS. 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 CKS.

2.5. 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 (Hissin and Hief, 1976). Malondialdehyde (MDA), the lipid peroxidation product in the cells, was assayed according to a thiobarbituric acid fluorometric method using 1,1,3,3-tetramethoxypropane as the standard (Giinther et al., 1995). Control tests indicated that CKS did not interfere with the GSH and lipid peroxidation assays. The protein concentration was determined by the method of Bradford, using bovine serum albumin as the standard.

2.6. 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 (Wang and Joseph, 1999). In the brief, the cells were treated with 25 μM of dichlorodihydrofluorescein diacetate for 20 min and the medium was replaced by fresh medium containing *t*-BHP (250 μM) and CKS. After 10 min of treatment, the intracellular reactive oxygen species were monitored using a fluorescence spectrophotometer (GeminiXS, Molecular Devices) by exciting at 485 nm and following the emission at 530 nm for 30 min.

2.7. 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 CKS 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 (Johnson and Loo, 2000). 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.

2.8. Assay of free radical-quenching capacity and superoxide scavenging activity

The free radical-quenching capacity of CKS was tested by a method involving the bleaching of stable DPPH (Ursini et al., 1994). Superoxide was generated using 25 μM xanthine and 0.02 U/ml xanthine oxidase with, 200 μg/ml salmon testes DNA, 100 μM dihydroethidine in 50 mM KH_2PO_4-KOH buffer, pH 7.4, with or without CKS. The oxidation of dihydroethidine to ethidium by superoxide was monitored using a fluorescence spectrophotometer (GeminiXS, Molecular Devices) by exciting at 475 nm and following the emission at 610 nm, as previously described (Benov et al., 1998). Superoxide dismutase (100 U/ml) was used as a reference inhibitor.

2.9. Animal treatment and hepatotoxicity assessment

Male Sprague–Dawley rats (200 ± 10 g) were used for the experiments. The rats 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:12 h dark/light cycle, and acclimatized for at least l week before use. To study its protective effect against *t*-BHP-induced hepatotoxicity, CKS in saline was administered intragastrically at 0.5–5 mg/kg once daily for three consecutive days. Three hours after the final treatment, the rats were treated with *t*-BHP (20 mg/kg, intraperitoneally, dissolved in saline). Twenty-four hours after the administration of *t*-BHP, the rats 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 (Giinther et al., 1995; Hissin and Hief, 1976).

2.10. 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.

2.11. Statistical analysis

All experiments were repeated at least three times. Results are reported as means±S.D. 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.

3. Results

3.1. Effect of CKS on the t-BHP-induced cytotoxicity

The protective effects of CKS against the *t*-BHP-induced cytotoxic injury of the cultured hepatocytes were quantified by LDH and MTT assay. To assess whether CKS would protect the hepatocytes from *t*-BHP-induced damage, the cells were simultaneously incubated with CKS and *t*-BHP. CKS, which is nontoxic even at a high concentration (5 μg/ml), afforded full protection from cell injury. To more precisely quantify the protective effects of CKS toward *t*-BHP, we also performed LDH assays, and that the results were consistent with those obtained from the MTT assay. Adding CKS to the primary cultured hepatocytes effectively protected the cells from the cytotoxicity induced by *t*-BHP as expressed by the leakage of LDH (Table 1).

3.2. Effects of CKS on t-BHP-induced depletion of GSH and lipid peroxidation on hepatocytes

GSH is known to have a protective role in *t*-BHP-induced toxicity (Joyeux et al., 1990). Therefore, the influence of CKS on the *t*-BHP-induced depletion of GSH in the cultured rat hepatocytes was investigated. As expected, exposure to 250 μM *t*-BHP resulted in a dramatic depletion (>80%) of GSH, which coincided with the onset of cytotoxicity. In the presence of CKS, the loss of intracellular GSH was largely prevented in a dose-dependent manner. These results suggest that CKS reduced *t*-BHP-induced oxidative stress in the cells. CKS alone did not alter basal GSH levels. The loss of intracellular GSH was accompanied by a corresponding increase in intracellular GSSG (data not shown). Lipid peroxidation has been recognized as a potential mechanism of cell injury. Therefore, to explore the consequences of

Table 1
Effects of CKS on *t*-BHP-induced oxidative hepatotoxicity in cultured rat hepatocytes

Treatment	LDH release (% of control)	MTT (% of control)	Lipid peroxidation (MDA: nmol/mg protein)	Glutathione (nmol/mg protein)
Control	100 ± 12[a]	100 ± 12[a]	1.65 ± 0.18[a]	63.8 ± 7.2[a]
CKS 5	114 ± 13[a]	96 ± 10[a]	1.46 ± 0.17[a]	64.2 ± 7.3[a]
t-BHP	320 ± 46[b]	32 ± 4.1[b]	7.55 ± 0.81[b]	12.3 ± 0.14[b]
CKS 0.5 + *t*-BHP	270 ± 34[b]	54 ± 7.1[a,b]	6.36 ± 0.52[a]	18.2 ± 1.6[a,b]
CKS 1.0 + *t*-BHP	232 ± 31[a,b]	82 ± 11[a,b]	2.91 ± 0.32[a,b]	41.6 ± 4.3[a,b]
CKS 5.0 + *t*-BHP	140 ± 21[a]	94 ± 12[a]	1.84 ± 0.95[a]	58.2 ± 6.2[a]

The cultured hepatocytes were treated with *t*-BHP (250 μM) and CKS (0.5, 1.0, or 5.0 μg/ml) for 6 h and the oxidative hepatotoxicities were evaluated using the LDH and MTT assays, and by assessing the lipid peroxidation and glutathione levels, as described in Section 2. Each value represents the mean ± S.D. of three independent experiments, performed in triplicate.

[a] $P < 0.01$, significantly different from the *t*-BHP.

[b] $P < 0.01$, significantly different from the control.

t-BHP-induced oxidative damage to cellular macromolecules and to determine the possible effects of CKS, we analyzed the formation of malondialdehyde (MDA) as a marker for membrane lipid peroxidation. CKS alone did not change the degree of MDA formation compared to the untreated controls. However, exposure to *t*-BHP alone for 6 h increased the amount of cell-associated MDA in the hepatocytes (Table 1). The presence of CKS significantly prevented the MDA production induced by *t*-BHP and this inhibition was strongly dependent upon the concentrations of CKS, indicating that CKS was able to inhibit the membrane lipid peroxidation triggered by the injurious peroxy radicals generated from *t*-BHP.

3.3. Effects of CKS on t-BHP-induced intracellular ROS production in hepatocytes

To confirm that CKS reduces *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 CKS in a dose-dependent manner (Fig. 1). These results demonstrate that the CKS exhibited significantly antioxidant activity.

3.4. Effects of CKS on t-BHP-induced oxidative DNA damage on hepatocytes

We further assessed the effects of CKS on the 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." 250 μM *t*-BHP treatment for 20 min increased four-fold the tail moments in the cells versus the control cells (Fig. 2). As shown in Fig. 1, *t*-BHP (250 μM) rapidly increased DCF fluorescence, indicating that *t*-BHP induced a rapid increase in intracellular ROS levels. These results further suggest that *t*-BHP increased DNA damage through a mechanism involving intracellular ROS production. However, the increased

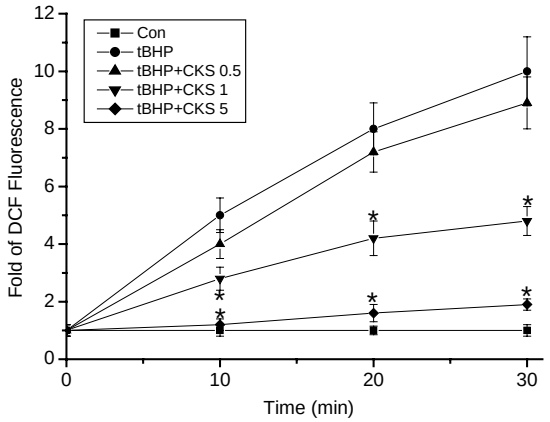

Fig. 1. Effect of CKS on the intracellular ROS formation induced by *t*-BHP. The cultured hepatocytes were treated with 25 μM of dichlorodihydrofluorescein diacetate for 20 min and the medium was replaced by fresh medium containing *t*-BHP (250 μM) and CKS (0.5, 1, or 5 μg/ml). After 10 min of treatment, the intracellular reactive oxygen species were measured by monitoring the fluorescence increases for 30 min. Each value represents the mean ± S.D. of three independent experiments, performed in triplicate. (*)$P < 0.01$, significantly different from the *t*-BHP-treated cells.

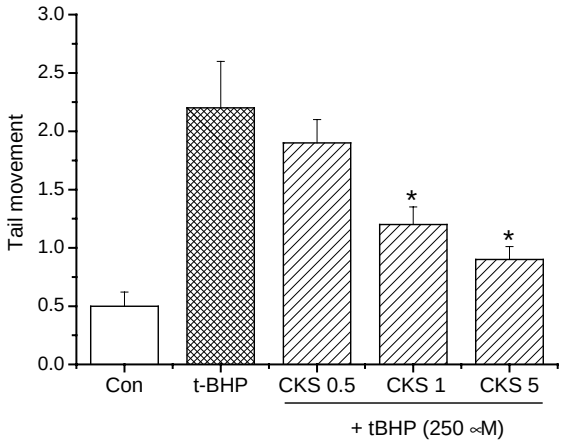

Fig. 2. Effects of CKS on *t*-BHP-induced oxidative DNA damage to hepatocytes. The cultured hepatocytes were treated with *t*-BHP (250 μM) and CKS (0.5, 1, or 5 μg/ml) for 20 min and the oxidative DNA damage was evaluated using the Comet assay, as described in Section 2. Each value represents the mean ± S.D. of three independent experiments, performed in triplicate. (*)$P < 0.01$, significantly different from the *t*-BHP-treated cells.

Table 2
Free radical-quenching activity of CKS on the DPPH and superoxide radicals

Treatment (μg/ml)	DPPH bleaching (%)[a]	Superoxide scavenging activity (%)[b]
Control	0	0
CKS 0.1	21.2 ± 3.4	28.2 ± 4.5
CKS 0.2	44.5 ± 5.2	51.7 ± 6.3
CKS 0.5	62.6 ± 7.1	75.6 ± 9.1
CKS 1.0	88.3 ± 9.2	93.1 ± 9.5

[a] CKS was mixed with DPPH (10 mM, 30 μl) in methanol (3 ml). The reaction mixtures were then colored by the addition of toluene, and read at 517 nm against a blank without CKS. The degree of DPPH bleaching is expressed as a percentage in relation to the absorbance of the control.

[b] Superoxide was generated using 25 μM xanthine and 0.02 U/ml xanthine oxidase with, 200 μg/ml salmon testes DNA, 100 μM dihydroethidine in the presence or absence of CKS. The oxidation of dihydroethidine to ethidium (ethidium-DNA fluorescence; excitation 475 nm/emission 610 nm) by superoxide was monitored using a fluorescence spectrophotometer. Superoxide dismutase (100 U/ml) was used as a reference inhibitor. The degree of superoxide scavenging activity is expressed as a percentage in relation to the control.

tail moments caused by *t*-BHP-induced oxidative DNA damage were decreased in the presence of CKS in a dose-dependent manner (Fig. 2). These results demonstrate that the increase of intracellular ROS production may mediate the *t*-BHP-induced DNA damage of the cells and that the CKS significantly prevented *t*-BHP-induced oxidative DNA damage through its anti-oxidative effects.

3.5. Free radical-quenching activity of CKS on the DPPH and superoxide radicals

In order to determine whether the protection afforded to the hepatocytes by CKS against *t*-BHP-induced oxidative cytotoxicity and DNA damage might be the consequence of the free radical-quenching capacity of CKS, the bleaching of DPPH and the superoxide radical scavenging activity by CKS was measured. The results, which are summarized in Table 2, showed that CKS was able to quench the DPPH free radicals in a dose-dependent manner. The superoxide scavenging properties of CKS were evaluated in the xanthine/xanthine oxidase system, in which the superoxide generated oxidizes dihydroethidine to fluorescent ethidium, which then binds to DNA (ethidium-DNA), further amplifying its fluorescence. Accordingly, the level of fluorescence is inversely proportional to the scavenging activity. CKS dose-dependently inhibited fluorescence formation (Table 2). Therefore, these results indicate that CKS has free radical-quenching activity.

3.6. Effect of CKS on t-BHP-induced hepatotoxicity in rats

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 rats, as indicated by the increase in their ALT and AST serum levels after *t*-BHP administration (Table 3). CKS pretreatment prevented the

Table 3
Effects of the pretreatment of rats with CKS on *t*-BHP-induced oxidative hepatotoxicity

Treatment	Serum ALT (U/l)	Serum AST (U/l)	Lipid peroxidation (MDA: nmol/g liver)	Glutathione (mg/g liver)
Control	43 ± 6[a]	96 ± 3[a]	81 ± 9[a]	1.82 ± 0.21[a]
CKS 5	38 ± 5[a]	93 ± 4[a]	78 ± 9[a]	1.88 ± 0.20[a]
t-BHP	275 ± 31[b]	762 ± 85[b]	186 ± 20[b]	1.23 ± 0.13[b]
CKS 0.5 + *t*-BHP	243 ± 24[b]	611 ± 65[b]	164 ± 18[b]	1.36 ± 0.16[b]
CKS 1.0 + *t*-BHP	122 ± 15[a,b]	324 ± 41[a,b]	121 ± 14[a,b]	1.74 ± 0.18[a]
CKS 5.0 + *t*-BHP	61 ± 7[a]	114 ± 12[a]	94 ± 10[a]	1.84 ± 0.19[a]

The rats were pretreated with CKS (0.5s, 1.0, or 5.0 mg/kg, i.g.) once daily for three consecutive days. The control rats were given saline. Three hours after the final treatment, the rats were treated with *t*-BHP (20 mg/kg, i.p.). 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 ± S.D. of five mice.

[a] $P < 0.01$, significantly different from the *t*-BHP.

[b] $P < 0.01$, significantly different from the control.

t-BHP-induced elevation of the ALT and AST serum levels in a dose-dependent manner. In a manner which was consistent with the effect on the serum levels of ALT and AST, the pretreatment with CKS significantly decreased *t*-BHP-induced lipid peroxidation in the liver (Table 3). As the oxidative stress of tissue generally involves the GSH system, we therefore, measured the level of GSH for each group of livers. CKS, by itself, did not affect the hepatic GSH levels. Whereas the administration of *t*-BHP significantly depleted the GSH level, pretreatment with CKS significantly protected the GSH depletion produced by *t*-BHP (Table 3). A dose-dependent protective effect on the depletion of the GSH level was observed on increasing the CKS pretreatment from 0.5 to 5 mg/kg.

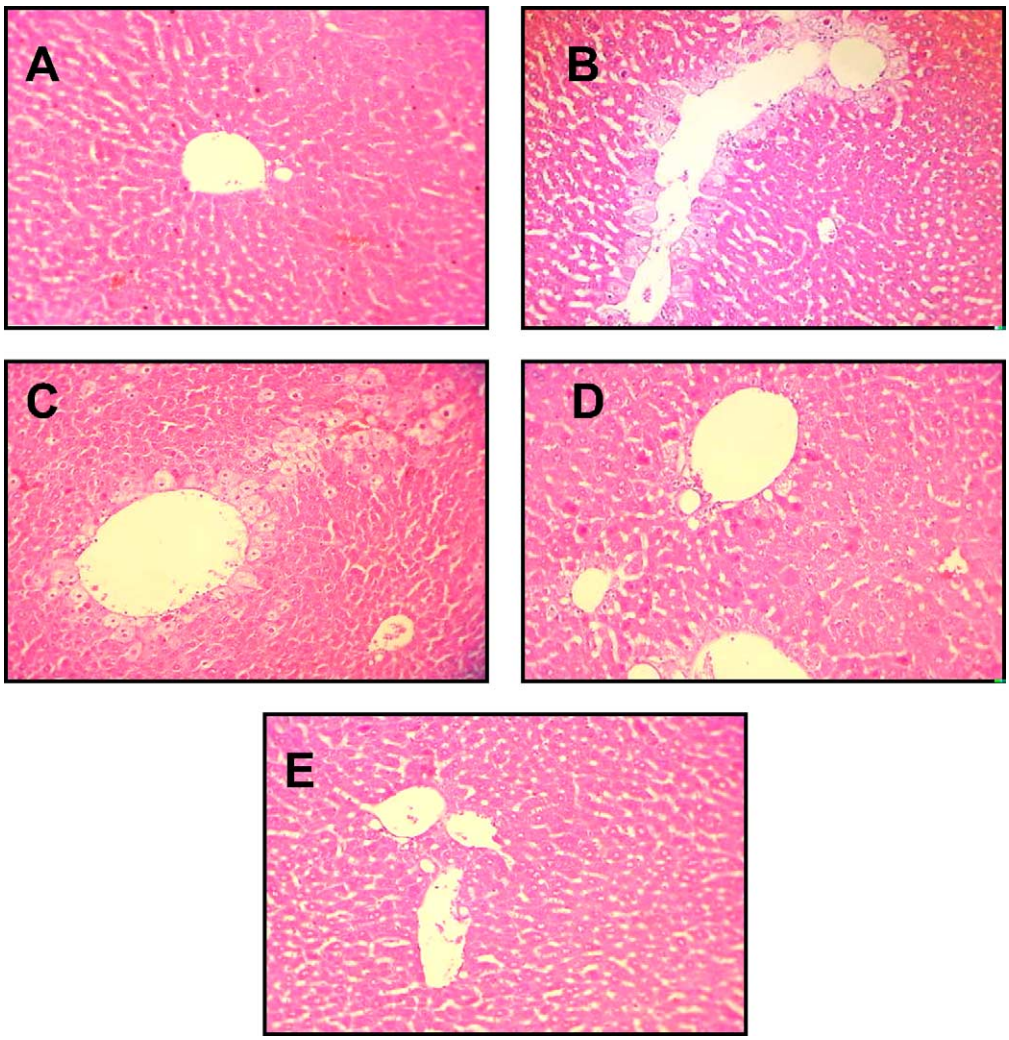

Fig. 3. Effects of CKS pretreatment on *t*-BHP-induced liver damage in rats. The rats were pretreated with CKS (0.5, 1, or 10 mg/kg, i.g.) once daily for three consecutive days. The control rats were given saline. Three hours after the final treatment, rats were treated with *t*-BHP (20 mg/kg, i.p.). The rats were sacrificed 24 h after the *t*-BHP administration. (A) Liver from rat treated with saline; (B) liver from rat treated with *t*-BHP; (C) liver from rat treated with CKS (0.5 mg/kg) plus *t*-BHP; (D) liver from rat treated with CKS (1 mg/kg) plus *t*-BHP; and (E) liver from rat treated with CKS (5 mg/kg) plus *t*-BHP X-100.

3.7. Histopathology of the liver

Histopathological studies 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. 3). According to microscopic examinations, severe hepatic lesions induced by *t*-BHP were remarkably reduced by the administration of CKS, which were in good agreement with the results of the serum aminotransferase activities and hepatic lipid peroxidation. Necrosis, which is a more severe form of injury was also markedly prevented and minimized by pretreatment with CKS. CKS (5 mg/kg) treatment alone did not cause any change in the liver histology (data not shown).

4. Discussion

Liver disease, especially viral and alcoholic hepatitis, occurs predominantly in the developing world (Simonsen et al., 1999; Loguercio and Federico, 2003) 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 (Mehendale et al., 1994; Stohs, 1995). Oxidants may generate lipid hydroperoxides, which subsequently decompose into alkoxyl and peroxyl radicals, which are able to oxidize cellular constituents (Halliwell and Gutteridge, 1999). 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 (Joyeux et al., 1990; Schiano, 2003).

The aim of this study was to evaluate the ability of CKS, with its inherent antioxidant activity, to have an effect on the cellular and DNA damage in rat primary hepatocyte cultures, and hepatotoxicity in rats caused by *t*-BHP-induced oxidative stress. In this study, we used *t*-BHP, which has frequently been employed as an oxidative stress inducer (Rush et al., 1985; Joyeux et al., 1990; Hwang et al., 1996; Soszynski and Bartosz, 1997) to induce oxidative hepatotoxicity in vitro and in vivo. The present study showed that *t*-BHP produced 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 CKS resulted in the protection of the hepatocytes against the *t*-BHP induced oxidative hepatotoxicities in a dose-dependent manner (Table 1). CKS also attenuated *t*-BHP induced cellular ROS production (Fig. 1) and DNA damage (Fig. 2) in the cultured hepatocytes. In addition, CKS exhibited the ability to quench DPPH and superoxide radicals effectively (Table 2).

An antioxidant action has been reported to play an important role in the hepatoprotective activity of many plants (Hwang et al., 1996; Luper, 1998). Silymarin, obtained from the plant *Silybum marianum* has been intensively studied, because of its hepatoprotective effects (Wellington and Jarvis, 2001). Silymarin is now used clinically in the treatment of many liver diseases (Giese, 2001). In our previous in vitro experiments, we showed that CK acts as an antioxidant, in such situations as in vitro lipid peroxidation, and has a strong capacity to quench superoxide radicals (Lee and Jeong, 2002). Further evaluation, in this study, demonstrated that CKS, the saponin fraction obtained from CK, was the component responsible for the antioxidant activity derived from CK in the cultured rat hepatocytes and rat liver. This provides biological evidence supporting the effectiveness of CKS for liver disorders. In previous studies, saponin compounds, which are widely distributed in plants, were considered to play an important role as dietary antioxidants for the prevention of oxidative liver damage (Jun et al., 2002; Yoshikawa et al., 2003).

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 (Thornalley et al., 1983; Rush et al., 1985). 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

(Altman et al., 1994). In the hepatotoxicity and lipid peroxidation experiments, CKS 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 (Table 1). 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 (Blair, 2001). These data not only confirm the hepatoprotective and anti-lipid oxidation activities of CKS, but also support the proposed function of CKS as a scavenger of radicals that ultimately attenuates the resulting cytotoxicity and genotoxicity. However, we cannot rule out the possibility that CKS may interact directly with *t*-BHP in the medium, in such a way as to alleviate the cell damage caused by *t*-BHP. Furthermore, in our previous study, we showed that the preventive effects of CK against acetaminophen and carbon tetrachloride-induced hepatotoxicity were associated with its inhibitory effect on the microsomal cytochrome P450-mediated bioactivation of these hepatotoxicants (Lee et al., 2001; Lee and Jeong, 2002). Therefore, the protective effects of CKS on *t*-BHP-induced oxidative hepatotoxicity, at least in part, could be associated with the inhibition of the 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 cell integrity and DNA damage and result in cell injury (Halliwell and Gutteridge, 1999). A relationship between GSH concentration and the extent of hepatocytes inhury has been demonstrated in experiments in which the hepatic concentration of GSH was altered by toxin treatment. This study showed that *t*-BHP reduced the level of GSH, an index of oxidative stress, in cultures hepatocytes and in the rat liver, and that treatment with CKS significantly prevented the *t*-BHP induced depletion of GSH levels, but that CKS alone did not affect the cellular level of GSH (Tables 1 and 3). In our previous in vivo studies, we also showed that pretreatment with CK significantly prevented the depletion of GSH content in the liver of acetaminophen or carbon tetrachloride-intoxicated mice, whereas, the hepatic GSH levels and glutathione-*S*-transferase activities were not affected by treatment with CK alone (Lee et al., 2001; Lee and Jeong, 2002). The formation of lipid peroxidation products and the depletion of the GSH level implicate oxidative stress. Therefore, the results showed that CKS attenuated the oxidative stress induced by *t*-BHP (Table 2).

Oxidative stress appears to be involved in the mechanism of various types of cell injury (Spector, 2000). 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 (Stohs, 1995). The DCF assay is widely used for measuring overall ROS formation in biological systems (Wang and Joseph, 1999). When rat 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 CKS, the generation of excess ROS by *t*-BHP was strongly reduced, and this resulted in the DCF fluorescence being significantly decreased. The ROS reducing effect of CKS was comparable to that exerted by *N*-acetylcysteine (data not shown). This antioxidant agent is well-known to act as a scavenger of ROS and as a precursor of GSH by providing cysteine (Cotgreave, 1997). There is accumulating evidence that the increased intracellular ROS occurring in cells act as a common mediator of DNA damage caused by physical or chemical stimuli, such as hydrogen peroxide or nitric oxide (Collins, 1999; Termini, 2000). 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 effect of CKS on this process. The results showed that *t*-BHP increased DNA damage through increased intracellular ROS production (Figs. 1 and 2), since the increased DNA damage induced by *t*-BHP was completely abolished using *N*-acetylcysteine (data not shown). The significant blockade of the *t*-BHP-induced DNA damage brought about by the treatment with CKS (Fig. 2), indicates that the increase of ROS production mediates the observed DNA-damage caused by *t*-BHP. It was reported

that, in isolated rat hepatocytes, *t*-BHP induced unscheduled DNA synthesis, a marker of DNA damage (Hwang et al., 1996). 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 CKS exerts an antioxidant action inside the cells, and is 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 CK-treated hepatocytes. The fact that DNA represents an important target molecule of the reactive species generated during oxidative stress prompted the present investigation. This study shows that the mechanism of action of CKS could be essentially a free radical scavenging mechanism. In addition, CKS exhibited an effective ability to quench DPPH and superoxide radicals (Table 2). The results obtained in this study indicate that the effects of CKS can be explained by free radical scavenging. The in vivo study also showed that the oral pretreatment with CKS significantly lowered *t*-BHP-induced serum levels of the hepatic enzyme markers (ALT and AST) and reduced oxidative stress of the liver, as determined by evaluating the lipid peroxidation and GSH levels (Table 3). Histopathological evaluation of the rat livers also revealed that CKS reduced the incidence of liver lesions and inflammation, including hepatocyte swelling, leukocyte infiltrations and necrosis, induced by *t*-BHP (Fig. 3). It has been proposed that ROS generated in inflamed tissue can cause injury to target cells (Maeda and Akaike, 1998). We therefore suggested that CKS could inhibit *t*-BHP-induced oxidative damage in the liver by blocking the cytotoxicity induced by *t*-BHP and decreasing the progressive damage caused during inflammation.

In conclusion, this study demonstrated that CKS showed protective effects against *t*-BHP-induced oxidative hepatotoxicity in rat primary cultured hepatocytes and the rat liver. One of the mechanisms contributing to its effectiveness as an antioxidant may involve the quenching of free radicals. However, further studies are needed in order to clarify the exact mechanism(s). Recently, much attention has been focused on the protective biochemical function of naturally occurring antioxidants in biological systems, and on their mechanism of action. This study therefore provides biological evidence supporting the use of CKS for the treatment of liver disorders and suggests that CKS could function as a chemopreventive agent in the living system.

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