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, sacurfate (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 sacurfate 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, acute stress, non-steroidal anti-inflammatory drugs, and ethanol. The lipid peroxide level in gastric mucosa was also apparently elevated. In the same case, the main source of reactive oxygen species seems to be xanthine-xanthine oxidase and activated polymorphonuclear leukocytes.

Otherwise, it was reported that cacao liquor contained potent antioxidants such as epicatechin, catechin, clovamide, quercetin, and their glucosides. Additionally, cacao liquor or extracts showed radical scavenging activities in vitro and in vivo.

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. 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, and the catechin content was measured by HPLC. 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 oxydase, and myeloperoxidase were purchased from Sigma Chemical Co. Sacurfate 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-

| Table 1. Concentrations of Total Polyphenols, Catechin, and Epicatechin in CWSP |
|-----------------|-------------|
| Concentrations (%) |
| Total polyphenols | 55.0        |
| Catechin         | 18.5        |
| Epicatechin      | 9.6         |

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 adminis-
tration 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 scan-
ning 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 Lowry et al.  

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.  

Measurement of xanthine oxidase (XOD) activity.  
XOD activity was measured by the method of Tanaka et  

al.  

Measurement of myeloperoxidase (MPO) activity.  
MPO activity was measured by the method of Thomas  
et al.  

bance at 655 nm of mixture was immediately recorded  
for 5 minutes. Activity was calculated from optical den-
sity 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.  

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 absorb-
ance 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

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 consist-
tently 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%, re-
spectively. 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 ad-
ministration. 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 inhib-
ited 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 ($r^2=0.634$).

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.1-8 In the case of gastric ulcers induced by ethanol, it was reported that lipid peroxide as TBARS in the mucosa increased greatly.9 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.10,11 However, recent studies2,12,13 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.\(^2,12,13\) 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 O\(_2^*\) and H\(_2\)O\(_2\) generation of human leukocyte including PMN activated by mitogen such as phorbol myristate acetate. CWSP also reduced mitogens as phytomagneticulin B 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.

\(\alpha\)-Tocopherol, which is a known potent antioxidant, had cytoprotective effects on various organs.\(^22,24\) However, in the case of experimental gastric ulcer, \(\alpha\)-tocopherol was not effective or showed only a slight effect.\(^25,26\) In this study, treatment with \(\alpha\)-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


