pISSN 1229-1153 / eISSN 2465-9223 *J. Food Hyg. Saf.*
Vol. 31, No. 1, pp. 1~7 (2016)
http://dx.doi.org/10.13103/JFHS.2016.31.1.1

Journal of Food Hygiene and Safety
Available online at http://www.foodhygiene.or.kr

Protective Effect of Green Tea Extract and EGCG on Ethanol-induced Cytotoxicity and DNA Damage in NIH/3T3 and HepG2 Cells

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(Received February 3, 2016/Revised February 14, 2016/Accepted February 19, 2016)

ABSTRACT - In the present study, our aim was to determine whether green tea extract (GTE) and its major constituent, epigallocatechin-3-gallate (EGCG) have a protective effect on ethanol-induced cytotoxicity and DNA damage in NIH/3T3 and HepG2 cells. The cell viability and DNA single strand breaks were examined by MTT assay and alkaline single cell gel electrophoresis (Comet assay), respectively. Ethanol decreased the cell viability and also increased DNA single strand breaks in a concentration-dependent manner. On the other hand, GTE showed the protective effect of cytotoxicity and DNA damage induced by ethanol in both cell lines. GTE and EGCG, were found to possess the anti-oxidative and anti-genotoxic activities by evaluation with DPPH test, LDL oxidation assay, oxidative DNA damage assay and 8OH-2'dG generation test. These results were also verified by the experimental results demonstrating the lower cytotoxicity and genotoxicity of commercial green tea liqueur compared to pure ethanol in same concentration. Thus it is concluded that the supplementation of GTE or EGCG may mitigate the ethanol-induced cytotoxicity and DNA damage.

Key words : Ethanol, Green tea extract, EGCG, cytotoxicity, DNA damage, chemoprevention

Ethanol-induced liver disease may be caused by oxidative stress produced by ethanol metabolism[1]. It was previously demonstrated that ethanol degradation in the liver transcriptionally induced the molecules associated with intracellular reactive oxygen species (ROS) generation, and activated the enzyme activities of cytochrome P450s[2,3] (Cederbaum, 2001; French, 2001). ROS generated in the liver produces lipid peroxidation, protein oxidation and DNA oxidative damage[4,5]. These cellular states called oxidative stress may eventually induce cellular necrosis or apoptosis[6]. Thus, ROS production and oxidative stress in liver cells are the main cause of acute and chronic alcoholic liver disease (ALD)[7]. Moreover, it is also well-known that ethanol reduces the cellular level of antioxidants scavenging ROS[8]. Therefore, it is reasonably thought that antioxidants having ROS scavenging property and/or increasing antioxidant levels in the cells may be beneficial to protect against alcohol-induced liver damage. In the present investigation, cytotoxicity and DNA damage provoked by ethanol-induced oxidative stress are compared with green tea extract (GTE) and related test compounds. And the protective effect of green tea extract and its major constituent, epigallocatechin-3-gallate (EGCG), are studied to find the potential protectants against ethanol-induced toxicity. The present study also provides the fundamental data for the protection against oxidative stress-induced toxicity.

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Materials and Methods

Chemicals

Ethanol, acetaminophen, vitamin C, Trolox, EGCG, vitamin E, butyl hydroxyaled toluene (BHT), low density lipoprotein (LDL) and DMSO are products of Sigma Chem. GTE was prepared from green tea (*Thea sinensis L., Theaceae*) cultivated in Bosung area in Chonnam province, Korea. From our previous study, this green tea (Woojeon® Daehandawon, Bosung, Korea) showed the highest content of total polyphenols and strongest antioxidant activities among five greens teas tested by harvest time[9]. Therefore, we decided to use it for recent studies.

Dried green tea (100 g) was extracted in 1 L of 100% ethanol for 3 days in room temperature and the extract was filtered and dried under vacuo. The yield of GTE was about 10.5% (w/w). Total polyphenol content in the extracts was determined by the Folin-Ciocalteu method[10]. The results were expressed as gallic acid equivalent for total polyphenols. The content of total polyphenols was 32.3% in the GTE[9], GTE and other test compounds were dissolved in DMSO and final concentration of DMSO was adjusted to

1% (v/v) and the same amount of DMSO was used for the control experiment. Commercial green tea liqueur (16% ethanol, Nokchaju®) was purchase from Millim Ind. Co. Ltd. (Suncheon, Korea).

Cell culture

NIH3T3 cells and HepG2 cells, human liver cancer cells, were purchased from ATCC (USA). NIH3T3 cells were routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO Co.) containing 10% fetal bovine serum (FBS) (GIBCO), 1% L-glutamine and 1% penicillin-streptomycin. HepG2 cells were cultured in Minimum Essential *Media* (MEM) containing 10% FBS (GIBCO), 1% L-glutamine and 1% penicillin-streptomycin.

Ethanol-induced cell cytotoxicity

MTT bioassay was used to measure the cytotoxic effects and protective effects against ethanol-induced toxicity[11]. In brief, cells was cultured in 25,000/well in 80-90 μL under CO_2 incubator for 24 h, and several concentrations of ethanol were added with/without ethanol in the presence/or absence of test compounds. The final volume of each culture was 100 μL. After 20 h incubation, MTT (15 μL) as added and incubated further for 4 h. Media was aspirated and DMSO (200 μL/well) was added and the absorbance was measured at 570 nm.

DNA damage (Comet assay)

To evaluate DNA damage, Comet assay using NIH3T3 and HepG2 cells was carried out[12,13]. DNA damage with ethanol treatment in the presence/or absence of antioxidants was examined. In brief, 1.5×10^6 cells were inserted into each well of 6-well culture plate and incubated at 37°C for 24 h.

Then, ethanol with/without antioxidants was treated and further incubated for 45 min. Media was removed and 3 mL fresh media was added and further incubated for 1 h. After adding 1 mL trypsin-EDTA, cells were harvested and centrifuged at 1,100 rpm for 3 min after adding 2 mL media.

The supernatants were discarded and 0.5% LMPA (low melting point agarose) (300 μL) was added to obtain cell suspension. This suspension was dropped on the pre-coated slide with 0.65% NMPA (normal melting agarose) and the slide was covered with cover slide. The slide was left on ice for 30 min and cover slip was removed. A final layer of agarose (130 μL of 0.5% LMPA) was applied in the same way. The slide without coverslip was immersed in ice-cold lysis solution (10 mM Tris, pH 10.0, containing 2.5 M NaCl, 100 mM EDTA, 10% DMSO and 1% Triton X-100) at 4°C for 30 min.

Electrophoresis was carried out in a tank containing 300 mM NaOH, 1 mM EDTA, pH 13.0 for 15 min under 25 V and 300 mA. Slides were then transferred to 0.4 M Tris buffer (pH 7.5), washed three times and gently dried. Ethidium bromide (2 mg/mL) was dropped onto the gel to stain DNA. Slides were examined at X400 magnification using a BH2 fluorescence microscope (Olympus, Japan) equipped with a 20BG-W2 dichromatic mirror (excitation filter: 515 nm, barrier filter: 590 nm). Image analysis was performed with the software Komet (version 5.5, Kinetic Imaging, Liverpool, UK) on 50 randomly selected cells. DNA damage was quantified by the increase of the tail moment, which was defined as a product of comet length (TL) and Olive tail moment (OTM) according to the original procedure[14].

Free radical scavenging effect

To examine free-radical scavenging activity, DPPH (1,1-diphenyl-2-picryl hydrazil) assay was used[15]. The radical scavenging activity was routinely determined using 60 mM DPPH. Absorbance was measured at 520 nm after incubation of test sample at 37°C for 30 min.

LDL oxidation

For evaluating antioxidative activity against low density lipoprotein (LDL) formation induced by lipid peroxidation, thiobarbituric acid (TBA) assay was used[16]. Test compounds (10 μL) was added to the solution of 10 μL LDL (2 μL/μL), 470 μL NaCl (150 mM) and 10 μL copper sulfate (100 μM). And final volume was adjusted to 500 μL. The solution was incubated at 37°C incubator for 3 h. To stop the reaction, 25 μL BHT ethanol solution (4%) was added to the 400 μL reaction solution and the final solution was heated at 95°C for 50 min after adding 500 μL phosphotungstic acid (10%) and 250 μL thiobarbituric acid (0.7%) dissolved in 0.5M sulfuric acid. Then the solution was cooled down with running water and 500 μL n-butanol was added, vortexed and centrifuged at 4,000 rpm for 15 min. And the absorbance of the supernatant was measured at 535 nm.

Formation of 8-OH-2'-deoxyguanosine (8-OH-2'dG)

Calf thymus DNA (1 mg/mL in 14 mM Na_2HPO_4, pH 7.4) (100 μL) was incubated at 37°C for 3 h in the presence or absence of test compounds. For the positive reference compounds, 1 mM H_2O_2 and 1 mM $FeCl_2$ were used. After incubation, 10 μL sodium acetate (3 M) and 2.5 ml ethanol were added and the tubes were maintained at −80°C for 1 h. After lysis, the pellet was obtained by centrifugation at 3,000 rpm for 15 min.

To the pellet, 10 mM Tris (pH 7.4 containing 1 mM EDTA) 0.3 mL, 0.5 M NaOAc 30 μL and 0.25 M $MgCL_2$ 10 μL were added and the solution was incubated with 10 μL nuclease P1 (0.5 mg/ml) at 50°C for 30 min, followed by 1 h incubation at 37°C after adding 10 μL 1 M Tris (pH 10.6) and 10 μL alkalne phosphatase (0.1 U/μL). Then the

solution was filtered with 0.2 um filter after adding 4 μL acetic acid (5.8 M).

The 8-OH-2'dG and 2'-dG levels were analyzed by HPLC with an electrochemical detector (ESA, model LC-4C, West Lafayette, IN, USA). A separation was carried out on a stainless steel C-18 ODS column (250 × 4.6 mm i.d., 5 mm spherical particle, Shimadzu, Japan), with 100 mM sodium acetate in 5% methanol (pH 5.2 with phosphoric acid) as a mobile phase at 1.0 mL/min. Oxidative damage was expressed as the molar ratio of 8-OH-2'dG to 10^5 molecules of deoxyguanosine (2'dG), which was calculated from the absorbance at 260 nm[17].

Statistical analysis

Statistical analysis was performed using the IBM® SPSS® Statistics, version 21. Student's t-test and one-way ANOVA with Dunnett's multiple comparison test. Significant different ($p < 0.05$, $p < 0.01$) compared to control.

Results

Ethanol-induced cytotoxicity and protection of GTE

As shown in Fig. 1, ethanol concentration-dependently exhibited cytotoxicity at 0-4.6% and at 4.6%, ethanol treatment showed 73.6% and 69.0% viability on NIH3T3 and HepG2 cells, respectively. There is no significant difference in cytotoxicity between these two cell lines. For further study, the concentration of 1, 2, and 4% ethanol may be used to evaluate cytoprotective effects of antioxidants.

Fig. 2a (NIH3T3) and 2b (HepG2) showed the cytoprotective effects of antioxidants (0.025-0.1%) against ethanol-induced cytotoxicity at the concentrations of ethanol (1.0, 2.0, 4.0%). Compared with ethanol-treated group, GTE treatment exhibited high cytoprotective effects. Among the antioxidants used, vitamin C showed highest protective effect, Trolox and BHT being relatively weak. Moreover, BHT (0.1%) rather increased cytotoxic effects by ethanol. These patterns were similarly observed in both cell lines.

Fig. 3 showed that GTE and vitamin C possess concentration-dependently protective effect against ethanol-induced cytotoxicity as expected. It is observed that GTE (0.2-0.6%) showed higher protective activity than those of vitamin C (0.05-0.2%).

Ethanol-induced DNA damage and protection of GTE

Fig. 4 clearly showed that ethanol treatment concentration-dependently increased DNA damage. In particular, at 0-2.5%, ethanol increasingly exhibited DNA damage and these effects are almost same in two different cell lines. At 2.5% ethanol, Comet assay demonstrated OTM 33.4 and 29.6 in NIH3T3 and HepG2 cells, respectively. However, 4.6% ethanol showed much more toxicity and changed cell morphology, and eventually OTM was decreased.

Under these experimental conditions, effects of GTE and

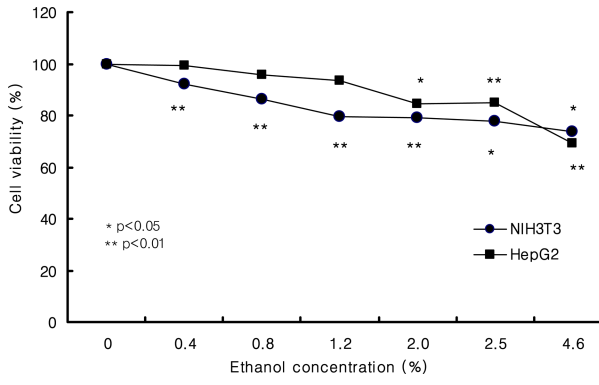

Fig. 1. Ethanol induced cytotoxicity in NIH/3T3 and HepG2 cells. 25,000 cell/well were seeded in each well of 96-well plates. MTT assay was performed in three independent of triplicate experiments. Results are expressed as mean value (%). Treated groups are compared to solvent control. Significant values are expressed by Student's t-test (*$p < 0.05$, **$p < 0.01$).

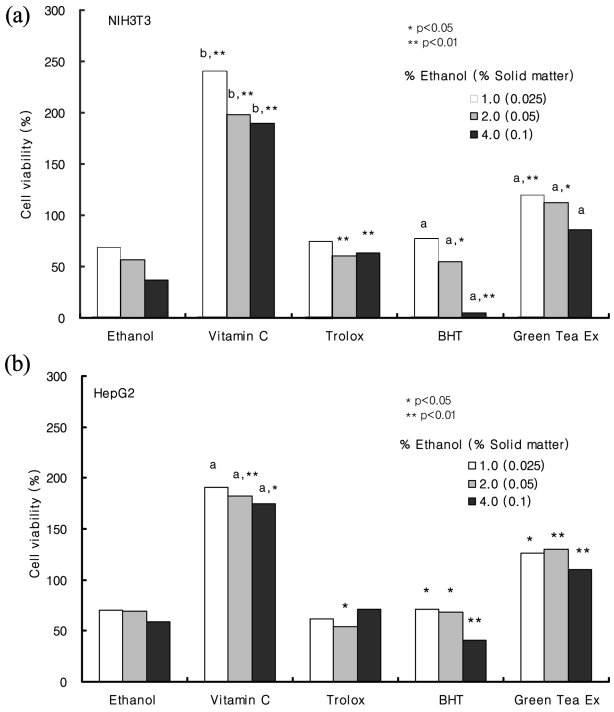

Fig. 2. Protective effect of GTE and some antioxidants on ethanol induced cell cytotoxicity in a) NIH/3T3 cells and b) HepG2 cells. 25,000 cell/well were seeded in each well of 96-well plates. MTT assay was performed in three independent of triplicate experiments. Results are expressed as mean value (%). Treated groups are compared to ethanol control. Significant values are expressed by Student's t-test (*$p < 0.05$, **$p < 0.01$) and One-way ANOVA and Dunnett's test ($^a p < 0.05$, $^b p < 0.01$). Solid matter means non-volatile material left after evaporation.

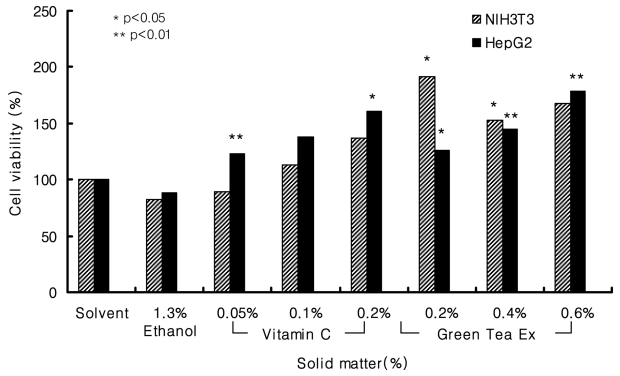

Fig. 3. Concentration dependent effect of GTE and vitamin C on ethanol induced cell cytotoxicity in NIH/3T3 and HepG2 cells. 25,000 cell/well were seeded in each well of 96-well plates. MTT assay was performed in three independent of triplicate experiments. Results are expressed as mean value (%). Treated groups are compared to each ethanol control. Significant values are expressed by Student's t-test (*p < 0.05, **p < 0.01). Solid matter means non-volatile material left after evaporation.

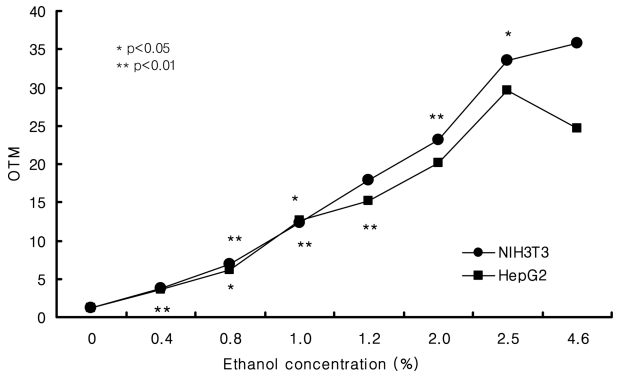

Fig. 4. Ethanol induced DNA damage in NIH/3T3 and HepG2 cells. 1.5×10^6 cell/well were seeded in each well of 6-well plates. Comet assay was performed in triplicate experiments. Results are expressed as mean value of olive tail moment (OTM). Treated groups are compared with solvent control. Significant values are expressed by Student's t-test (*p < 0.05, **p < 0.01).

vitamin C were measured. As shown in Fig. 5, GTE (0.2-0.6%) and vitamin C (0.05-0.2%) significantly inhibited DNA damage induced by 1.3% ethanol treatment in NIH3T3 and HepG2 cells. Even though all concentration groups tested with GTE and vitamin C showed the inhibition of DNA damage, it is worth to note that in HepG2 cells, GTE and vitamin C inversely protected DNA damage at 0.2-0.6% and 0.05-0.2%, respectively. The protective effects of GTE and vitamin C in HepG2 cells were highest at lowest concentration tested by 0.2% for GTE and 0.05% for vitamin C.

The anti-oxidative and anti-genotoxic effects of GTE and EGCG

For finding the active principle(s) of GTE on the protective effects against cytotoxicity and DNA damage induced by ethanol treatment, anti-oxidative action of EGCG, one of well-known major constituents, was initially examined. As shown in Fig. 6a, GTE showed significant inhibition of DPPH assay and LDL oxidation by 56.0% ($p < 0.05$) and 94.8% ($p < 0.01$), respectively at 150 μg/mL. EGCG also showed significant inhibition of DPPH assay and LDL

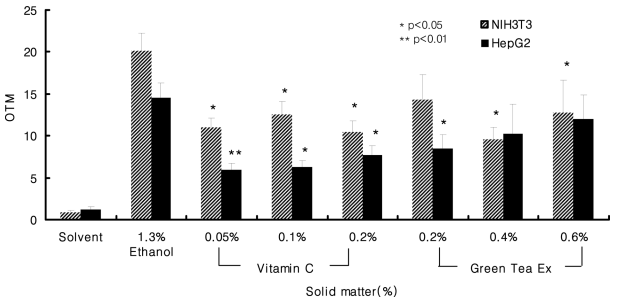

Fig. 5. Protective effect of GTE and vitamin C on ethanol induced DNA damage in a) NIH/3T3 cells and b) HepG2 cells. 1.5×10^6 cell/well were seeded in each well of 6-well plates. Comet assay was performed in triplicate experiments. Results are expressed as mean value ± SD of olive tail moment (OTM) from triplicate experiment. Treated groups are compared to each ethanol control. Significant values are expressed by Student's t-test (*p < 0.05, **p < 0.01). Solid matter means non-volatile material left after evaporation.

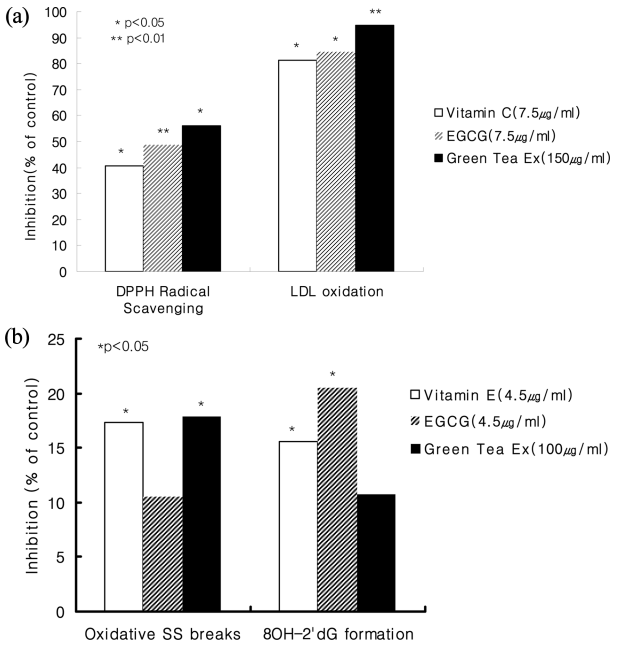

Fig. 6. (a) Antioxidant activities of GTE and EGCG on the DPPH radical scavenging effect and LDL oxidation inhibition. (b) Inhibition of H_2O_2-induced oxidative single strand (SS) breaks and 8OH-2'dG formation. All data are expressed as mean value ± SD from triplicate experiment. Treated groups are compared to ethanol control. Significant values are expressed by Student's t-test (*p < 0.05, **p < 0.01).

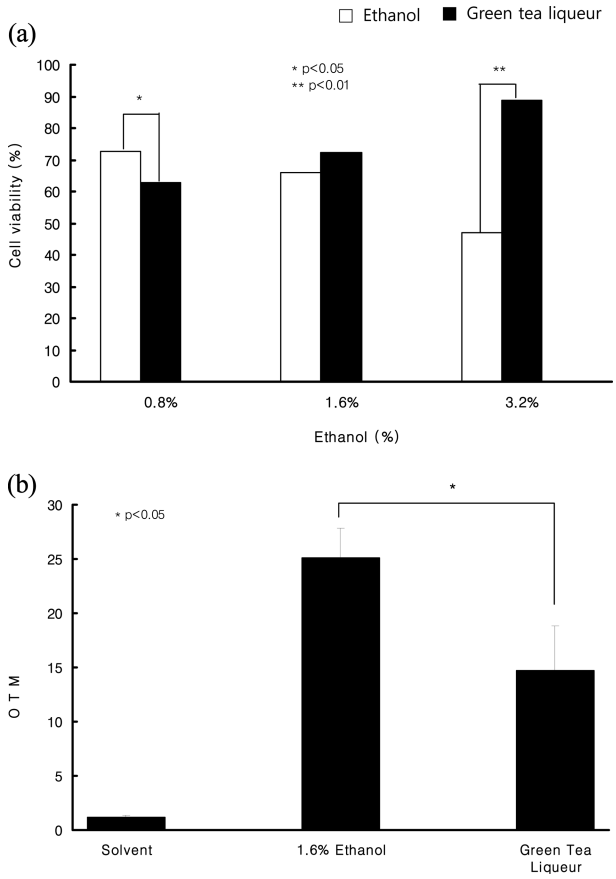

Fig. 7. Comparison of commercial green tea liqueur and pure ethanol with same ethanol concentration on the cell viability by (a) MTT assay and on the single strand breaks by (b) Comet assay. MTT assay was performed in three independent of triplicate experiments. Comet assay was performed in triplicate experiments. Results are expressed as mean value ± SD. Green tea liqueur groups are compared to each pure ethanol control. Significant values are expressed by Student's t-test (*$p < 0.05$, **$p < 0.01$).

oxidation by 48.7% ($p < 0.01$) and 84.6% ($p < 0.05$), respectively at 7.5 μg/mL, while the reference compound, vitamin C exhibited 40.8% ($p < 0.01$) and 81.2% ($p < 0.05$), respectively at 7.5 μg/mL[18].

In addition, GTE and EGCG inhibited H_2O_2-induced genotoxicity. As shown in Fig. 6b, GTE (100 μg/mL) inhibited DNA single strand breaks by 17.9% ($p < 0.05$) and 8-OH-2'dG formation by 10.7% while EGCG also inhibited 10.4% ($p < 0.05$) and 20.5% ($p < 0.05$), respectively at 4.5 μg/mL. The reference compound, vitamin E, showed 17.3% ($p < 0.05$) and 15.6% ($p < 0.05$) inhibition, respectively at 4.5 μg/mL.

The anti-oxidative and anti-genotoxic effects of commercial green tea liquor

To verify the protective effects of green tea, anti-oxidative and anti-genotoxic activity of commercial green tea liqueur was measured. Green tea liqueur showed higher cell survival rate as shown in Fig. 7a. At 1.6% and 3.2%, green tea liqueur exhibited 6.6% and 41.5% ($p < 0.01$) higher cell survival than those of the pure ethanol-treated group. It is important to note that green tea liqueur also increased cell cytotoxicity along with the amounts added as same as pure alcohol, but cell survival rate also increased depending on the concentrations added compared to the group having the same concentration of ethanol alone. These results were also well matched with the results of DNA damage experiments in Fig. 7b. Green tea liqueur showed 41.4% ($p < 0.05$) higher protective effect against DNA damage compared with same concentration of pure ethanol-treated group.

Discussion

The purpose of investigation was to evaluate the protective activity of antioxidants against ethanol-induced cell toxicity and genotoxicity. Previously, it was reported that oxidative stress induced by ethanol may cause liver toxicity[17,19]. Additionally, It was also reported that lipid peroxidation of unsaturated fatty acids and excess of micronutrient factors such as iron and copper augmented alcohol-induced liver toxicity[20-22]. However, the protective activity of antioxidants such as green tea related compounds against alcohol-induced liver toxicity is still obscure.

As shown in Fig. 1 and 4, ethanol exhibited concentration-dependent cytotoxicity (MTT assay) and genotoxicity (Comet assay). On the other hand, antioxidants including GTE, vitamin C, Trolox and BHT showed protective effects on cell toxicity. Among the antioxidants tested, GTE, showed high protective effects (Fig. 2). BHT which is synthetic and strong antioxidant showed more cytotoxicity at high concentration of treatment.

During our preliminary screening experiment using 120 plant extracts including *pueraria* extract[18], GTE was found to possess high protective effect against cell toxicity and DNA damage induced by ethanol in mouse epithelial cells, NIH3T3 and human liver cells, HepG2. 13% ethanol solution having GTE (solid matter 2-6%) or vitamin C (solid matter 0.5-2%) was formulated and tested since the ethanol concentration of most popular alcoholic beverage is 13%. These formulations were also found to exhibit the protective effects on cell toxicity and DNA damage at the test concentration of 0.05-0.2% for vitamin C and 0.2-0.6% for GTE together with 1.3% ethanol (Fig. 3 and 5).

The results of Comet assay (Fig. 4) detecting DNA single strand break suggests that ROS formed by ethanol may damage the cellular DNA, thereby decreases the genomic stability. These genotoxicic effects are deeply associated with cancer formation in chronically-exposed tissues by

ethanol such as stomach and liver and may be potentiated or repressed by nutrition factors[23]. Thus, the antioxidative plant extracts including GTE may protect DNA damage induced by ethanol and may contribute to the genome stability.

When protective effect of EGCG was examined to find the active principle(s) of GTE, this compound was found to possess the desirable protective effects on cellular toxicity as well as DNA damage (Fig. 6). These results strongly suggest that EGCG contributes, at least in part, to the anti-oxidative and anti-genotoxic effects of GTE against ethanol-induced toxicity.

Our results are well correlated with the previous observations that GTE reduced liver toxicity in alcohol-induced liver disease model in rats[24] and CYP2E1-dependent toxicity in HepG2 cells[25], indicating the detoxifying properties of GTE and polyphenols such as EGCG through their anti-oxidative action. And based on the results of Fig. 7, it is also suggested that the congeners such as EGCG in the alcoholic beverage products may reduce ethanol-induced oxidative stress. The content of total polyphenols including EGCG, ECG and EGC etc. was 32.3% in the GTE[10]. It was well-known that major tea catechin is EGCG in the tea polyphenols[26]. Therefore, EGCG may contribute its cytoprotective[27] and anti-genotoxic[28] effects on ethanol-induced oxidative stress.

The ethanol concentrations on mucosal layer in the stomach, and in blood and liver cells after first-circulation of liver largely depend on the ethanol concentrations ingested. When the alcoholic beverage having high ethanol concentration (whiskey, liqueur etc.) is ingested, the ethanol concentration on stomach epithelial cells is high, which may produce inflammation and/or ulcer on stomach mucosa. In liver cells, the remaining alcohol due to the deficiency of antioxidative enzymes during ethanol metabolism produces necrotic lesion. Therefore, it is reasonably thought that liquors having GTE, EGCG or vitamin C as a supplement, or antioxidant administration after ethanol ingestion probably reduce the ethanol-induced toxicity on stomach mucosal layer and liver cells. Further investigation of the effects of GTE and EGCG on CYP2E2 expression and antioxidative enzymes may be in due course to unveil the cellular mechanism of the protective effect.

Acknowledgements

This study was supported by 2015 Research Grant from Kangwon National University (No. 520150300) and The Institute of New Drug Development, KNU.

국문요약

본 연구의 목적은 NIH3T3와 HepG2 세포에서 에탄올 유도 세포독성 및 유전독성에 대하여 녹차엑기스(GTE)와 epigallocatechin-3-gallate (EGCG)의 보호작용을 평가하는데 있다. 세포생존율은 MTT assay를 실시하였으며 DNA 손상도는 Comet assay로 실시한 결과 에탄올은 농도의존적인 세포독성과 유전독성을 나타내었다. 한편 GTE와 EGCG는 에탄올 유도 세포독성 및 DNA 손상에 대하여 유의성 있는 억제효과를 나타내었으며 DPPH시험과 LDL oxidation 및 8OH-2'dG 생성시험에서 항산화효과를 나타내었다. 한편 녹차성분 함유 시판 리큐르주도 순수 에탄올에 비하여 세포독성억제 및 DNA 손상억제효과를 나타내었다. 이상의 시험결과 GTE와 함유 EGCG는 항산화성 유전독성억제기전을 통한 에탄올독성저감 물질로 판단된다.

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