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Transfected HepG2 Cells for Evaluation of Catechin Effects on Alcohol-Induced CYP2E1 Cytotoxicity

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Abstract To evaluate the toxicological properties of human cytochrome P450 2E1 (CYP2E1) induced by ethanol and possible protective effects of various green tea catechins on alcohol-induced toxicity, transfected HepG2 cells that stably and constitutively express human CYP2E1 were established using the recombinant retroviral expression vector. Exposure of the CYP2E1-expressing HepG2 cells to high concentration of ethanol (200 mM) for 5 days resulted in a more than 50% increase of cytotoxicity, assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction, lactate dehydrogenase (LDH) release, and reactive oxygen species (ROS) production, and loss of normal morphology, in comparison with HepG2 cells containing control vector. Treatment of the cells with various catechins increased cell viability by more than 2-fold. (−)-Epicatechin gallate and (−)-catechin gallate at the lowest concentration (5 μM) attenuated cell death induced CYP2E1 by 60–65%. Therefore, the results showed that the catechins, including epimerized catechins, have strong protective effects against alcohol-induced CYP2E1 toxicity, and it is correlated with antioxidant effect.

Key words: HepG2 cells, cytochrome P450 2E1, ethanol, catechins

Alcoholic liver disease is the most common form of liver disorder in the western world, and it is continuously increasing in eastern countries [6]. Chronic ethanol consumption enhances oxidative stress in the liver, and the role of oxidative stress has been considered as one of the key mechanisms responsible for alcoholic liver injury [16, 17, 22, 36]. Ethanol-induced cytochrome P450 2E1 (CYP2E1) appears to be correlated with the generation of oxidative stress and formation of reactive metabolites. HepG2 cells have served as models for studying the hepatotoxicity of chemicals and drugs, although their usefulness is partly limited owing to their low ability to express CYP2E1 [10, 11, 35]. Many research groups have approached the transient and stable transfection of CYP2E1 to mammalian cell lines to clarify drug interaction including ethanol [20, 24, 28, 32, 35, 37]: Transduction of human CYP2E1 cDNA to a HepG2 cell line and establishment of enzymatically active cells overexpressing CYP2E1 are essential for investigating the protective effects of appropriate antioxidant against alcohol-induced CYP2E1 toxicity *in vitro*.

Green tea is widely consumed in Asia, especially in Korea, Japan, and China. The major components of tea, such as (−)-epicatechin (EC), (−)-epigallocatechin (EGC), (−)-epicatechin gallate (ECG), and (−)-epigallocatechin gallate (EGCG), have been reported to have various physiological functions [1, 8, 35]. Although the mechanism underlying these effects is not fully elucidated, many studies have shown that the induction or inhibition of cytochrome P450 by tea components may have a significant contribution. Muto *et al.* [25] demonstrated the nonspecific inhibitory effects of tea polyphenols toward several human cytochromes P450 (CYPs). These studies suggested that inhibition of specific CYPs by green tea may be one of the protection mechanisms against genotoxicity. The antioxidant activities of individual tea catechins have been studied in the field of hepatoprotection against lead-induced cytotoxicity in HepG2 cells [8, 15], polysaturated fat and iron-induced hepatotoxicity in HepG2 cells overexpressing CYP2E1 [35], and in alcohol-induced oxidative stress in the rodent liver [30]. However, the protective effect of catechins against alcohol-induced oxidative stress in the HepG2 cell system expressing CYP2E1 has not yet been reported.

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In this study, we transduced human CYP2E1 cDNA into a HepG2 cell line to screen reliable antioxidants against alcohol-induced oxidative stress and also investigated the protective effect of various catechins against alcohol-induced liver cell damage.

MATERIALS AND METHODS

Cell Culture and Treatment

The 293GPG retrovirus packaging cell line was provided by Clontech (San Jose, CA, U.S.A.), and the HepG2 cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA, U.S.A.). The 293GPG cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), 0.3 mg/ml G418, 2 mg/ml puromycin, and 1 μg/ml tetracycline. HepG2 cells were grown in Minimum Essential Medium (MEM) containing 10% FBS and 1% penicillin/streptomycin antibiotics mixture (Gibco/BRL, Rockville, MD, U.S.A), and genetically engineered HepG2 cells were cultivated in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 20% FBS and 1 mg/ml G418.

Construction of the Expression Vector and Transfection

Human CYP2E1 cDNA was amplified from a human liver cDNA library (Clontech, Palo Alto, CA, U.S.A.) by PCR using P*fu* polymerase and primers (forward 5'-CCATATG-TCTGCCCTCG GAGTGAC and reverse 5'-AGTGCTTC-CAGCAGG AAG TGG). The full length of human CYP2E1 cDNA was inserted into the *Hin*dIII and *Nde*I restriction sites of modified pLNCX (inserted *Nde*I site) expression vector (Clontech) and mapped (Fig. 1). The HCYP2E1F-LNCX, a retroviral vector containing human CYP2E1 cDNA, was used to transfect the packaging cell line 293GPG by lipofectamin (Gibco/BRL), generating a stable transfected tool to produce CYP2E1 in the virus. The pLNCX contains elements derived from Moloney murine leukemia virus (MoMuLV) and Moloney murine sarcoma virus (MoMuSV), and is designed for retroviral gene delivery and expression. The 5' viral LTR in this vector contains viral promoter/enhancer sequences that control expression of the neomycin (G418) resistance gene for selection in HepG2 cells. Virus infection of HepG2 cells was carried out by supplying a medium previously collected and filtered. Infected HepG2 cells were seeded in a 100-mm culture dish, and resistant colonies were formed in IMDM containing 1 mg/ml G418 for selection. Colonies were isolated, grown on a large scale, and maintained in IMDM containing G418 (1 mg/ml) [28].

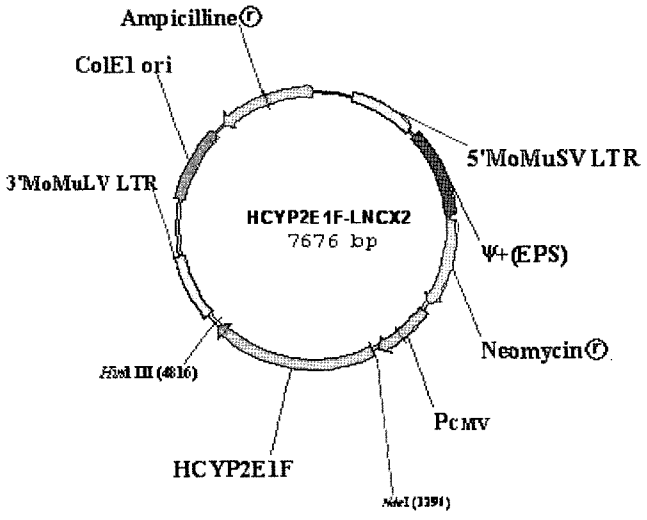

Fig. 1. Restriction map and construction of HCYP2E1F-LNCX.

RT-PCR

Total RNA was isolated by EasyBlue reagent (Intron, Seoul, Korea). A CYP2E1 5' primer (GCATCTCTTGCCTATCCTT) corresponding to positions 1,042–1,024, and a 3' primer (ATGGACCTACCTGGAAGGACAT) complementary to bases 353–374 were used for amplification of 690 bp of the CYP2E1 product. The reaction mixture was heated at 94°C for 2 min, followed by 40 cycles at 94°C for 30 s, 62°C for 30 s, and 72°C for 30 s, and extension at 94°C for 5 min [37]. RT-PCR products were separated on 1% agarose gels.

Western Blotting

Cells were lysed with Passive Lysis Buffer (Promega, Madison, WI, U.S.A.) and centrifuged at 15,000 rpm for 20 min at 4°C. Protein concentration was determined by the Bradford protein assay kit (Biorad, CA, U.S.A.). Protein separation was carried out on a 12% SDS-PAGE gel and transferred to nitrocellulose membrane. CYP2E1 was detected using goat anti-rabbit CYP2E1 (1:10,000), with goat anti-rabbit secondary antibody conjugated with horseradish peroxidase (1:15,000). Protein was visualized by chemiluminescence (Pierce, Rockford, IL, U.S.A.).

Enzymatic Analysis of CYP2E1

The catalytic activity of CYP2E1 in cells was determined with whole-cell lysates by *p*-nitrophenol (PNP) hydroxylation and *N*,*N*-dimethylnitrosamine (DMN) oxidation using colorimetric methods [10]. PNP oxidation was performed in a reaction system containing 0.1 M PBS (pH 7.4), 1 mM PNP, and 2 mg/ml cell lysates. The reaction was initiated with an NADPH-generating system (1 mM glucose 6-phosphate, 0.5 mM NADP+, and 1 U glucose-6-phosphate dehydrogenase) and stopped by the addition of 0.2 volume of 20% TCA solution after 60 min at 37°C. Final products were analyzed at 535 nm after centrifugation at 10,000 ×*g* for 5 min and addition of 0.5 volume of 2 M NaOH. To determine the oxidation of DMN, the reaction system was

the same as above, except for DMN (1 mM) in place of PNP. The reaction was initiated with the NADPH-generating system and stopped by the addition of 20% TCA after 60 min. After centrifugation at 3,000 ×g for 5 min, the aliquot of supernatant was added to 0.5 volume of Nash reagent [30% (w/v) ammonium sulfate, 0.4% (v/v) acetylacetone, 0.6% (v/v) glacial acetate] and incubated for 30 min at 50°C. The product was measured at 420 nm after cooling at room temperature.

Measurements of Ethanol Cytotoxicity

The cytotoxicity of ethanol was evaluated by formazan production and lactate dehydrogenase (LDH) release. To measure the formazan production, 5×10^4 of transfected cells were plated onto 24-well plates, and the respective treatments were initiated after 20 h of incubation. IMDM, containing 5% FBS without G418, was added, followed by ethanol treatment. Following exposure to ethanol for 5 days, 250 μl of 0.5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution in PBS was added, and the plates were incubated for 2 h. At the end of incubation, the medium was removed, and blue formazan formed was solubilized with DMSO. Absorbance at 570 nm was measured with background subtraction at 630 nm.

The LDH release was determined in transfected cells exposed to ethanol for 5 days. The medium was collected to determine LDH activity released in the medium, and cells were lysed to measure LDH activity in the cell. The *In Vitro* Toxicology Kit (Sigma) was used to determine LDH activity. LDH release was expressed as the percentage of LDH in the medium to the sum of medium LDH plus cell lysate LDH.

Determination of Reactive Oxygen Species

The intracellular reactive oxygen species (ROS) level was detected using fluorescence probe, 2',7'-dichlorofluorescein diacetate (DCF-DA): DCF-DA, which enters the cell, is cleaved to form DCF-H. Trapped DCF-H is then oxidized by oxygen free radicals to produce fluorescent DCF. Therefore, DCF-DA was added to the culture plates at a final concentration of 25 μM and incubated for 30 min at 37°C. The fluorescence intensity in the cells was measured on a spectrofluorometer (TECAN, Salzburg, Austria) with an excitation wavelength of 485 nm and emission wavelength of 530 nm.

Treatments with Various Catechins

To evaluate the effects of various catechins, cells were plated on 24-well dishes at a density of 5×10^4 cells/well without G418, and incubated for 20 h under normal culture condition. Stock solutions of various catechins were freshly prepared during the experiments. IMDM containing 5% FBS without G418 was added, and the cells were treated with various concentrations of catechins for 30 min before ethanol exposure. After 5-day consecutive treatments, the medium was removed, and cell viability was evaluated by MTT test. Absorbance at 570 nm was measured with background subtraction at 630 nm. Cell viability was expressed as a percentage of corresponding control.

Statistical Analysis

Data are expressed as means±SEM or S.D. Statistical analysis was performed using one-way ANOVA, followed by Student-Newman-Keul's test for multiple comparisons between treatment groups. A value of $p<0.05$ was considered to be significant.

Results and Discussion

Characterization of HepG2 Cell Lines Expressing Human CYP2E1

After transfection of the HepG2 cells and colony selection, the expression of mRNA and protein of CYP2E1 in cells were determined, using RT-PCR and Western blot analyses (Fig. 2). The clear and strong expression of CYP2E1 was detected only in HepG2/2E1 transfected cells, whereas HepG2/Cont cells did not express CYP2E1. In mRNA expression, β-actin was used as an internal control to evaluate the expression of CYP2E1. In Western blot analysis, HepG2/2E1 cells treated with phorbol 12-myristate 13-acetate (PMA) had enhanced expression of CYP2E1 protein. Wu and Cederbaum [32] reported that PMA increases CYP2E1 content by 3-fold via activation of a retroviral vector with no effect on CYP2E1 turnover or substrate stability. The increased expression of CYP2E1 by PMA treatment would be expected to enhance the toxicity of ethanol.

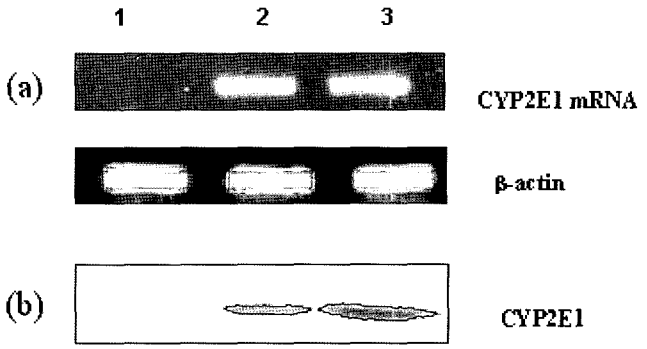

Fig. 2. RT-PCR (a) and Western blot (b) analyses of HepG2/cont and HepG2/2E1.
Lane 1, HepG2/Cont cells; Lane 2, HepG2/2E1 cells; Lane 3, PMA-treated HepG2/2E1 cells. (a) β-Actin was used as an internal control. (b) Western blot was carried out with 35 μg of S9 fraction protein. HepG2/2E1 cells in Lane 3 were incubated in the presence of 0.01 μg/ml of PMA for 48 h. HepG2/Cont is a control cell line containing pLNCX plasmid only. HepG2/2E1 cells were an established cell line overexpressing CYP2E1 in this study.

Table 1. Catalytic activities of CYP2E1 in HepG2/Cont and HepG2/2E1 cells.

	PNP[a]	DMN[b]
	nmol/min/mg protein	
HepG2/Cont[c]	0.076±0.001	0.189±0.001
HepG2/2E1[d]	0.152±0.002	0.449±0.007

Each value is mean±S.D. (n=3).
[a]*p*-Nitrophenol (PNP) and [b]*N*,*N*-dimethylnitrosamine (DMN) were the substrates to determine CYP2E1 enzyme activities.
[c]HepG2/Cont was a control cell line containing pLNCX plasmid only.
[d]HepG2/2E1 was an established cell line overexpressing CYP2E1 in this study.

Enzyme Activity of the Transfected HepG2 Cell

Since PNP and DMN are typical CYP2E1 substrates, PNP hydroxylation and DMN oxidation were determined to demonstrate the CYP2E1 activity of the transfected HepG2/2E1 cell line. The HepG2/2E1 cells are able to oxidize PNP to 4-nitrocatechol and DMN to formaldehyde. As shown in Table 1, the enzyme activities of PNP hydroxylation and DMN oxidation in whole cells were approximately 2–3-folds higher than the basal levels of the control cell line (HepG2/Cont), indicating that the HepG2/2E1 cells established in this study were enzymatically active. The PNP hydroxylation activity was similar to that of HepG2-CYP2E1 cells (0.162 nmol/min/mg S9 fraction) [37], a little lower than those of HepG2-CYP2E1 E43 and E47 cells (0.19 and 0.34 nmol/min/mg microsome) [9]. Using 7-hydroxycoumarin and transfected cell line, ER-181, Nakagawa *et al.* [26] showed that CYP2E1 enzyme activity was 102 pmol/10^6 cells.

Ethanol Cytotoxicity of Transfected HepG2 Cell Line Expressing CYP2E1

Alcoholic hepatotoxicity has been considered to be related to oxidative stress, which is caused by increased ROS and decreased antioxidants [18, 33, 36]. Alcohol-induced CYP2E1, in chronic ethanol consumption in particular, is one of the main sources of ROS production, and it is poorly coupled with NADPH-cytochrome P450 reductase [31, 34]. Free-radical production has been detected in ethanol-fed rat liver microsomes, brains [2, 12], and ethanol-added cell lines expressing CYP2E1 [33, 35, 36]. The CYP2E1-related ROS formations have been implicated to play an important role in the production of very reactive 1-hydroxyethyl radical (HER) from ethanol, which interacts with lipids, proteins, and biological systems [5, 23, 36]. In order to determine appropriate experimental conditions in the present study, ethanol toxicity was evaluated after 50, 100, and 200 mM ethanol treatment for 5 days: The toxicity of ethanol was evaluated by MTT assay, LDH release, and ROS formation. As seen in Table 2, the toxicity of ethanol increased as a function of time over a period of 1–5-day exposure. The MTT reduction was lowered by

Table 2. Cytotoxicity of ethanol in HepG2/Cont and HepG2/2E1 cells.

	Formazan production (A_{570}-A_{630})	LDH release (% leakage)	Fluorescence intensity (arbitrary unit)
HepG2/Cont	0.401±0.02	9.99±0.39	442.88±5.625
HepG2/2E1	0.230±0.04	30.10±0.07	957.13±74.346

Each value is mean±S.E.M. (n=5).

50%, and the LDH release increased 3-fold by ethanol treatment in HepG2/2E1 cells. The ROS production assessed by DCF-DA was higher in HepG2/2E1 cells, indicating that the formation of ROS generated by ethanol-induced CYP2E1 is one of the main reasons for the ethanol toxicity. Figure 3 shows the concentration dependency of ethanol toxicity in HepG2/2E1 cells. Cytotoxicity increased dependently in HepG2/2E1 cells, whereas no significant change was found in HepG2/Cont cells. The morphological changes were observed by light microscopy (10×20 magnification) after treatment with 200 mM ethanol for 48 h. As shown in Fig. 4, HepG2/2E1 cells lost their normal shape, became more round, and showed unclear cell membranes. Furthermore, the cell number was markedly reduced by ethanol treatment. These results indicate that ethanol was more toxic to HepG2/2E1 cells than to HepG2/Cont cells.

The ethanol concentration (200 mM) applied in the present study is very high, compared with blood alcohol concentration of heavy alcoholics; however, the concentrations of ethanol used in *in vitro* studies are generally higher than those required to produce similar effects *in vivo*. Many studies found the cytotoxic effects of ethanol at concentrations

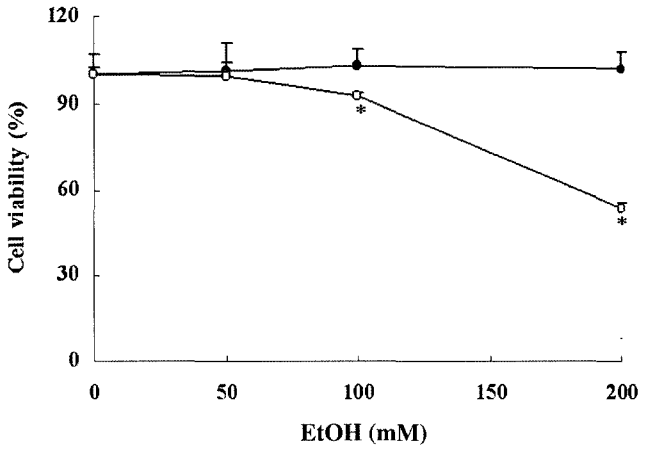

Fig. 3. Ethanol toxicity of HepG2/Cont and HepG2/2E1 cells.
Cells were plated on 24-well dishes at a concentration of 5×10^4 cells/well and incubated in IMDM containing 20% FBS for 24 h. Ethanol was added at 50–200 mM to fresh medium containing 5% FBS with 0.01 μg/ml PMA for 5 d. Values are means±SEM of separate experiments in triplicate. The asterisks (*) indicate significant differences between HepG2/Cont cells (●), HepG2/2E1 cells (○) (*p<0.01 versus HepG2/Cont cells).

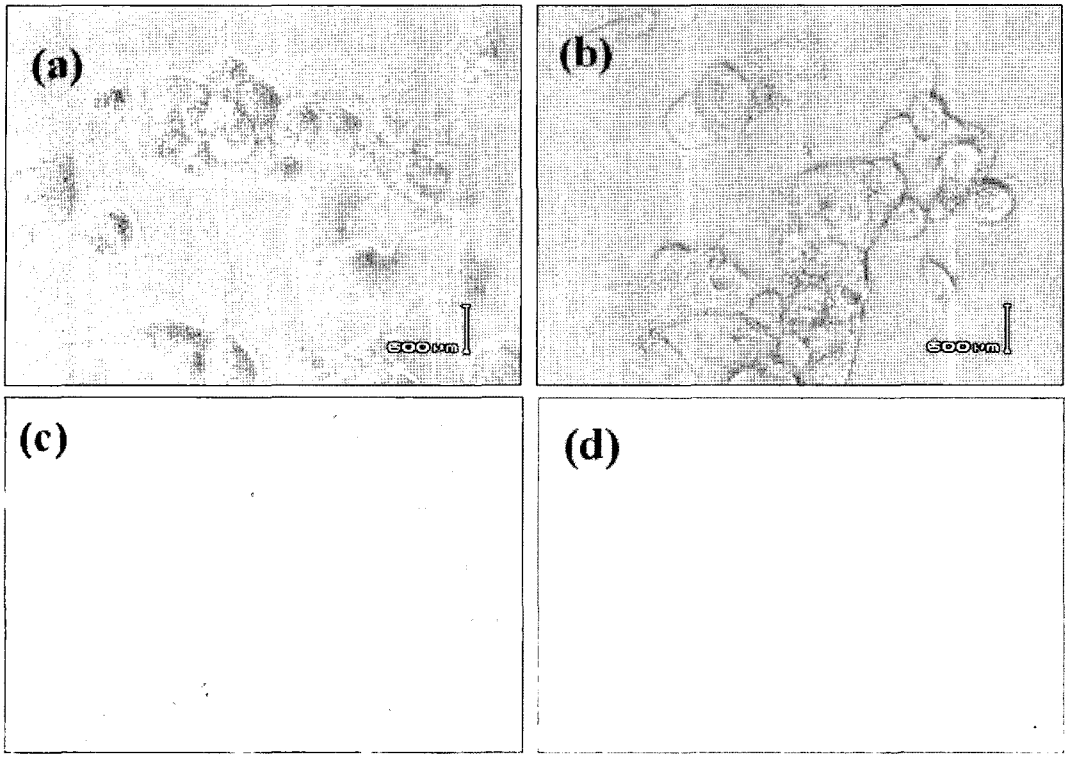

Fig. 4. The morphology of HepG2/Cont and HepG2/2E1 cells in the absence and presence of ethanol.
Cells were exposed to 200 mM ethanol for 48 h with 0.01 μg/ml PMA (10×20 magnification). (a) HepG2/Cont cells; (b) HepG2/Cont cells plus ethanol; (c) HepG2/2E1 cells; (d) HepG2/2E1 cells plus ethanol.

from 40 mM to 200 mM, and a few studies observed the toxicity at lower ethanol concentrations (40–60 mM) with 30–40% loss of viability in HepG2 cells [4, 7, 19, 26]. However, we could not observe any significant loss of cell viability after 5-days of treatment with 100–200 mM ethanol in HepG2/Cont cells.

Protective Effects of Various Catechins Against Ethanol Toxicity

The possible protective effect of various catechins on ethanol toxicity was evaluated in HepG2/2E1 cells (Fig. 5). With the exception of (−)-CG and (−)-ECG that prevented cell death by 60–65%, most catechins were not effective at the lowest concentrations used (5 μM). However, all catechins showed significant protective effects at 25 μM ($p<0.01$), and (−)-EGCG was the most effective. At the highest concentrations of catechins examined (50 μM), the protective effects were rather decreased except for (−)-CG. Moreover, ROS production in HepG2/2E1 cells was significantly lowered (over 50%) by all catechins at 25 μM and 50 μM (data not shown). These results suggest that the possible mechanism of catechins to protect against ethanol cytotoxicity is the inhibition of ROS production, especially at low concentration, and that the hydroxyl group of the B-ring and the galloyl moiety of catechins [i.e., (−)-ECG, (−)-CG] are the main contributors to their antioxidative activities [3]. Furthermore, the present study clearly showed that epimerized catechins as well as main catechins were very effective protectors against ethanol-induced CYP2E1 toxicity. Consumption of canned and bottled tea drinks is increasing in Asian countries. These products are generally

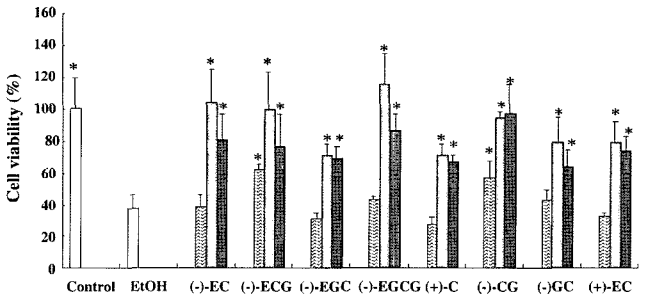

Fig. 5. Dose-dependent effects of catechins against CYP2E1-dependent ethanol toxicity.
HepG2/2E1 cells in 24-well plates were incubated with 200 mM ethanol together with 5, 25, or 50 μM various catechins for 5 d. Each value is mean±SEM. The asterisks (*) indicate significant differences from ethanol treatment only (n=5, *$p<0.01$, ethanol vs. ethanol plus catechins in HepG2/2E1 cells). No addition (□), 5 μM (▨), 25 μM (▩), and 50 μM (■) of catechins. EtOH, ethanol; (−)-EC, (−)-epicatechin; (−)-ECG, epicatechin gallate; (−)-EGC, epigallocatechin; (−)-EGCG, (−)-epigallocatechin gallate; (+)-C, (+)-catechin; (−)-CG, (−)-catechin gallate; (−)-GC, gallocatechin; (+)-EC, (+)-epicatechin.

processed at above 100°C for several minutes, and it is known that, during processing, considerable amounts (around 50%) of catechins are epimerized at the 2-position, and (−)-gallocatechin (GC), (-)-catechin gallate (CG), and (−)-gallocatechin gallate (GCG) are formed [13].

In this *in vitro* study, we demonstrated the protective effects of various catechins against alcohol-induced CYP2E1 toxicity, whereas other research groups studied the protective effects of catechins against arachidonic acid (AA) or AA plus iron for induction of CYP2E1 cytotoxicity. Ethanol is a weaker prooxidant than AA or AA plus iron, and also requires a longer exposure time to observe the toxicity [14, 35]. However, direct ethanol treatment is the most effective model to evaluate ethanol toxicity in humans. During 5 days of treatment periods, we did not observe any significant ethanol toxicity, when treated with 5–25 μM catechins (data not shown).

In summary, the present study demonstrates that human CYP2E1-overexpressing HepG2/2E1 cells appear to be a useful *in vitro* model to characterize the toxicological properties of ethanol-induced CYP2E1, and the possible protection provided by chemicals. The catechins, including epimerized catechins, have strong protective effects against alcohol-damaged liver, and it is correlated with an antioxidant effect.

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