Effects of Polyphenol Substances Derived from Theobroma cacao on Gastric Mucosal Lesion Induced by Ethanol

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The antiulcer activity of cacao liquor water-soluble crude polyphenols (CWSP) was examined. CWSP, α-tocopherol, sucralflate (500 mg/kg), and cimetidine (250 mg/kg) were orally administered to male SD rats 30 minutes before ethanol treatment. 5 ml/kg of ethanol given intragastrically caused lesions in mucosa of the glandular stomach. CWSP caused a reduction of such hemorrhagic lesions as well as cimetidine and sucralflate which are typical antiulcer drugs, but α-tocopherol was less effective. Thiobarbituric acid reactive substances in gastric mucosa significantly increased with ethanol administration. CWSP treatment significantly reduced this change. The administration of ethanol extensively increased myeloperoxidase (MPO) but not xanthine oxidase (XOD) activity. CWSP reduced the activities of both enzymes; they were considered the main sources of oxygen radicals. According to an in vitro study, CWSP directly reduced XOD but not MPO. These results suggest that the antiulcer mechanism of CWSP was not only radical scavenging but also modulation of leukocyte function.

Key words: cacao liquor; polyphenols; antioxidant; gastric mucosal injury; ethanol

Introduction

It has been reported that free radicals are important in the pathogenesis of gastric lesions in some models. Enzymatic antioxidants such as superoxide dismutase (SOD), catalase, and combinations of them are effective in ulcers induced by ischemia-reperfusion,¹² acute stress,³⁴ non-steroidal anti-inflammatory drugs,⁵⁶ and ethanol.⁷⁻⁹ The lipid peroxide level in gastric mucosa was also apparently elevated.⁵⁻⁶ In the same case, the main source of reactive oxygen species seems to be xanthine-xanthine oxidase¹⁰⁻¹¹ and activated polymorphonuclear leukocytes.¹²⁻¹³ Otherwise, it was reported that cacao liquor contained potent antioxidants such as epicatechin, catechin,¹⁴ cloveamide, quercitin, and their glucosides.¹⁵ Additionally, cacao liquor or extracts showed radical scavenging activities in vitro¹⁶ and in vivo.¹⁷

The purpose of this work was to study cacao liquor-derived polyphenols as antioxidant in gastric mucosal injury induced by ethanol administration.

Materials and Methods

Materials. Cacao liquor water-soluble polyphenols (CWSP) were prepared as in a previous report.¹⁶ Cacao liquor was defatted with ethyl ether and extracted with 5-fold boiling water for 30 min. The extract was concentrated in vacuo and put on a Sephadex LH20 (Pharmacia Co. Ltd.) column for chromatography, and eluted with water with stepwise increases in the ratio of acetone. The 30% acetone elution was collected and freeze-dried. Total polyphenols concentration of this fraction was measured by the Prussian blue method,¹⁸ and the catechin content was measured by HPLC.¹⁹ A typical HPLC pattern is shown in Fig. 1. This fraction contained approximately 55% polyphenols, with catechin concentrations at about 28% (Table 1). Quercetin and other phenolic substances were not detected.

α-Tocopherol, cimetidine, thiobarbituric acid, xanthine, xanthine oxidase, and myeloperoxidase were purchased from Sigma Chemical Co. Sucralflate was obtained from Chugai Pharmaceutical Ltd. Japan. The other chemicals were reagent grade, obtained from Wako Pure Chemical Industries.

In vivo study. Animals Male Sprague-Dawley rats, 9 weeks old and weighting 250-270 g, were used. The animals were obtained from Clea Japan Inc.

Experimental ulcers. Twenty-four hours before the experiment, the rats were deprived of food. They had free access to drinking water. During the period of starva-

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<th>Table 1. Concentrations of Total Polyphenols, Catechin, and Epicatechin in CWSP</th>
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<td>Concentrations (%)</td>
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<td>Total polyphenols</td>
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Abbreviations: CWSP, cacao liquor water-soluble crude polyphenols; TBARS, thiobarbituric acid reactive substances; MPO, myeloperoxidase; XOD, xanthine oxidase; PMN, polymorphonuclear leukocyte.

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tion, animals were housed individually to avoid coprophagy. Test chemicals were dissolved or suspend-
ed in 0.1 w/v% carboxyl methyl cellulose (CMC). Five ml/kg body weight of test solution were given to the animals intragastrically 30 minutes before the administration of 5 ml/kg of ethanol. After 60 minutes, animals were killed under anesthesia and their stomachs were removed, opened along the curvature and rinsed with physiological saline. The degree of gastric mucosal damage was evaluated by a computerized video scanning system (PCA-II System Science Co.). The gastric mucosa membrane was collected and frozen at −80°C until use.

Measurement of protein. Protein was measured by the method of Lowly et al.19

Measurement of lipid peroxidation. The gastric mucosa was homogenized with 1.15% KCl solution to obtain a 10% homogenate solution. The level of thiobarbituric acid reactive substances (TBARS) in the gastric mucosa was measured by the method of Ohkawa et al.20

Measurement of xanthine oxidase (XOD) activity. XOD activity was measured by the method of Tanaka et al.21 Gastric mucosa homogenate was centrifuged at 4000 g for 10 minutes at 4°C, and the supernatant was collected. XOD-catalyzed uric acid formation in the gastric mucosa was monitored spectrophotometrically at 550 nm. The reaction mixture consisted of 30 mM of a phosphate boric acid buffer (pH 8.2), 0.6 mM xanthine, and 1 mg protein of the sample. The level of uric acid in the reaction mixture was measured by a Wako uric acid test kit after incubation for 3 hours at 37°C.

Measurement of myeloperoxidase (MPO) activity. MPO activity was measured by the method of Thomas et al.21 The reaction mixture consisted of 117 mM acetate buffer (pH 5), 0.4 mM tetramethyl benzidine, 0.3% H₂O₂, and 1 mg protein of the sample. Absorbance at 655 nm of mixture was immediately recorded for 5 minutes. Activity was calculated from optical density per minute.

In vitro study Effects of CWSP on XOD activity. The direct effects of CWSP on XOD were studied by the method of Maccio et al.22 Fifteen μM xanthine and various concentrations of CWSP in 0.1 mM phosphate buffer were poured into a quartz cuvette and measured for reference. The reaction was started by adding 10 U of XOD, and the absorbance was recorded at 295 nm for 3 min at room temperature.

Effects of CWSP on MPO activity. The assay was done by the method of Thomas et al. with a slight modification. Various concentrations of CWSP were put in a reaction mixture with 5 U of MPO and absorbance at the 655 nm was measured as described.

Statistical analysis. Results were expressed as mean ± S.D. All analyses were done using SPSS Statistical Software. Mean values were calculated by ANOVA and multiple range comparisons or Student’s t-test. Values of p < 0.05 were considered significant.

Results
In vivo study
Experimental ulcer
1) Dose finding study. To decide on the appropriate CWSP dosage, the following study was done. Five animals of each group were treated with 250, 500, or 1000 mg/kg of CWSP. The reduction rates of gastric mucosal lesion were 32.5 ± 19.3, 79.6 ± 10.3, and 80.5 ± 5.4% compared with the 0.1% CMC treatment group. Almost all of the animals of the 1000 mg/kg treatment group were observed to have CWSP remains in the stomach.

2) Main study. Male SD rats were divided into five groups of 10 animals. Each groups were treated with 0.1% CMC, 500 mg/kg of CWSP, α-tocopherol, sucralfate or 250 mg/kg of cimetidine.

Five ml/kg of ethanol given intragastrically consistently caused lesions in the mucosa of the glandular stomach in the control group. As shown in Fig. 2, CWSP, cimetidine, and sucralfate given orally 30 minutes before the administration of ethanol markedly reduced the damaged area by 82.7, 73.2, and 90.6%, respectively. α-tocopherol, a typical antioxidant, slightly reduced the damaged area by 39.2%. The level of TBARS in the gastric mucosa, as the index of lipid peroxidation, increased 60 minutes after ethanol administration. This change was significantly inhibited by CWSP treatment (Fig. 3).

As shown in Fig. 4, XOD activity did not change even after ethanol administration, and CWSP greatly inhibited the activity of this enzyme. The level of TBARS in the stomach did not correlate with the XOD activity in the stomach (r² = 0.232).

Changes in MPO activity are shown in Fig. 5. MPO activity increased significantly according to the mucosal damage. Treatment with of CWSP significantly prevent-
Antiulcer Effect of Cacao Polyphenols

Fig. 2. Effects of α-Tocopherol, CWSP, Cimetidine, and Sucralfate on Gastric Mucosal Injury by Ethanol Administration.
Significantly different from ethanol; *: p<0.01, **: p<0.001.

Fig. 3. Effects of CWSP on TBARS in Gastric Mucosa after Administration of Ethanol.
Significantly different from no treatment; +: p<0.01.
Significantly different from ethanol; *: p<0.01.

Fig. 4. Effects of CWSP on XOD Activity in Gastric Mucosal Lesions Induced by Ethanol Administration.
Significantly different from ethanol; *: p<0.001.

The rise of MPO activity. The correlation between MPO activity and TBARS was positive (r²=0.634).

In vitro study
All experiments were done three times, and a typical result is shown.

Effects of CWSP on XOD activity
As shown in Fig. 6, CWSP inhibited XOD activity in a dose-dependent manner up to 100 μg/ml. Fifty percent of the inhibition concentration was 39.78 μg/ml.

Effects of CWSP on MPO activity
CWSP did not inhibit MPO activity directly up to 1000 μg/ml (data not shown).

Discussion
Recently, many reports suggest the important role of active oxygen radicals in gastric lesions. In the case of gastric ulcers induced by ethanol, it was reported that lipid peroxide as TBARS in the mucosa increased greatly. We confirmed that phenomenon in this study (Fig. 3). Treatment with CWSP, which have potent antioxidative activity, inhibited the rise of TBARS. At the same time, CWSP reduced hemorrhagic lesions, like cimetidine and sucralfate, which are typical antiulcer drugs (Fig. 2).

Much of the work on the source of active oxygen radicals in gastric lesions has been focused on XOD. However, recent studies suggest the possibility that XOD was not the main source of radicals. In our study, XOD activity in the mucosa did not rise even after ethanol administration. The result showed that the major source of radicals was not xanthin-XOD systems, at least in this model. CWSP treatment markedly inhibited XOD activity not only in vitro (Fig. 4) but also in...
vivo (Fig. 6). We found that a high concentration of CWSP was in the stomach just after administration, thus XOD in the mucosa might be inhibited under these conditions.

Contrarily, glanocytes, especially PMN, are considered to cause gastric mucosal injury by infiltrating into the inflammation area and generating oxygen radicals.2,12,13 The activity of MPO, which is a marker enzyme of leukocytes, was thought to represent leukocyte migration to the injured tissue.12,13 In order to estimate the number of leukocytes, we measured MPO activity in the gastric mucosa. We found a significant increase in MPO activity after ethanol treatment (Fig. 5). In addition, CWSP did not inhibit MPO activity in vitro. Therefore, activated PMN, which infiltrated into the inflammation area, seems to have contributed to the rise of lipid peroxide by generating active oxygen. According to the previous report,16 polyphenolic substances derived from cacao liquor inhibited O2− and H2O2 generation of human leukocyte including PMN activated by mitogen such as phorbol myristate acetate. CWSP also reduced mitogens as phytotoxemagglutinin that induced proliferation of human leukocytes.

In conclusion, the results of this study indicate that the mechanism of the antulcer effect of CWSP is reduction of migration of activated leukocytes to the inflammation area and so, the following attack of oxygen radicals generation by these cells.

α-Tocopherol, which is a known potent antioxidant, had cytoprotective effects on various organs.23,24 However, in the case of experimental gastric ulcer, α-tocopherol was not effective or showed only a slight effect.25,26 In this study, treatment with α-tocopherol also had only weak activity. We summarized that the mechanism of CWSP was not only inhibition of lipid peroxidation, according to these findings.

Further studies are required to discover of physiological effects and their mechanisms of cacao polyphenols.

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References