ORIGINAL ARTICLE

Green Tea Extract Co-administered with a Polymer Effectively Prevents Alcoholic Liver Damage by Prolonged Inhibition of Alcohol Absorption in Mice

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Abstract — Aims: Alcohol toxicity can induce multiple organ dysfunction, including the liver. Gallated catechins (GCs), the components of green tea extract (GTE), have been known to inhibit intestinal lipid absorption. This study was designed to investigate the inhibitory effect of GC on the absorption of the lipid-soluble ethanol in normal mice. In addition, the effectiveness of prolonging the GC-mediated effect was evaluated as a means of preventing alcoholic liver damage. Methods: GTE was administered orally immediately or 90 min before ethanol administration and the blood ethanol and acetaldehyde levels were measured. Binge ethanol administration (by gavage every 6 h for 24 h) was used to induce acute liver injury, and GTE was administered 90 min prior to every ethanol administration. Results: When GTE, but not GC-decreased GTE, was administered immediately before ethanol intake, the blood ethanol and acetaldehyde levels were significantly lower than those in the control. On the other hand, GTE has no effect when GTE was administered 90 min before ethanol intake. When GTE was co-administered with polyethylene glycol (PEG) or poly-γ-glutamate (PGA) 90 min before ethanol intake, the lowering effect of GTE on the blood ethanol and acetaldehyde levels was maintained in contrast to the GTE-alone-treated group. After binge ethanol administration, liver weight decreased, and serum alanine aminotransferase and aspartate aminotransferase levels were elevated. Additionally, histopathological changes, such as macrovesicular steatosis and necrosis, were induced in the liver, together with reactive oxygen species generation. When GTE + PEG or GTE + PGA, but not GTE alone, was administered 90 min before ethanol intake, acute liver injury was ameliorated. Conclusion: These findings support the development of GTE + PEG or GTE + PGA as an inhibitor of intestinal alcohol absorption for the preventative treatment of acute alcohol toxicity.

INTRODUCTION

Alcohol has become the most socially accepted addictive drug worldwide. Drinking alcohol beverages is a common feature of social gatherings. However, many societies experience serious medical, social and economic problems as a result of alcohol abuse (Room et al., 2005; Guo and Ren, 2010), even though drinking in moderation may not be harmful (Cordain et al., 1997; Frid, 2000; Gaziano et al., 2000; Elkind et al., 2006). A long-term alcohol misuse or binge drinking can result in life-threatening physical and mental health hazards and cause detrimental damage to human organs, including the brain, liver, heart, lungs, skeletal muscles and bones. For example, memory disorders (Adolfsson and Karlsson, 1987; Bondi et al., 1998), liver cirrhosis (Bird and Williams, 1988), ventricular dysfunction (Li and Ren, 2008), dilated cardiomyopathy (Li and Ren, 2008), ventricular arrhythmias (Lang et al., 2005), stroke and hypertension (Jones et al., 2005) are likely to occur. Alcohol abuse is closely associated with societal problems, such as car accidents, social violence, broken homes, productivity losses, child abuse and other crimes (Moeller et al., 1998; Moeller and Dougherty, 2001). Additionally, it increases the economic burden on society (Baumberg, 2006; Rehm et al., 2009).

Currently, there are three medications for the treatment of alcohol abuse and alcohol dependence that have been approved by the US Food and Drug Administration (FDA): disulfiram, naltrexone and acamprosate. For >50 years, disulfiram has been approved for the treatment of alcohol dependence and has been shown to improve the rates of alcohol abstinence (Krampe et al., 2006), but the continued use of disulfiram requires a significant level of patient motivation because of the severe discomfort that is experienced when alcohol is consumed. Unlike disulfiram, which relies on aversion, naltrexone and acamprosate directly target the learned association of experiencing euphoria following the alcohol use. However, naltrexone and acamprosate have some side effects, such as gastrointestinal problems (e.g. abdominal pain, nausea and diarrhea) and symptoms associated with decreased central nervous system arousal (e.g. daytime sleepiness and fatigue) (Bouza et al., 2004; Rosner et al., 2010; Witkiewitz et al., 2012). Moreover, many natural substances, which have less toxicity and fewer side effects, have been known to have preventive and therapeutic effects for ethanol intoxication. For instance, Pueraria lobata (Keung and Vallee, 1993; Xie et al., 1994; Overstreet et al., 1996), Tabernanthe iboga (Glick et al., 1994; Sershen et al., 1994; Rezvani et al., 1995) and Hypericum perforatum (Ciccocioppo et al., 1999) have been reported to reduce voluntary alcohol intake. Moreover, saponins (Yoshikawa et al., 1996a,b,c; Yoshikawa et al., 1999), Panax ginseng (Lee et al., 1993) and Laurus nobilis (Yoshikawa et al., 2000) have been reported to reduce alcohol absorption from the gut, although their effective time in the gut has not been properly considered. These natural substances were absorbed into the blood circulation within a few minutes, and the blood concentrations of these natural substances are known to peak at ~1 h after ingestion (Yang et al., 1998; Lee et al., 2002). Especially, at social gatherings, a single dose of a natural substance just before drinking an alcohol beverage may be insufficient to inhibit alcohol toxicity. Therefore, to maximize the anti-alcohol effect of natural substances in the intestinal lumen, these natural substances need to remain longer in the intestinal lumen. The present study investigated the effect of gallated catechins (GCs) of green tea extracts (GTE) on ethanol absorption, because GC is known to inhibit intestinal...
lipid absorption (Raederstorff et al., 2003). Based on the hypothesis that the resins polyethylene glycol (PEG) and poly-\(\gamma\)-glutamate (PGA) selectively inhibit the intestinal absorption of green tea-derived GC through combining with GC (Park et al., 2009), the present study also investigated whether the inhibitory effect of GTE on ethanol absorption could be prolonged by a polymer co-treatment for a sufficient time to ameliorate ethanol-induced acute liver damage.

MATERIALS AND METHODS

Chemicals and materials

PGA was gifted from NUC Electronics (Daegu, Korea). PEG was a kind gift from Kukjeon Pharma (Seoul, Korea). Green tea leaves (BOSUNG SEIJAK) were purchased from Bosung Green Tea Co. (Jeonnam, Korea). Epigallocatechin-3-gallate (EGCG), epicatechin-3-gallate (ECG), epigallocatechin (EGC) and epicatechin (EC) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2',7'-Dichlorodihydrofluorescein diacetate (H\(_2\)DCFDA) was purchased from Molecular Probes (Eugene, OR, USA). All chemicals were purchased from Sigma-Aldrich unless otherwise noted.

Preparation of GTE

Green tea leaves (20 g) were added to 1000 ml of nanopure water. After being stirred for 5 min at 80°C, the tea leaves were removed by filtration using filter paper (Advantec 2 filter paper, Hyundai Micro Co., Seoul, Korea) under reduced pressure. The extract was dried by lyophilization. A total of 3 g of GTE was collected, in which the EGCG, ECG, EGC and EC contents were 100, 53, 56 and 31 mg/g GTE, respectively. An equal amount of the GTE solution was mixed with 2 g of PEG beads for 5 min at room temperature. After filtration, the supernatant was lyophilized (the GC concentration of the GTE solution was partially reduced the amount of GC in the GTE solution.

Analysis of catechins from GTE with HPLC

HPLC analysis was conducted on a Waters Alliance 2695 liquid chromatography equipped with a model 2487 dual-absorbance detector (Waters Co., Milford, MA, USA). A Waters symmetry C18 reverse phase packing column (4.5 mm \(\times\) 250 mm, 5 m) was used at 25°C for separation. The catechins were detected simultaneously at 235 nm. An elution gradient was performed by varying the proportion of solvent A (water:trifluoroacetic acid, 99.9:0.1 v/v) to solvent B (acetonitrile:trifluoroacetic acid, 99.9:0.1 v/v), with a flow rate of 1 ml/min. The mobile phase composition changed linearly from 9.5 to 14% solvent B in 10 min. This composition was maintained for 10 min and was followed by a linear increase in solvent B to 27.5% within 15 min. Over a period of 5 min, the mobile phase composition was returned to the initial conditions to prepare for the next run. All of the prepared solutions were filtered through 0.45-\(\mu\)m membranes (Sartorius, Maisemore, UK), and the mobile phase was degassed before injection into the HPLC.

Animals and treatments

Male C57BL/6 mice aged 9 weeks were purchased from Jung-Ang Experimental Animals (Seoul, Korea) and housed in an air-conditioned room at 22°C for 7 days to adapt to the new housing conditions. Standard laboratory chow (Hyochang Science, Seoul, Korea) and tap water were given ad libitum. The animals were fasted for 12 h prior to experiments, but were supplied with water ad libitum. The animals were treated in the manner approved by the Keimyung University institutional ethics committee, Daegu, Korea, for supervising animal research (KM-2010-42).

Measurement of blood ethanol and acetaldehyde levels in ethanol-treated mice

Water, epicatechins (EGCG, EGC, ECG and EC), PEG, PGA, GTE, GTE + PEG and GTE + PGA were administered orally to the fasted (12 h) mice. Epicatechins (each 90 mg/kg), PEG (90 mg/kg), PGA (90 mg/kg), GTE (900 mg/kg), GTE + PEG (900 and 90 mg/kg, respectively) and GTE + PGA (900 and 90 mg/kg, respectively) were suspended in water and given orally prior to the administration of ethanol. EC was dissolved in 10\(\mu\)l of DMSO in advance before the addition of water. Either immediately or 90 min later, 8 ml of ethanol (20 or 40% w/v, in tap water/kg of mice was given orally. Blood samples were collected from the infra-orbital venous plexus at 15, 30, 60, 120, 180 and 300 min after the administration of ethanol. The blood ethanol and acetaldehyde concentration in the venous blood sample obtained before the administration of ethanol was considered the concentration at time 0. Then, blood was immediately mixed with a 10-fold volume of ice-cold 0.33 N perchloric acid and centrifuged at 1000 \(\times\) g at 4°C for 10 min. Blood ethanol and acetaldehyde levels in the supernatants were assessed by an enzymatic method (R-biopharm, Darmstadt, Germany). The areas under the venous blood ethanol and acetaldehyde concentration–time curve between 0 and 300 min were determined.

Ethanol-induced acute liver injury model by a binge-type ethanol administration

To induce an acute liver injury model by binge drinking ethanol, 40% (w/v) ethanol was administered at 8 ml/kg body weight by gavage every 6 h for 24 h (Yang et al., 2003). Water, PEG, PGA, GTE, GTE + PEG and GTE + PGA were administered 90 min prior to every administration of ethanol. At 4 h after the last ethanol administration, the mice were anaesthetized with sodium pentobarbital (0.05 mg/g body weight). An aliquot of 300-\(\mu\)l blood sample was collected from the orbital venous plexus into a heparinized tube and immediately chilled on ice. The plasma was then separated and stored at \(-80^\circ\)C before analysis. After blood sampling, the livers were removed, weighed and apportioned. One part of each liver was frozen in liquid nitrogen, whereas another part was fixed with 4% phosphate-buffered paraformaldehyde and embedded in paraffin.

Biochemical detection

Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were used as indicators of...
Fig. 1. The effect of GTE on blood ethanol and acetaldehyde levels in ethanol-loaded mice (a & c). Mice were fasted for 12 h, and 900 mg/kg GTE was administered orally with 8 ml/kg ethanol (20% w/v, in tap water). Blood samples were collected from the infra-orbital venous plexus at 15, 30, 60, 120, 180 and 300 min after the administration of ethanol. The AUC are depicted in (b & d) as the percentage of the control value. *$P<0.05$ and **$P<0.01$ compared with the control value. Each group contained 5–7 mice.

Fig. 2. The effect of GC on blood ethanol levels in ethanol-loaded mice. Mice were fasted for 12 h, and each epicatechin (90 mg/kg) or GC-decreased GTE (900 mg/kg) was administered orally with 8 ml/kg ethanol (20% w/v, in tap water). Blood samples were collected from the infra-orbital venous plexus at 15, 30, 60, 120, 180 and 300 min after the administration of ethanol (a & c). The AUC are depicted in (b & d) as the percentage of the control value. *$P<0.05$ and **$P<0.01$ compared with the control value. Each group contained 5–7 mice.
hepatotoxicity. These assays were performed on mice sera using a diagnostic kit obtained from Asan Pharmaceutical (Seoul, Korea).

Liver histopathology
Embedded liver tissue blocks were cut into 6-μm sections and stained with haematoxylin and eosin. Mounting media and cover slips were placed on the slides, which were then left to dry overnight. A diagnosis of fatty liver was made on the basis of the presence of macro- or microvesicular fat in >5% of the hepatocytes in a given slide.

Measurement by confocal microscopy of generated reactive oxygen species (ROS)
Intracellular ROS were measured by using a cell permeable fluorescent dye, H$_2$DCFDA. Frozen liver tissue was sectioned, stained with 5 μM H$_2$DCFDA and incubated for 30 min at 37°C. Subsequently, the tissue was washed twice with the Kreb-Ringer bicarbonate buffer to remove all unbound dye in the medium that contained the cells. Mounting media and cover slips were placed on the slides, which were then left to dry overnight. The images were obtained by subjecting the cells to confocal laser microscopy (LSM 5 EXCITER; Carl Zeiss, Jena, Germany), using excitation and emission wavelengths at 488 and 525 nm, respectively.

Statistical analysis
The results are expressed as the means ± SEM. SPSS (version 14.0; SPSS, Inc., Chicago, IL, USA) was used for the statistical analysis. The area under the curve was calculated using Microcal Origin software (version 7.0; Northampton, MA, USA). Comparisons between the two groups were performed with Student’s two-tailed t-test. For comparisons of more than two groups, significance was tested using analysis of variance with Bonferroni’s correction to analyse a relatively small number of samples. P-values of <0.05 were considered significant.

RESULTS

Effect of GTE on blood ethanol and acetaldehyde levels in ethanol-loaded mice
As shown in Fig. 1, when GTE was administered orally immediately before ethanol administration, GTE maintained blood ethanol and acetaldehyde at lower levels than in the controls during the experiment. The area under the curve (AUC) of the blood ethanol and acetaldehyde levels of the GTE group was ~40% lower (P < 0.01) than that of the control group (Fig. 1b and d). The difference between the GTE and control groups was statistically significant.

EGCG and ECG are critical for the effect of GTE on blood ethanol levels in ethanol-loaded mice
To clarify which GTE components were responsible for the effect of GTE on blood ethanol levels in ethanol-loaded mice, EGCG, ECG, EGC and EC were each administered orally (90 mg/kg) with ethanol (Fig. 2a). The AUC of the blood ethanol level of the ECG and EGCG groups was ~20% lower (P < 0.05) than that of the control group, whereas the AUC for the EC and EGC groups was similar to that of the control group (Fig. 2b). The difference between the ECG and EGCG groups and the control group was statistically significant.

To confirm that GC, including EGCG and ECG, is critical for the inhibitory effect of GTE on blood ethanol levels in ethanol-loaded mice, GC-decreased GTE (contained 23.4 mg EGCG/g GTE and 12.6 mg ECG/g GTE) was administered...
orally with ethanol. The GC-decreased GTE group exhibited a similar blood ethanol elevation to the control group. These results suggested that the effect of GTE on blood ethanol levels in ethanol-loaded mice was primarily caused by two GC, i.e. EGCG and ECG (Fig. 2c and d).

**Effect of GTE co-administered with a polymer on blood ethanol and acetaldehyde levels in ethanol-loaded mice**

Figures 1a and 3a illustrate that the effects of GTE intake on blood ethanol levels depended on the interval between GTE and ethanol administration. The lowering effect of GTE on blood ethanol levels was absent when GTE was administered 90 min before the administration of ethanol (Fig. 3a), indicating that the effect of GTE on blocking ethanol absorption persists only when GTE is present in the intestinal lumen. The 90 min interval between GTE and ethanol intake was chosen because the blood concentrations of tea ingredients, especially catechins, are known to peak at ~1 h after GTE ingestion (Yang et al., 1998; Lee et al., 2002). Based on the assumption that the resins PEG and PGA selectively inhibit the intestinal absorption of green tea-derived GC by combining with the GC (Park et al., 2009), a GTE + PEG or GTE + PGA combination was administered 90 min before the administration of ethanol. In these mice, the lowering effect of GTE on the blood ethanol and acetaldehyde levels was maintained in contrast to the GTE-alone-treated group (Figs. 3 and 4). There was no difference in the blood ethanol and acetaldehyde levels between the control group and the PEG- or PGA-alone-treated group, indicating that PEG- or
PGA-alone did not affect ethanol absorption or intestinal motility at the range of concentrations used.

Protective effects of GTE co-administered with a polymer on ethanol-induced acute liver injury

The administration of 40% (w/v) ethanol at 8 ml/kg body weight by gavage every 6 h for 24 h was used as a model of acute liver injury (Yang et al., 2003). The liver weights decreased, and serum ALT and AST levels significantly elevated in these animals compared with the normal group that was treated with tap water instead of ethanol (Fig. 5). The prominent histopathological changes induced in control mouse livers by this binge-type-ethanol administration were macrovesicular and microvesicular steatosis; however, small necrotic changes were observed, including cell enlargement, sheet-like patterns, neutrophil infiltration and nuclear dissolution (Fig. 6). When a GTE + PEG or GTE + PGA combination was administered 90 min before the administration of ethanol, there was less acute liver injury than in the GTE-alone-treated group (Fig. 5). Additionally, ethanol administration caused only minor microvesicular steatosis in the livers of mice treated with GTE + PEG or GTE + PGA (Fig. 6).

Suppression of hepatic ROS generation by GTE co-administered with a polymer

Ethanol-induced hepatic ROS generation was measured by using a cell permeable fluorescent dye, H$_2$DCFDA. Ethanol administration caused approximately a 2-fold increase in ROS generation in the liver compared with the normal group that was treated with tap water instead of ethanol (Fig. 7). In the GTE + PEG- or GTE + PGA-treated mice, the ethanol-induced levels of ROS generation were significantly lower than in the ethanol-treated control group.

DISCUSSION

As the blood alcohol concentration increases (Dubowski, 1985; Pratt et al., 1990; Gentry, 2000), a person’s response to stimuli decreases, speech becomes slurred, the gait becomes unsteady and trouble with walking is experienced. At high blood alcohol concentrations, a person can become comatose and die (Welling et al., 1977; Dubowski, 1985; Pratt et al., 1990). The consumption of a large volume of alcohol can induce hepatocellular death (Israel et al., 1975; Zhou et al., 2001; Yang et al., 2003), which causes transient hepatic mitochondrial DNA depletion (Demeilliers et al., 2002) and ROS formation (Cunningham et al., 1990; Bautista and Spitzer, 1996). Alcohol, which is lipid soluble, is absorbed epithelially in the gastrointestinal tract largely by simple diffusion into the blood. Moreover, alcohol, like many drugs, is absorbed more rapidly from the small intestine than the stomach (Haber et al., 1996); In a fasting individual, it is generally agreed that 10–20% of a dose of alcohol is absorbed from the stomach and 75–80% is absorbed from the small intestine (Dubowski, 1985; Haber et al., 1996; Gentry, 2000). The blood alcohol concentration depends on the amount of alcohol consumed, the rate of absorption, the first-pass metabolism, the distribution dynamics, the hepatic metabolism and the blood flow rate (Haber et al., 1996; Gentry, 2000). Because alcohol is metabolized at a fairly constant rate, the amount of alcohol consumed and the
absorption rate can be the most important factors in determining the blood alcohol concentration.

The present study has demonstrated that green tea-derived GC, such as EGCG and ECG, acutely reduce blood ethanol levels mainly through their inhibitory effects on ethanol absorption in the gastrointestinal tract, whereas there are no effects on the blood ethanol level when the GC is mainly present in the circulatory system. Green tea is one of the most popular traditional teas in Asian countries, and it is currently consumed worldwide as a dietary supplement or beverage. Water-soluble GTE contains various catechins, of which EGCG is the most abundant (Yang et al., 1998; Lambert and Yang, 2003). It has been reported that orally administered GTE may inhibit glucose (Kobayashi et al., 2000; Zhu et al., 2001; Johnston et al., 2005) and cholesterol (Raederstorff et al., 2003) absorption from the gastrointestinal tract. GC, including EGCG and ECG, appears to be responsible for these inhibitory effects. These reports suggest that the effects of GC are likely to be the result of steric hindrance, which is caused by the incorporation of GC into the cell membrane with subsequent disruption of the surrounding lipid bilayer, as demonstrated previously by using transfected Xenopus oocytes as an expression vector (Hossain et al., 2002). Hence, one may speculate that GC might hinder various activities in the plasma membrane, including simple diffusion. Moreover, GC has been demonstrated to interact with various plasma-membrane proteins and lipid rafts (Patra et al., 2008). Many natural extracts, including Panax ginseng (Lee et al., 1993), Radix puerariae (Keung and Vallee, 1993; Xie et al., 1994), Laurus nobilis (Yoshikawa et al., 2000), Senegae radix (Yoshikawa et al., 1995) and Salvia miltiorrhiza extract (Colombo et al., 1999), have demonstrated the ability to inhibit intestinal alcohol absorption. These natural extracts generally have an ~20% inhibitory effect on alcohol absorption, whereas GTE had an ~40% inhibitory effect. However, natural GTE and other natural extracts, which could be inhibitors of alcohol absorption in the intestinal lumen, enter the circulatory system over time and might therefore lose the ability to inhibit intestinal alcohol absorption, as observed in the present study. In this way, prolonging the gastrointestinal effect of alcohol absorption inhibitors would be beneficial.

The polymers PEG and PGA at the concentrations used in the

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Fig. 7. ROS generation in the liver induced by acute ethanol administration. Mice were fasted for 12 h, and 40% (w/v) ethanol was administered at 8 ml/kg body weight by gavage every 6 hr for 18 h. PEG (90 mg/kg), PGA (90 mg/kg), GTE (900 mg/kg), GTE + PEG (900 mg/kg GTE and 90 mg/kg PEG) or GTE + PGA (900 mg/kg GTE and 90 mg/kg PGA) was administered 90 min prior to every administration of ethanol. At 4 h after the last ethanol administration, the livers were removed and frozen. The frozen livers were sectioned and stained with 5 μM H2DCFDA, and cellular ROS levels were measured by confocal laser microscopy (a). For the quantification of ROS levels, fluorescence intensity was analysed by LSM 5 EXCITER software (b). The results are expressed as the means ± SEM; n = 5 for each group. When **P < 0.01 compared with the normal group that was treated with tap water instead of ethanol. *P < 0.05 compared with the control group that was treated with 40% ethanol. Scale bar = 50 μm.
present experiments appeared to absorb active ingredients of green tea physically through a dipole–dipole interaction to give retardation of intestinal alcohol absorption. Co-administration of GTE with PEG or PGA, particularly to alleviate the effects of binge drinking in situations where this is not preventable, could maximize GC-mediated anti-alcohol effects, including the alleviation of alcohol-induced hepatotoxicity. Although GTE + PEG or GTE + PGA mixture effect- ively retarded intestinal alcohol absorption in animals, the effect of GTE + PEG or GTE + PGA mixture in the human body is yet to be determined. Future clinical studies could evaluate whether GTE + PEG or GTE + PGA can decrease alcohol absorption and thereby lead to amelioration in acute alcohol toxicity.

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