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Direct in vivo Evidence of Protective Effects of Grape Seed Procyanidin Fractions and Other Antioxidants against Ethanol-Induced Oxidative DNA Damage in Mouse Brain Cells

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Ethanol is a principle ingredient of alcoholic beverages with potential neurotoxicity and carcinogenicity, and the ethanol-associated oxidative DNA damage in the central nervous system is well documented. The present work studied the possible protective effects of grape seed oligomer and polymer procyanidin fractions against ethanol-induced toxicity and compared these with resveratrol and other well-known antioxidants (ascorbic acid and vitamin E). By using the single cell gel electrophoresis (comet assay), a simple and sensitive technique for genotoxicity studies, the potential genotoxicity of acute and chronic ethanol administration in the different brain regions was investigated. Acute ethanol administration, at the dose of 2.5 or 5.0 g kg^{-1} i.p., could induce significant DNA damage in cerebellum and hippocampus. Chronic administration of ethanol at the dose of 2.5 or 5.0 g kg^{-1} p.o. for 30 days could induce significant DNA damage in cerebellum, hippocampus, hypothalamus, and cortex, which could be auto-repaired at least 3 days after ethanol withdrawal. Oral administration of grape seed oligomer and polymer procyanidins and resveratrol (25, 50, and 100 mg kg^{-1}) for 3 days before acute ethanol (5.0 g kg^{-1}, i.p.) or repeated administration of these substances together with ethanol (5.0 g kg^{-1}, p.o.) for 30 consecutive days could significantly inhibit DNA damage in brain cells induced by ethanol. As compared, ascorbic acid (50, 100, and 200 mg kg^{-1}) and vitamin E (100, 200, and 400 mg kg^{-1}) could also present protective effects on ethanol-induced DNA damage. Furthermore, the concentrations of ethanol and acetaldehyde in brain regions of the mice were detected by gas chromatography after administration of ethanol plus antioxidants. All of the results indicated that ethanol could induce region-specific oxidative DNA damage in which the cerebellum and hippocampus were more vulnerable, but intake of grape seed procyanidins or other natural antioxidants could protect the brain against ethanol-induced genotoxicity.

KEYWORDS: Procyanidins; ethanol; antioxidant; DNA damage; mouse

INTRODUCTION

Ethanol abuse is one of the most costly health problems in the world (*1*). One of the main toxic targets of ethanol is the central nervous system. A number of in vivo and in vitro studies has shown a wide range of structural and functional alterations in brain neurons. For example, altered cell membrane fluidity and structural alterations have been observed (*2*). The genotoxic effect of ethanol has also been demonstrated, especially its neurotoxicity (*3*). It has been shown that ethanol induces DNA single-strand breaks in brain cells of the rats (*4*). In vitro, acute exposure of cultured neurons to ethanol results in reversible DNA single-stand breaks, whereas chronic exposure causes loss of cell viability (*5*).

The mechanism by which ethanol exerts its genotoxicity is not well understood. It is generally considered that free-radical generation plays an important role in the genotoxicity of ethanol (*6*). Inhibition of DNA damage in target organs by antioxidants, therefore, is expected to be effective for prevention of radical-mediated genotoxicity, which will be helpful to understand the mechanism of the genotoxic action of ethanol.

Ascorbic acid and vitamin E are well-known natural antioxidants, which present neuroprotective effects and could protect cells against free-radical-mediated damage (*7*, *8*). Recently, catechins, procyanidins (**Figure 1**), and resveratrol, the major phenolic compounds of grape and red wine (*9*−*11*), have been also reported to possess potent antioxidant activities (*12*, *13*). Procyanidins are oligomers and polymers of flavan-3-ol mono-

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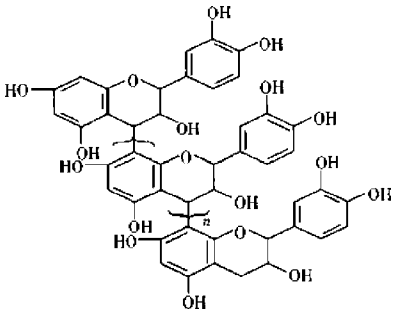

Figure 1. General chemical structure of procyanidins; $n = 2$ to 12–15 in oligomer procyanidins, $n = 10$ to 30–33 in polymer procyanidins.

mer units (catechins) linked most by acid-labile 4 → 8 and, in same cases, by 4 → 6 bonds, distributing widely in the plant kingdom, from ferns and their allies to the most advanced di- and mono-cotyledonous plants (*12*). Grape seeds are among the richest sources of these compounds. In our previous work, freeze-dried oligomer and polymer procyanidin fractions were isolated from grape seeds (*14*), permitting us to further study their chemical and biological properties. Thus, the main objective of the present work was to study the protective effects of these procyanidin fractions against ethanol-induced oxidative DNA damage in mouse brain cells. For comparison purposes, other antioxidants, that is, ascorbic acid, vitamin E, and resveratrol, were also tested.

Single cell gel electrophoresis (SCGE), more commonly known as the comet assay, is a simple, sensitive, and rapid method for the detection and quantification of DNA damage by strand breaks at the individual cell level (*15*−*18*). Following gel electrophoresis under alkaline conditions, damaged DNA is released from the nucleus, forming a comet "head" and "tail". Studies have shown that the alkaline comet assay has been widely used in both in vivo and in vitro studies to detect DNA damage and to measure DNA repair capability (*19*, *20*). Furthermore, this system is particularly suitable in studying antioxidant activity of natural molecules (*21*).

In the present study, in order to detect the DNA damage induced by ethanol and to detect which brain regions are more vulnerable to the genotoxic action of ethanol, the alkaline single cell gel electrophoresis was used to detect the effects of acute and chronic ethanol in various brain regions of the mice, and the concentrations of ethanol and acetaldehyde in brain regions were detected by gas chromatography (GC). Furthermore, the tested compounds were administered to mice before or together with ethanol treatment to investigate their potential protective effects against ethanol-induced genotoxicity in the central nervous system.

MATERIALS AND METHODS

Animals. Male Swiss mice (20 ± 2 g), obtained from the Experimental Animal Center of Shenyang Pharmaceutical University, were used in the present study. The animals were housed under standard conditions (22 ± 2 °C temperature, 50 ± 10% relative humidity, 12L: 12D light/dark cycles). Food and water were available ad libitum. All animal use procedures were in accordance with the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of People's Republic of China, 1988.

Chemicals. *trans*-Resveratrol (3,4′,5-trihydroxy-*trans*-stilbene (purity 99% GC)) was purchased from Sigma Chemical Company (St. Lois, MO). Ascorbic acid (VC) was from Tianjin Bodeya Chemical Agencies, China. Vitamin E was from Guangzhou Baiyunshan Pharmaceutical Com., China. Low-melting agarose (LMA) and normal-melting agarose (NMA) were purchased from AMRESCO Inc. (Solon, OH) and Shanghai Yito Inc. (Shanghai, China), respectively. Ethidium bromide (EtBr) was obtained from Sigma Chemical Company (St. Louis, MO). Ethanol (EtOH), dimethyl sulfate (DMS), carboxymethyl cellulose sodium (CMC), dimethyl sulfoxide (DMSO), and isopropanal were used as an internal standard (IS) and were obtained from Tianjin Fuchen Chemical Agencies, China. All other chemicals were of reagent grade and purchased from commercial sources.

Preparation of Procyanidin Fractions from Grape Seeds. *Isolation of Monomeric, Oligomeric, and Polymeric Procyanidin Fractions.* Grape seeds (*Vitis vinifera*, cv. Fernão Pires) were ground finely (i.d. ≤ 1 mm) using an ultra-centrifugal mill ZM 100 (Retsch GmbH & Co. KG, Haan, Germany). The grape seed powder was immediately extracted using first methanol−water (80:20, v/v) followed by acetone−water (75:25, v/v) to obtain a crude phenolic extract, as described earlier (*22*). After removing organic solvents, the crude phenolic extract was chromatographed by column on Lichroprep RP-18 (200 × 25 mm i.d.; 25−40 μm particle size) to isolate oligomeric and polymeric procyanidin fractions, named as F2 and F3, respectively, with the procedures similar to those already described (*14*). Briefly, elution began with distilled water adjusted to pH 7.0 to eliminate phenolic acids followed by ethyl acetate to elute the oligomeric procyanidin fraction. The polymeric procyanidins adsorbed at the top of the bed were eluted with methanol. The oligomeric procyanidin fraction was then redeposited onto the same preconditioned column to isolate the monomeric procyanidin fraction and oligomeric procyanidin fraction by elution first with diethyl ether and then with methanol. Both oligomeric and polymeric procyanidin fractions were evaporated at less than 30 °C to dryness and dissolved in water prior to lyophilization. The powders obtained were stored at −20 °C until used.

Chemical Characterization of Monomeric, Oligomeric, and Polymeric Procyanidin Fractions. Chemical characterization of the isolated fractions were realized by formaldehyde−HCl precipitation test, ash, sugar, and mineral analysis, elemental analysis, thioacidolysis HPLC, normal-phase HPLC, reverse-phase HPLC, and ESI−MS analysis. The detailed operating procedures of these methods were presented in our recent work (*13*).

Dosage and Treatment. *Ethanol.* (i) *Acute Treatment*. Thirty-two mice were randomly divided into two groups. The animals received an i.p. injection of 20% (v/v) ethanol at 2.5, 5.0 g kg^{-1} (0.13 mL/10 g b.wt., 0.25 mL/10 g b.wt.) ad un vic. Mice from controls were administered with saline. After 0, 2, 4, and 6 h of administration, animals were sacrificed by decapitation.

(ii) *Chronic Treatment*. Sixty-four mice were randomly divided into 4 groups of 16 mice in each group, which included control, positive, ethanol 2.5 g kg^{-1}, and ethanol 5.0 g kg^{-1} groups. The animals in the ethanol groups were treated by gavage with 20% (v/v) ethanol at 2.5 and 5.0 g kg^{-1} every day for 30 consecutive days. The control groups and the positive groups were treated with distilled water for 30 days. At the last day, the positive groups received an i.p. injection of DMS at 20 mg kg^{-1}. Sixteen animals, four mice from each group respectively, were sacrificed by decapitation 4 h after the last treatment. The other animals were sacrificed at 24 h and at the 3rd and 7th day after ethanol withdrawal.

Ethanol was diluted to 20% (v/v) with saline for intraperitoneal injection or with redistilled water for oral treatment. DMS was diluted with saline. The doses and the administration route of ethanol were based on the study which reported that 4 g kg^{-1} of orally administered ethanol caused DNA damage in the rat brain (*4*), and for the chronic treatment, they were based on Luczaj and Skrzydlewska's report (*23*).

Antioxidants. On the basis of the results of the experiment above, the protocol for studying the protective actions of antioxidants against ethanol was as follows.

(i) *Acute Treatment*. Thirty-two mice were randomly divided into eight groups of four mice each group. Twenty-four animals were orally treated with ascorbic acid at the doses of 50, 100, and 200 mg kg^{-1}, vitamin E at 100, 200, and 400 mg kg^{-1}, resveratrol at 25, 50, and 100 mg kg^{-1}, or procyanidins at 25, 50, and 100 mg kg^{-1} for 3 consecutive days. Thirty minutes after the last treatment, the antioxidants plus ethanol groups and the ethanol group were treated with an i.p. injection of 20% ethanol at 5.0 g kg^{-1}. Four hours after ethanol administration, the animals were sacrificed by decapitation.

Table 1. Chemical Composition and Purity of Procyanidin Fractions Isolated from Grape Seeds

					elemental composition % (w/w)			mineral composition (mg/g sample)					
procyanidin fraction	major constituents	range of DP	mDP	%*G*	C	H	N	Fe	Cu	Pb	Cd	sugar	purity (w/w %)
oligomer	oligomer procyanidins	2 to 12–15	7.2 ± 0.2	28.8 ± 0.3	54.1 ± 0.2	4.4 ± 0.2	<0.2	0.06	0.12	<0.003	0.0004	trace	93.0 ± 1.3
polymer	polymer procyanidins	10 to 30–33	25.2 ± 1.5	35.1 ± 0.7	53.9 ± 0.0	5.0 ± 0.0	<0.2	0.05	0.10	0.007	0.0002	trace	92.2 ± 1.8

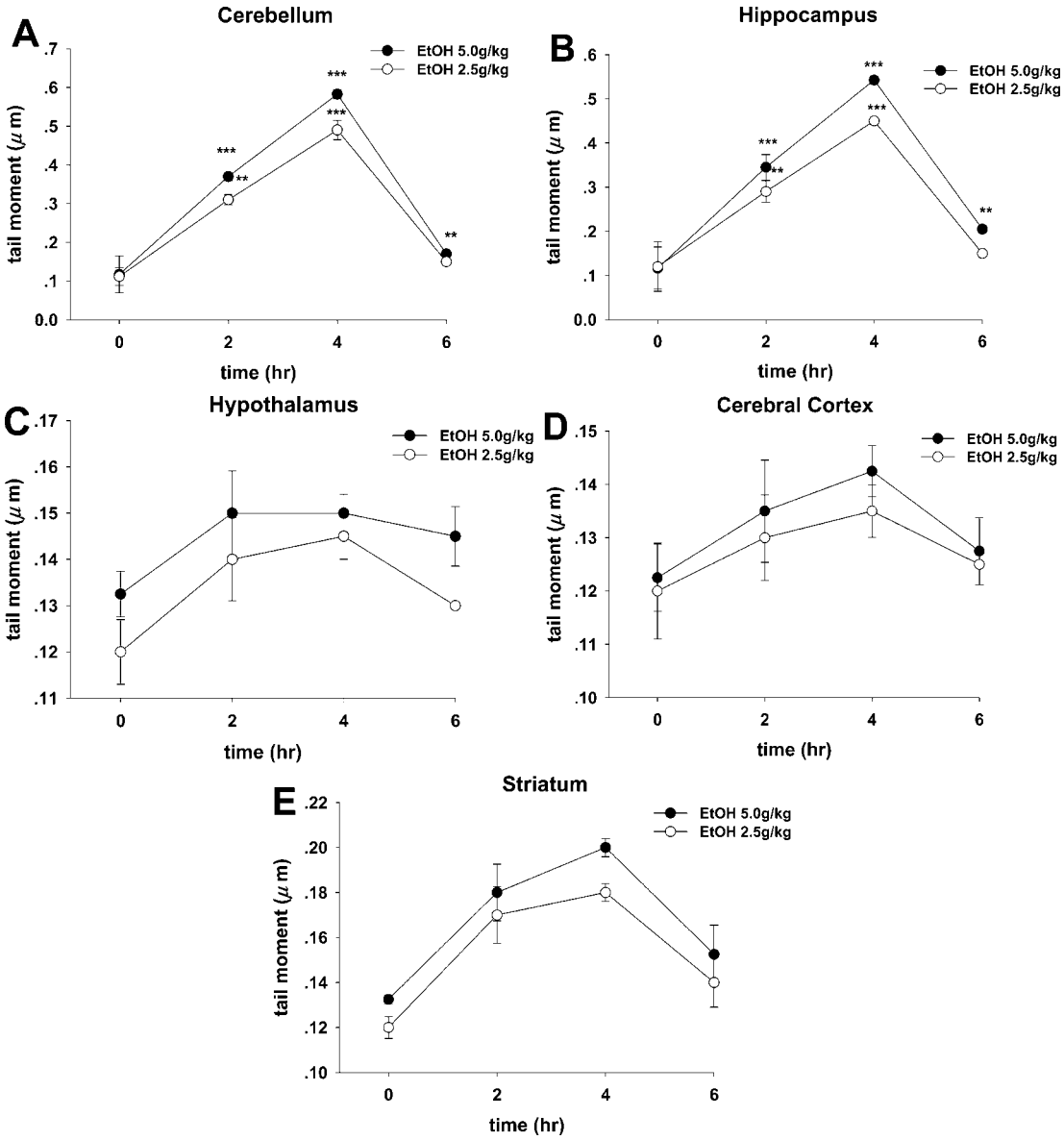

Figure 2. Time course of effects of ethanol on brain cell DNA in five brain regions of the mice following acute administration of ethanol (2.5 and 5.0 g kg^{-1}, i.p.). (**A**) Cerebellum (CB); (**B**) hippocampus (HIP); (**C**) cerebral cortex (CC); (**D**) hypothalamus (HT); (**E**) striatum (ST). Average tail moment lengths of DNA damage of brain cells at different times, post ethanol administration, are shown. Two hundred cells were examined in duplicate for each condition, and the tail moments are expressed as the mean ± S.E.M. **, $P < 0.01$ and ***, $P < 0.001$ compared with the ethanol 0 h group; $n = 4$.

(ii) *Chronic Treatment*. Animals were orally treated with ascorbic acid, 25, 50, and 75 mg kg^{-1}, vitamin E, 25, 50, and 100 mg kg^{-1}, resveratrol, 10, 20, and 40 mg kg^{-1}, or procyanidins, 10, 20, and 40 mg kg^{-1}. The antioxidants plus ethanol groups and the ethanol group were simultaneously treated with 20% (v/v) ethanol at 5.0 g kg^{-1} for 30 consecutive days. Animals were sacrificed by decapitation 4 h after the last treatment.

(iii) *Ethanol and Acetaldehyde Determination.* Forty-two mice were randomly divided into six groups of seven mice each group, which included an ethanol group and the antioxidants plus ethanol groups. Mice in the antioxidants plus ethanol groups were orally treated with ascorbic acid at the dose of 200 mg kg^{-1}, vitamin E at 400 mg kg^{-1}, resveratrol at 100 mg kg^{-1}, or procyanidins at 100 mg kg^{-1} for 3 consecutive days. Thirty minutes after the last treatment, the antioxidants

plus ethanol groups and the ethanol group were treated with an i.p. injection of 20% ethanol at 5.0 g kg^{-1}. Animals were sacrificed by decapitation 40 min after the ethanol treatment.

Ascorbic acid and procyanidins were dissolved in redistilled water. Vitamin E was dissolved in soybean oil. Resveratrol was suspended with 0.5% CMC.

Sample Preparation. After sacrifice, the mouse brain was removed and immediately dissected on ice. The brain was separated into five regions, cerebellum, hippocampus, hypothalamus, striatum, and cerebral cortex. The brain regions were minced, suspended at 1 mL/g in chilled homogenizing buffer (containing PBS/NaCl, 8.01 g; KCl, 0.2 g; Na_2HPO_4, 2.9 g; KH_2PO_4, 0.2 g/L), and gently homogenized manually. To obtain nuclei, the homogenate was centrifuged at 1000*g* for 5 min, and the precipitate was resuspended in chilled homogenizing buffer for the comet assay.

In the experiment of the determination of ethanol and acetaldehyde concentrations by GC, the mouse was decapitated, and the brain was quickly dissected on ice and separated into five regions. The different regions of brain tissues were added into 100 μL/10 mg of chilled artificial cerebrospinal fluid (aCSF: 145 mM NaCl, 2.8 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, 1.55 mM Na_2HPO_4, 0.45 mM NaH_2PO_4, pH 7.4) and homogenized at 4 °C with a Potter−Elvehjem homogenizer with a Teflon pestle, and the homogenate was centrifuged at 4 °C and 12000*g* for 3 min. Then, 20 μL of the supernatant solution was mixed vigorously with 20 μL of 0.05% isopropanol (v/v) (IS solution).

Comet Assay. The comet assay was performed under alkaline conditions essentially according to the previous reports (*24*, *25*) with a slight modification. In brief, normal-melting-point (NMA) and low-melting-point agarose (LMA) were dissolved in PBS using water bath heating. Then, 100 μL of 1% NMA was added to fully frosted slides for a firm attachment, and the slides were allowed to solidify with cover slips in the refrigerator for 5 min. After solidification of the gel, the cover slips were removed, and 50 μL of brain cells mixed with 50 μL of 0.5% LMA was added. The cover slips were added to the layer, and the slides were again allowed to solidify in the refrigerator for 15 min. After removing the cover slips, the slides were submersed in the lysing solution (2.5 mol/L of NaCl, 100 mmol/L of Na_2EDTA, 10 mmol/L of tris-HCl, pH 10; 1% TritonX-100 and 10% DMSO were added freshly) for 1 h. The slides were then placed in unwinding buffer (1 mmol/L of Na_2EDTA, 300 mmol/L of NaOH, pH 13) for 30 min, and electrophoresis was carried out using the same solution for 20 min at 0.92 V/cm (11 V and 300 mA). All of the steps were conducted under dimmed light. After electrophoresis, the slides were neutralized via three washings with 0.4 M tris-HCl (pH 7.4), 5 min each, and were submersed in 100% ethanol for 1 h of dehydration. After the slides were dried by airing, the slides were stained with 4×10^{-6} g/mL of ethidium bromide (EtBr).

The objects were observed at 200× magnification in a fluorescence microscope (Olympus, Japan) attached to a Pulnix video camera (assembled in the U.S.A.) and connected to a personal-computer-based image analysis system (LUCIA Comet Assay system 4.8). For each treatment group, 200 cells were examined. The parameters were automatically calculated by the Lucia image analysis system.

Determination of Ethanol and Acetaldehyde by GC (26). All samples were analyzed on a GC-17A gas chromatograph (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector (FID). Chromatographic conditions, in short, were as follows: column, injector, and detector temperatures 125, 180, and 230 °C, respectively. The separation column was a Poropak-Q column (2.6 mm × 2 m, U.S.A.). Nitrogen was used as the carrier gas at a flow rate of 30 mL/min. The standard curves were drawn from standard ethanol or acetaldehyde samples using the ratio of ethanol or acetaldehyde to isopropanol and were used to calculate the concentration of ethanol or acetaldehyde in the samples of the brain tissue homogenate.

Statistical Analysis. Data were expressed as the mean ± S.E.M. calculated from four mice of every group. Statistical comparisons of protective effects of the procyanidin fractions and other antioxidants on brain cell DNA damage induced by ethanol administration were made by means of one-way analysis of variance (ANOVA) followed by the Fisher's least significant difference (LSD) test.

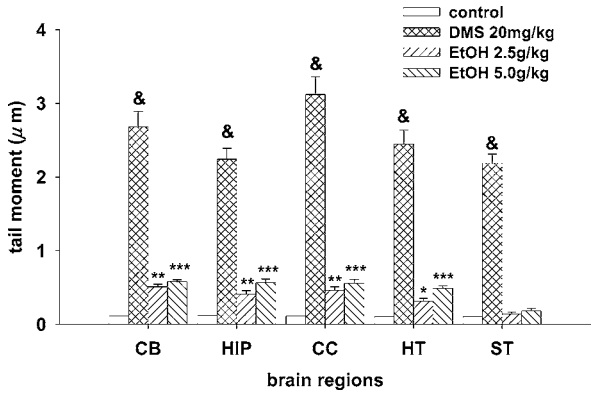

Figure 3. Effects of ethanol on DNA damage in five brain regions of the mice following chronic administration of ethanol (2.5 and 5.0 g kg^{-1}, p.o.) for 30 days. The extent of DNA damage was calculated from relative changes in tail moment length. Two hundred cells were examined in duplicate for each condition and the tail moments are expressed as the mean ± S.E.M. &, $P < 0.001$; ∗, $P < 0.05$; ∗∗, $P < 0.01$; ∗∗∗, $P < 0.001$ compared with the control group; $n = 4$.

Statistical analysis of the effects of ethanol on DNA single-strand breaks in different time points was performed using repeated measurements of ANOVA with the group as the between factor and time as the within factor, followed by a post hoc LSD test. On each time point, a one-way ANOVA followed by a post hoc LSD test was used to determine the difference between groups.

The level of significant was taken as $P < 0.05$. All statistical procedures were performed using the SPSS 13.0 software for windows (SPSS Inc., U.S.A.).

RESULTS

Chemical Composition and Purity of Grape Seed Procyanidin Fractions. Table 1 summarized the compositional data of the two procyanidin fractions isolated from grape seeds. All of these procyanidin fractions presented high purity (93.0 ± 1.3%, 92.2 ± 1.8%, respectively), with only trace amounts of minerals, sugar, and nitrogenous compounds (including proteins). Normal-phase HPLC and HPLC thiolysis analysis of this fraction permitted determination of the range of DP of procyanidins (from 2 to 12−15) and mDP (7.2). No catechins or other simple phenolics were detected in this fraction. The fraction of polymeric procyanidins had the degree of polymerization (DP) ranging from 10 to 30−33 (mean DP = 25.2), and the purity was over 92%. No catechins or other simple phenolics were detected in this fraction. These results provide base data for further studying their chemical or biological properties.

Effect of Acute Ethanol Administration on Brain Cells. Acute administration of ethanol, at the doses of 2.5 and 5.0 g kg^{-1} i.p., resulted in DNA single-strand breaks of brain cells in the cerebellum and hippocampus. The increase in DNA single-strand breaks in brain cells reached a peak at 4 h of post-ethanol administration when compared with that of the control group (one-way ANOVA; $p < 0.001$). No significant DNA damages were detected in the cerebral cortex, hypothalamus, and striatum. (**Figure 2**).

Effect of Chronic Ethanol Administration on Brain Cells. After chronic administration of ethanol, at the doses of 2.5 and 5.0 g kg^{-1} p.o., for 30 consecutive days, the DNA damages were detected in the cerebellum, hippocampus, cerebral cortex, and hypothalamus. The values of tail moment length (μm) were significantly increased when compared with those of the control groups (one-way ANOVA; $p < 0.001$). However, no significant DNA single-strand breaks were observed in the striatum. (**Figure 3**).

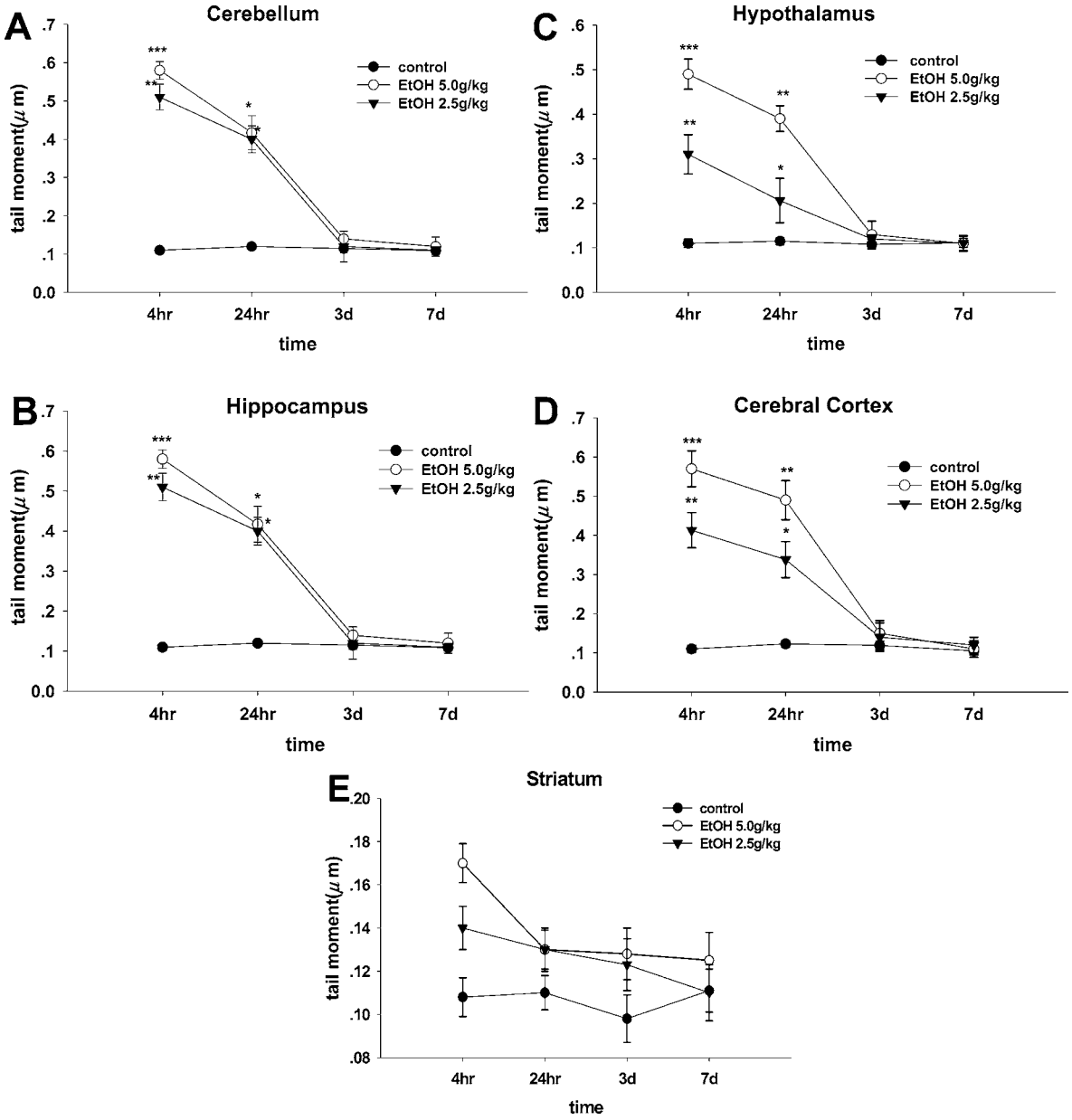

Figure 4. Time course of effects of ethanol withdrawal on brain cell DNA damage in five brain regions of the mice after 30 days of chronic ethanol (2.5 and 5.0 g kg^{-1}, p.o.) treatment. (**A**) Cerebellum (CB); (**B**) hippocampus (HIP); (**C**) cerebral cortex (CC); (**D**) hypothalamus (HT); (**E**) striatum (ST). Average tail moment lengths of DNA damage of brain cells at different times, post ethanol administration, are shown. Two hundred cells were examined in duplicate for each condition, and the tail moments are expressed as the mean ± S.E.M. ∗, $P < 0.05$; ∗∗, $P < 0.01$; ∗∗∗, $P < 0.001$ compared with the control group; $n = 4$.

Twenty-four hours after ethanol withdrawal, the DNA damages could also be detected in the cerebellum, hippocampus, cerebral cortex, and hypothalamus ($p < 0.001$ or $p < 0.05$). Three days after ethanol withdrawal, DNA damages returned to control levels. (**Figure 4**).

Protective Effects of the Procyanidin Fractions and Other Antioxidants on Brain Cell DNA Damage Induced by Acute Ethanol Administration. The protective effects of the isolated procyanidin fractions, ascorbic acid, vitamin E, and resveratrol on the DNA damages in the mouse cerebellum and hippocampus induced by acute ethanol administration at 5.0 g kg^{-1} i.p. are presented in **Figure 5**. The results show that all of these compounds significantly reduced ($p < 0.001$) the tail moment lengths induced by ethanol.

Protective Effects of the Procyanidin Fractions and Other Antioxidants on Brain Cell DNA Damage Induced by Chronic Ethanol Administration. Repeated treatment with the isolated procyanidin fractions, ascorbic acid, vitamin E, and resveratrol showed no adverse effects on DNA. However, all of these compounds could significantly protect DNA damage induced by chronic ethanol (5.0 g kg^{-1} p.o.) in the cerebellum, hippocampus, cerebral cortex, and hypothalamus ($p < 0.001$) (**Figure 6**).

Ethanol and Acetaldehyde Concentrations in Mouse Brain Regions after Acute Ethanol Administration. Our previous results showed that the peak time of ethanol concentration in the brain was 40 min after ethanol administration, detected by the microdialysis technique with GC (data not shown). Accordingly, 40 min after ethanol administration was chosen as the time point to detect the ethanol and acetaldehyde concentrations in brain tissue homogenate. The concentrations of ethanol and acetaldehyde in five brain regions are presented in **Figure 7**. The results show that the disposition of ethanol in brain regions was different after acute ethanol administration and the cerebel-

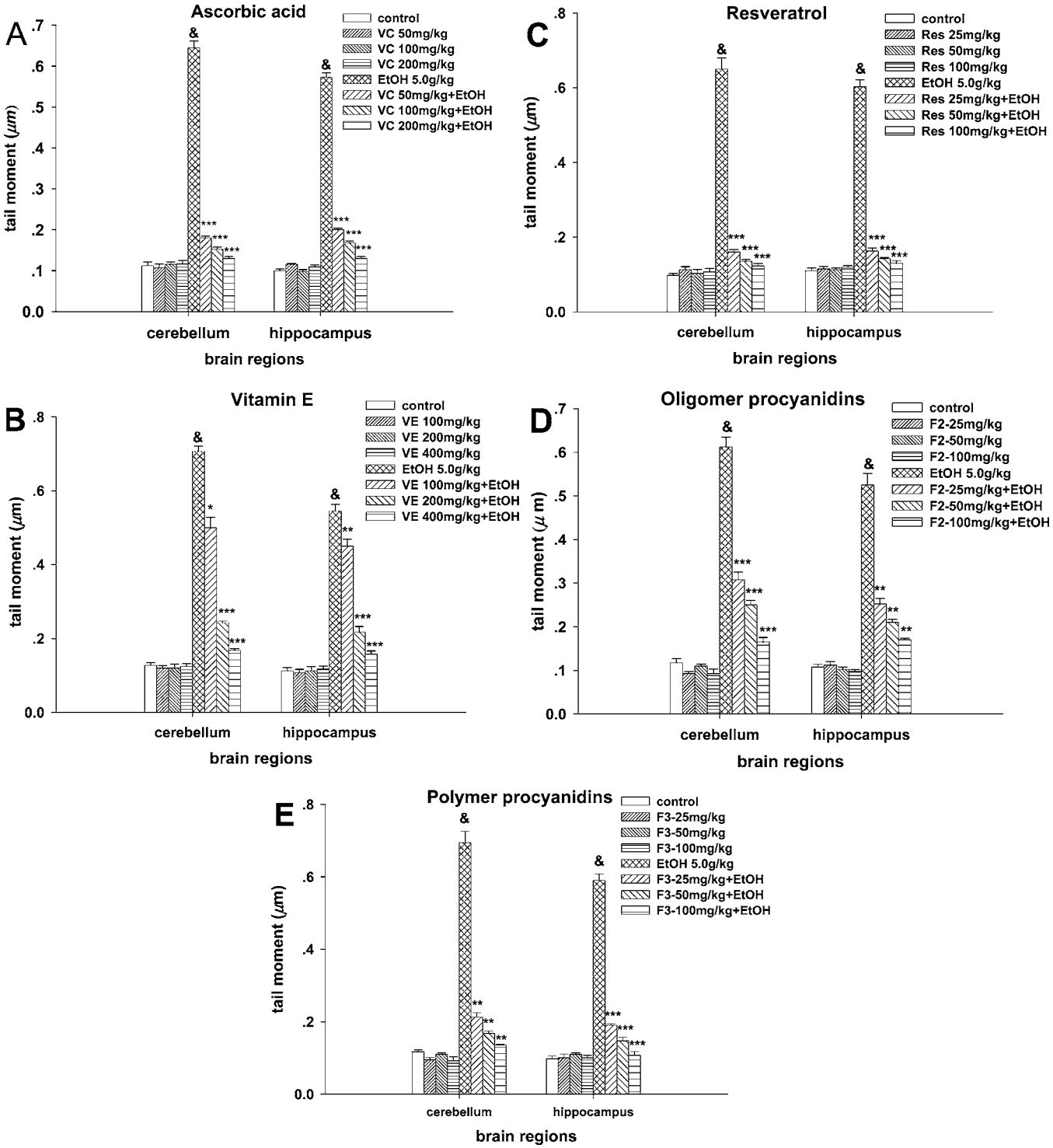

Figure 5. Protective effects of antioxidants on DNA damage in brain cells induced by acute ethanol (5.0 g kg^{-1}, i.p.) administration in the cerebellum and hippocampus of mice. (**A**) Ascorbic acid (VC); (**B**) vitamin E (VE); (**C**) resveratrol (Res); (**D**) oligomer procyanidins (F2); (**E**) polymer procyanidins (F3). Average tail moment lengths of DNA damage of brain cells at different times, post ethanol administration, are shown. Two hundred cells were examined in duplicate for each condition, and the tail moments are expressed as the mean ± S.E.M. ∗, $P < 0.05$; ∗∗, $P < 0.01$; ∗∗∗, $P < 0.001$ compared with the ethanol group. &, $P < 0.001$ compared with the control group; $n = 4$.

lum and hippocampus had higher ethanol concentration than other regions.

Ethanol and Acetaldehyde Concentrations in Mouse Brain Regions after Ethanol Plus Antioxidants Administration. After antioxidant treatment, the ethanol concentration in the cerebellum and hippocampus were lower than the single ethanol administration group. The results showed that procyanidins significantly reduced the ethanol concentration in the brain ($p < 0.001$) (**Figure 8A**), but there are no effects on acetaldehyde concentration (**Figure 8B**).

DISCUSSION

In this study, significant increases in DNA damage were observed in the cerebellum and hippocampus in mice by acute ethanol administration, and the peak increase in DNA damage was reached at approximately 4 h of post ethanol administration.

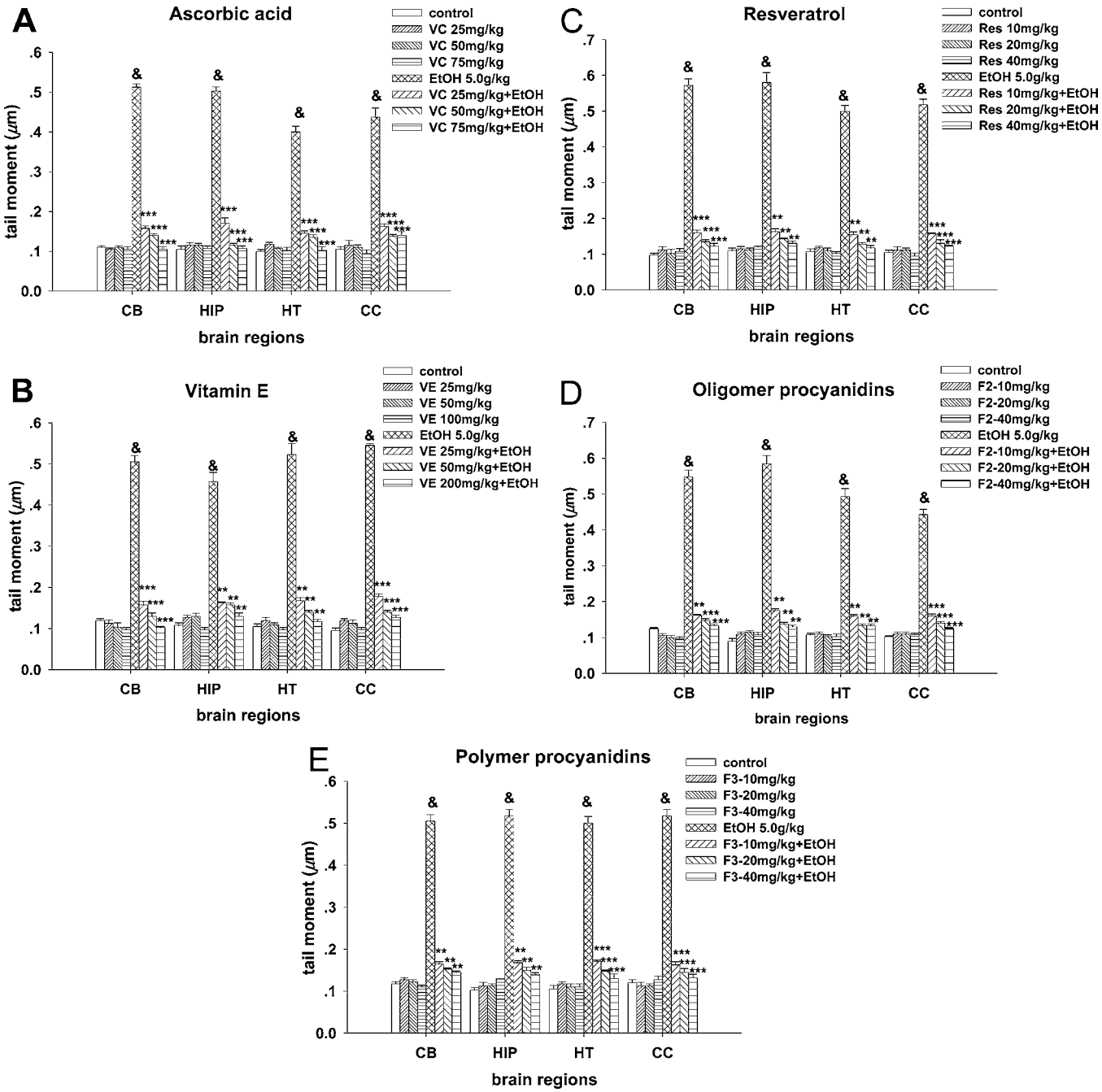

Figure 6. Protective effects of antioxidants on DNA damage in brain cells induced by chronic ethanol (5.0 g kg^{-1}, p.o.) administration in the cerebellum, hippocampus, hypothalamus, and cerebral cortex of mice. (**A**) Ascorbic acid (VC); (**B**) vitamin E (VE); (**C**) resveratrol (Res); (**D**) oligomer procyanidins (F2); (**E**) polymer procyanidins (F3). Average tail moment lengths of DNA damage of brain cells at different ethanol administration times are shown. Two hundred cells were examined in duplicate for each condition, and the tail moments are expressed as the mean ± S.E.M. ∗, $P < 0.05$; ∗∗, $P < 0.01$; ∗∗∗, $P < 0.001$ compared with the ethanol group. &, $P < 0.001$ compared with the control group; $n = 4$.

These results are similar to the previous reports, which showed that DNA damage peaked at 4 h after administration of ethanol at the dose of 4 g kg^{-1} in the rat (*4*, *27*). Chronic ethanol administration induced the DNA damages in the cerebellum, hippocampus, hypothalamus, and cortex, which could be considered as the targets of chronic ethanol toxicity in the brain. Renis et al. also showed significant DNA strand breaks in rat cerebellum and hippocampus using agarose gel electrophoresis after chronic ethanol administration (*28*). Interestingly, 3 days after ethanol withdrawal, DNA damage induced by chronic ethanol administration could be almost completely repaired, suggesting that the DNA auto-repairing ability in the brain is quite strong. However, even though the DNA damage would be auto-repaired, it is generally considered that DNA damage caused by chemicals increases the possibility of mutation and cancer (*27*).

Brain neurons are highly energetic cells with a high rate of production of reactive oxygen species which are known to be harmful to DNA. Biochemical characteristics and metabolic rates varied from region to region can lead to region-specific oxidative DNA damage, which could increase the vulnerability of specific brain regions to ethanol-induced oxidant stress. The present study showed that the disposition of ethanol in brain regions was different, and the cerebellum and hippocampus but not the striatum were more vulnerable to the effects of acute or chronic ethanol intake, suggesting the region-specific genotoxic action

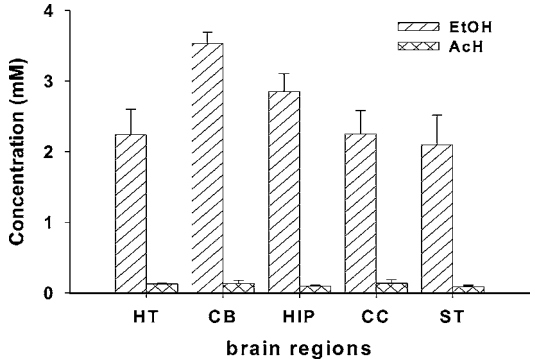

Figure 7. Ethanol and acetaldehyde concentrations in mice brain regions after acute ethanol (5.0 g kg^{-1}, i.p.) administration. CB: cerebellum; HIP: hippocampus; CC: cerebral cortex; HT: hypothalamus; ST: striatum; EtOH: ethanol; AcH: acetaldehyde. The concentrations of ethanol and acetaldehyde are expressed as the mean ± S.E.M. ($n = 7$ per group).

of ethanol in the brain. One of the possibilities for the striatum that is tolerant to ethanol toxicity, in terms of the parameters in the present study, might be the existence of high levels of ascorbic acid in this structure. Studies have shown that ethanol induces endogenous ascorbic acid release in the striatum (*29*, *30*), which as an antioxidant, suppresses ethanol-induced hydroxyl radical production (*31*).

Oxygen-derived free radicals have been implicated in many disease processes, including ethanol-induced neurotoxicity. Ethanol exposure has been shown to induce the generation of reactive free radicals in vitro (*32*) as well as in vivo (*31*, *33*). Therefore, it is reasonable to assume that antioxidants might have protective effects on ethanol-induced oxidative DNA damage. In the present study, it was clearly demonstrated that the isolated procyanidin fractions and other antioxidants such as ascorbic acid, vitamin E, and resveratrol could markedly decrease the levels of DNA single-strand breaks induced by ethanol.

Ascorbic acid, also called vitamin C, is a water-soluble antioxidant which takes a part in intracellular oxidation/reduction systems and in the binding of oxidants produced endogenously (*34*). Ascorbic acid can act as a first defense against free radicals formed after ethanol treatment and produces the protection against DNA damage. It is reported that ascorbic acid protects against ethanol-induced neurotoxicity in embryonic rat hippoc-

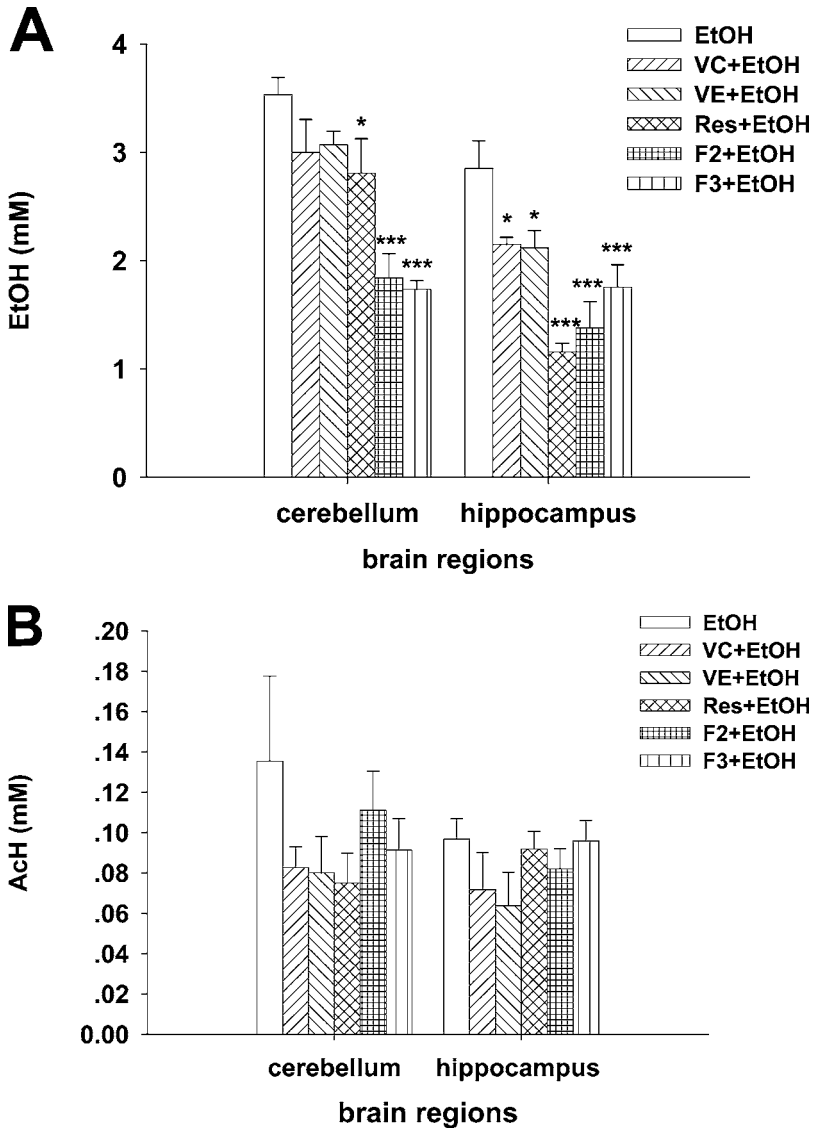

Figure 8. Ethanol and acetaldehyde concentrations in mice brain regions after ethanol (5.0 g kg^{-1}, i.p.) plus antioxidants administration. VC: ascorbic acid; VE: vitamin E; Res: resveratrol; F2: oligomer procyanidins; F3: polymer procyanidins; EtOH: ethanol; AcH: acetaldehyde. The concentrations of ethanol and acetaldehyde are expressed as the mean ± S.E.M. ($n = 7$ per group). ∗, $P < 0.05$ and ∗∗∗, $P < 0.001$ compared with the ethanol group.

ampus cultures (*7*) or in human brain glial cells (*35*). The protective action of ascorbic acid can be related to its scavenging of oxygen radicals and chemical repairing of damaged DNA. The present study using in vivo comet assay further demonstrated the protective action of ascorbic acid against ethanol-induced DNA damage in the brain, implicating the importance of keeping high levels of ascorbic acid in the brain after either acute or chronic intake of ethanol.

Vitamin E (a-tocopherol) is a most active lipid-soluble antioxidant and localizes in biological membrane. Its major function is attributed to protection of the cellular membrane from oxidative damage by free radicals (*36*, *37*). A number of studies have demonstrated the protective action of vitamin E, in vivo and in vitro, on DNA damage (*38*, *39*). DNA is damaged not only directly by free radicals but also by lipid peroxidation products (*40*, *41*). In the present experiments, vitamin E showed a significant protective effect against DNA damage when administered before ethanol treatment, suggesting that vitamin E protects not only cellular membrane but also DNA from oxidative damage.

Resveratrol (3,5,4′-trihydroxystilbene), a phytoalexin present in some food products including grapes (*42*), has recently attracted more attention for its high health protective activities, especially the protective effects against oxidative stress (*43*−*45*). The effect of resveratrol against oxidative DNA damage was also reported (*46*, *47*). In the present study, repeated oral administration of resveratrol could significantly protect against ethanol-induced DNA single-strand breaks, which was superior to the other antioxidants. The mechanism of action for resveratrol is related to its potential antioxidative activity and its interference with oxidative metabolism. For example, resveratrol likely reduces oxidative damage to purified DNA by scavenging the $^{\bullet}OH$ (*48*).

Procyanidins chemically belong to polyphenols and exist in many plant materials, including grapes. Grape seed extract has been shown to have an inhibiting effect on the accumulation of age-related oxidative DNA damage in the spinal cord and in various brain regions (*49*). The monomeric units of procyanidins, that is, catechins, have been reported to possess protective effects against DNA damage. For example, epigallocatechin 3-O-gallate (EGCG), the main catechin in green tea, reduces radical-induced DNA damage in primary leucocytes (*50*). An antioxidant cocktail containing β-catechin shows the free-radical scavenging activities and antagonizes DNA damage (*51*). It is suggested that catechins contribute to the protection of oxidative damage in biological systems by quenching $^1O_2$ (*52*). In the present experiments, the effects of oligomeric and polymeric procyanidin fractions isolated from grape seeds on ethanol-induced DNA damage were also verified. Previous studies have demonstrated that these compounds could significantly inhibit ethanol-induced hydroxyl radical production in the striatum (*53*). Therefore, it is reasonable to assume that scavenging of the ethanol-induced formation of oxidative species mainly contributes to the protective activities of procyanidins for the DNA damage induced by acute and chronic ethanol administration.

In the present studies, it was found that both lipid-soluble and water-soluble antioxidants showed the protective effects against ethanol-induced DNA damage. Thus, it is likely that the possible mechanisms of DNA damage induced by ethanol include processes both in the lipid and water compartments of the brain cells. A similar hypothesis was mentioned for antioxidants against $KBrO_3$-induced oxidative DNA damage (*45*). Furthermore, all of the antioxidants could decrease the ethanol concentrations in brain regions after acute ethanol administration, which suggests that one of the potential protective mechanisms of the antioxidants is the modification of the pharmacokinetics of ethanol.

In conclusion, the present study provided the direct in vivo evidence that acute or chronic intake of ethanol induced the oxidative DNA damage in certain brain regions, while the grape seed procyanidins and other antioxidants such as ascorbic acid, vitamin E, and resveratrol could prevent the ethanol-induced genotoxicity in the brain. In other words, the grape seed procyanidins and other antioxidants can be used in the prevention and treatment of the ailments in the central nervous system induced by alcohol abuse. Because grape seed is a byproduct of vinification and it is very rich in procyanidins (*9*−*10*, *14*), the use of grape seed procyanidins as alternative natural antioxidants to, for example, protect DNA damage would have importance from the practical and economical point of view.

ABBREVIATIONS USED

SCGE, single cell gel electrophoresis; LMA, low-melting agarose; NMA, normal-melting agarose; DP, degree of polymerization; mDP, mean degree of polymerization; GC, gas chromatography.

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