Kurosu, a Traditional Vinegar Produced from Unpolished Rice, Suppresses Lipid Peroxidation in Vitro and in Mouse Skin

Shoko Nishidai, Yoshimasu Nakamura, Koji Torikai, Mikako Yamamoto, Nobuhiro Ishihara, Hirotaka Mori, and Hajime Ohigashi

Research Center, Tamanoi Vinegar Co. Ltd., 100 Nishimachi, Yamatokiyama, Nara 639-1038, Japan
* Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, Kyoto 606-8502, Japan

Received March 15, 2000; Accepted April 28, 2000

The in vitro antioxidative activities of various kinds of vinegar were investigated by using a linoleic acid autoxidation model detected by the thiobarbituric acid (TBA) method and the 1,1-diphenyl-2-picrylhydrazyl radical system. An ethyl acetate extract of Kurosu (EK), a vinegar made from unpolished rice, exhibited the highest antioxidative activity in both systems. EK (5 mg) inhibited 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced edema formation (14%) and myeloperoxidase activity (52%, P < 0.01) in female ICR mouse skin. Furthermore, EK significantly suppressed double TPA application-induced H2O2 generation (53%, P < 0.01) and lipid peroxidation determined by the TBA-reacting substance level (95%, P < 0.01). In a two-stage carcinogenesis experiment with dimethylbenz[a]anthracene/TPA, EK significantly reduced the number of tumors per mouse by 36% (P < 0.05) at 15 weeks after promotion. These results suggest that the antitumor-promoting effect may be partially due to the antioxidative properties of EK such as the decomposition of free radicals and interference with free radical-generating leukocytes.

Key words: antioxidative activity; Kurosu; vinegar; phorbol ester; tumor promotion

Vinegar, which can be made from rice, apple, wine and various other materials, is a widely used acidic seasoning.2) Vinegar also has medicinal uses due to such physiological functions as digestive, appetite stimulating and exhaustion recovering effects.2) Kurosu, which is produced from unpolished rice through stationary surface acetic acid fermentation, is one of the most common traditional vinegars in Japan and is characterized by the higher contents of amino acids and organic acids than other vinegars.

This type of vinegar has recently been shown to improve blood fluidity, which leads to the prevention of hypertension.3) Thus, the bioregulatory functions of Kurosu have recently been attracting a great deal of attention.

Reactive oxygen species (ROS) induce membrane damage, DNA base oxidation, DNA strand breaks, chromosomal aberrations and protein alterations.4) Some ROS attack unsaturated fatty acids and cause oxidative damage to the cell membrane. Therefore, lipid peroxidation is thought to be closely associated with aging, atherosclerosis and carcinogenesis.5) There is increasing interest in the protective function of foods and their constituents against oxidative stress caused by ROS.6) However, there have been few studies on the antioxidative activity of vinegar. In this study, we found that the antioxidative activity of an ethyl acetate extract of Kurosu (EK) was much higher than that of the extracts of other types of vinegar in vitro. We also demonstrate that EK showed anti-inflammatory and antitumor promoting effects in mouse skin.

Materials and Methods

Materials and chemicals. Vinegar samples; Kurosu, rice vinegar, grain vinegar, apple vinegar and wine vinegar were obtained from Tamanoi Vinegar Co. (Nara, Japan). 12-O-Tetradecanoylphorbol-13-acetate (TPA), 1,1-diphenyl-2-picrylhydrazyl (DPPH) and dimethylbenz[a]anthracene (DMBA) were purchased from Nacalai Tesque (Kyoto, Japan). 6-Hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid (Trolox) was purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.), all other

1 To whom correspondence should be addressed. Tel: +81-75-753-6281; Fax: +81-75-753-6284; E-mail: ohigashi@kais.kyoto-u.ac.jp

Abbreviations: ROS, reactive oxygen species; EtOAc, ethyl acetate; EK, EtOAc extract of Kurosu; TPA, 12-O-tetradecanoylphorbol-13-acetate; DPPH, 1,1-diphenyl-2-picrylhydrazyl; DMBA, dimethylbenz[a]anthracene; Trolox, 6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid; TBA, thiobarbituric acid; TBARS, TBA-reacting substances; MPO, myeloperoxidase; IE, inhibitory effect
chemicals being purchased from Wako Pure Chemical Industries (Osaka, Japan).

**Extraction of the vinegar.** Vinegar (1 l) was extracted with EtOAc (1 l) at room temperature. The EtOAc-soluble fraction was mixed with a saturated sodium hydroxide carbonate solution (1 l) to remove the organic acids. The EtOAc phase was filtered and evaporated *in vacuo* and the aqueous phase was evaporated and lyophilized. Each concentrated sample was dissolved in dimethyl sulfoxide and used for the antioxidative assays.

**Total phenolic content.** The total phenolic content of the extract from each vinegar sample was analyzed by the method reported by Swain. The value for total phenolic content is expressed in terms of the gallic acid equivalent.

**Antioxidative activity in a linoleic acid autoxidation system.** The antioxidative activity in a linoleic acid autoxidation system was determined by the colorimetric thiobarbituric acid (TBA) method. A test sample (5 μl) was added to a mixture of linoleic acid (7 μl), 99.0% distilled ethanol (150 μl), and a 0.05 M phosphate buffer (pH 7.0, 1.35 ml). The solution was mixed in a test tube and incubated at 37°C under aerobic condition. To this reaction mixture (100 μl) were added 4% 3(2)-t-butyl-4-hydroxyanisole in ethanol (25 μl), 0.67% TBA (750 μl) and a 50 mM acetate buffer (pH 3.5, 750 μl). The mixture was then heated at 100°C for 1 hour. The absorbance of the solution at 532 nm was measured, and the degree of peroxidation was determined against a standard line obtained from TBA-reactive substances (TBARS) formation with authentic malondialdehyde.

**Measurement of DPPH radical scavenging activity.** The DPPH radical scavenging activity was evaluated as reported previously with slight modifications. Trolox was dissolved in ethanol. A test sample mixed with a 100 mM Tris-HCl buffer (pH 7.4, 1 ml) was added to 0.5 mM DPPH in ethanol (1 ml), and the mixture was shaken vigorously and left to stand for 20 min at room temperature in the dark. The DPPH radical scavenging activity is expressed as the ratio of the relative decrease in the absorbance of the test sample mixture at 517 nm to that of the 1 mM Trolox solution: DPPH radical scavenging activity (%) = ((vehicle) – (test compound)) / ((vehicle) – (1 mM Trolox)) × 100.

**Treatment of the animals.** Female ICR mice (7 weeks old) were obtained from Japan SLC (Shizuoka, Japan). The mice were fed with fresh tap water and rodent pellets and were maintained in a room with a controlled temperature of 25 ± 2°C under a 12-h light/dark cycle. The back of each mouse was shaved with surgical clippers 2 days before each experiment. EK (0.5 or 5 mg/100 μl in acetone) was topically applied to the shaved area of dorsal skin 30 min before the application of a TPA solution (5 μg/100 μl in acetone). In the double-treatment protocol, the same doses of TPA and of the test sample were applied twice at an interval of 24 h. The experiments were carried out under the Kyoto University guidelines for the ethical treatment of laboratory animals.

**Anti-inflammatory test on mouse skin.** The anti-inflammatory effects were examined as reported previously. The mice were killed 18 h after the TPA treatment and two markers for skin inflammation, *i.e.*, skin edema formation and myeloperoxidase (MPO) activity, were measured by the method of Nakamura et al.

**Determination of H₂O₂ in the mouse skin.** Determination of the H₂O₂ level was performed as reported previously. In the double-treatment protocol, the mice were sacrificed 1 h after the second TPA treatment. The H₂O₂ content was determined by the phenol red-horseradish peroxidase method.

**Determination of TBARS in mouse epidermis.** The TBARS level in the mouse epidermis was determined as reported previously. In the double-treatment protocol, the mice were sacrificed 1 h after the second TPA treatment. The final results are expressed as equivalents of nanomoles of malondialdehyde per cm².

**Two-stage carcinogenesis experiment in mouse skin.** The two-stage carcinogenesis experiment was performed as reported previously. One group was composed of 15 female ICR mice. The shaved backs of 7-week-old mice were treated with DMBA (50 μg/100 μl in acetone). One week after initiation, the mice were treated with TPA (1 μg/100 μl in acetone) twice a week for 20 weeks. In the inhibitor-treatment test, the mice were treated with EK (0.1 mg or 1 mg/100 μl in acetone) 40 min before the TPA treatment. The antitumor promoting activity was evaluated by both the ratio of tumor-bearing mice and by the number of tumors (more than 1 mm in diameter) per mouse.

**Statistical analysis.** A statistical analysis was performed by Student’s t-test.

**Results and Discussion**

**EtOAc extraction of the vinegar samples and total phenolic contents**

Partitioning with EtOAc yielded the EtOAc extract and the EtOAc-insoluble fraction. The total phenolic contents of the EtOAc extracts are shown in Table 1,
Table 1. Yields of the EtOAc Extract, EtOAc-insoluble Fraction, Total Phenolic Content and Color

<table>
<thead>
<tr>
<th></th>
<th>EtOAc extract (mg/l)</th>
<th>EtOAc-insoluble fraction (mg/l)</th>
<th>Total phenolic content (µg/mg of extract)</th>
<th>Color</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kurosu</td>
<td>71</td>
<td>24,000</td>
<td>112</td>
<td>dark brown</td>
</tr>
<tr>
<td>Rice vinegar</td>
<td>9.2</td>
<td>72,000</td>
<td>64</td>
<td>yellow</td>
</tr>
<tr>
<td>Grain vinegar</td>
<td>12</td>
<td>5,600</td>
<td>56</td>
<td>yellow</td>
</tr>
<tr>
<td>Apple vinegar</td>
<td>7.0</td>
<td>23,000</td>
<td>84</td>
<td>brown</td>
</tr>
<tr>
<td>Wine vinegar</td>
<td>12</td>
<td>36,000</td>
<td>34</td>
<td>brown</td>
</tr>
</tbody>
</table>

Antioxidative activity in a linoleic acid autoxidation system

The antioxidative activities of EK and of the rice, grain, apple and wine vinegar samples in a linoleic acid autoxidation system were determined by the TBA method. The level of TBARS is a well-known biomarker for the overall oxidative damage to cellular constituents including membrane lipids. As shown in Fig. 1, EK (0.1 mg/ml) completely inhibited linoleic acid autoxidation (inhibitory effect; IE = 97%). This activity was significantly higher than that of the EtOAc extract from any other vinegar sample (P < 0.001). The antioxidative activity of EK was comparable to that of α-tocopherol. The EtOAc-insoluble fraction from any of the vinegar samples showed little activity, even at 6 mg/ml (data not shown). These results suggest that Kurosu must contain some lipophilic antioxidants. The antioxidative activity of EK (0.3 mg/ml) continued for up to 9 days (Fig. 2). This strong and sustainable inhibitory activity against nonenzymatic lipid oxidation suggests the potential utilization of Kurosu constituents as effective antioxidative agents in food.

Radical scavenging activity in a DPPH radical system

Figure 3 shows the dose-dependent radical scavenging activity of EtOAc extracts from all the vinegar samples by using a representative colorimetric system with a stable DPPH radical. The radical scavenging activities of EK and of the EtOAc extract of apple vinegar were enhanced with increasing concentration (50% radical scavenging activity = 154 and 288 µg/ml, respectively), while the extracts from the rice, grain and wine vinegar samples showed little ac-

![Fig. 1. Antioxidative Activity of EtOAc Extracts of the Vinegar Samples against Lipid Autoxidation.](image1)

![Fig. 2. Time-dependent Changes in the TBARS Level in a Lipid Autoxidation System and in the Inhibitory Effects of EK and α-Tocopherol.](image2)
Table 2. Inhibitory Effect of EK on the TPA-induced Inflammatory Response and H₂O₂ Generation in Mouse Skin

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Edema (mg/punch) m ± SD*</th>
<th>IE (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone/TPA</td>
<td>84.0 ± 10.9</td>
<td>—</td>
</tr>
<tr>
<td>EK/TPA</td>
<td>72.6 ± 15.7</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>MPO (units/punch) m ± SD</td>
<td>IE (%)</td>
</tr>
<tr>
<td></td>
<td>1.62 ± 0.46</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>0.78 ± 0.03b</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>H₂O₂ (nmol/punch) m ± SD</td>
<td>IE (%)</td>
</tr>
<tr>
<td></td>
<td>4.32 ± 1.25</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>2.02 ± 0.87b</td>
<td>53</td>
</tr>
</tbody>
</table>

* Each value is presented as the mean ± standard deviation (n = 5).

**b Significantly different from acetone/TPA as determined by Student’s t-test, P < 0.01.

Fig. 3. Radical Scavenging Activities of EK (●), Rice (○), Grain (▲), Apple (△), and Wine (■) Vinegar Extracts.

Each value is shown as the mean (n = 3). The maximal SD for each experiment was 5%.

tivity (50% radical scavenging activity > 500 μg/ml). EK again exhibited the highest radical scavenging activity among the vinegar samples tested. The higher content of phenolic compounds in EK (Table 1) may, at least in part, contribute to this radical scavenging activity and thus to the antioxidative activity toward lipid peroxidation. While ferulic acid, a well-known antioxidant in rice and in fermented rice products, has recently been isolated from EK (unpublished results), its level in EK is thought to be too low to explain the strong antioxidative activity of EK. Purification and further identification of the antioxidative constituents are currently underway in our laboratory.

Inhibitory effect on oxidative stress in mouse skin

As EK showed potent antioxidative activities in vitro, we examined its inhibitory effect on the TPA-induced inflammatory responses and oxidative stress in mouse skin. As shown in Table 2, pretreatment with EK at a dose 1000-fold higher than that of TPA (5 μg) 30 min before TPA application inhibited skin edema formation (IE = 14%), and also significantly reduced MPO activity (IE = 52%, P < 0.01). These results indicate that EK effectively suppressed the infiltration of ROS-producing leukocytes in the inflamed regions. Next, the inhibitory effect of EK on double TPA application-induced H₂O₂ generation in mouse skin was evaluated. A marked decrease in the H₂O₂ level was observed by a double pretreatment with EK at a dose 1000-fold higher than that of TPA (IE = 53%, P < 0.01). We subsequently examined the inhibitory effect on TPA-induced lipid peroxidation, as quantified by the amount of TBARS, which was potentially formed downstream of the H₂O₂ generation. As shown in Fig. 4, EK at 100- or 1000-fold doses of TPA potently inhibited TBARS formation in the mouse epidermis (IE = 85%, P < 0.01 and IE = 95%, P < 0.001, respectively). MPO is a lysosomal enzyme which catalyzes the formation of hypochlorous acid from a substrate H₂O₂. Hypochlorous acid is highly toxic, mutagenic, and also activates carcinogens. The observation that EK potently inhibited both MPO activity and H₂O₂ generation strongly suggests that EK may effectively suppress the generation of hypochlorous acid in an inflamed region, and thus inhibit oxidative damage.
Antioxidative Activity of Kurosu

Antitumor promoting activity in mouse skin

Oxidative stress is widely accepted as important in the mechanisms for tumor promotion. Since EK inhibited the oxidative stress in mouse skin, we evaluated in vivo the antitumor promoting activity of EK by a two-stage carcinogenesis experiment in mouse skin. The mice were treated with EK at the dose 100- or 1000-fold higher than that of TPA (1 μg in 100 μl acetone) 40 min before each TPA treatment. The antitumor promoting activity was determined by both the tumor incidence and the number of tumors per mouse. EK at both a 100- and 1000-fold dose relative to TPA did not reduce the tumor incidence (data not shown). In the group treated with EK at 1000-fold dose of TPA, the average number of tumors per mouse was markedly reduced by 36% (P<0.01) at 15 weeks, although this effect was not significant from 16 weeks. The average number of tumors per mouse in the control group reached a maximum of 23 per mouse at 15 weeks, and the increase stopped at this time point. However, the tumor numbers in the EK-treated group increased slightly from 16 weeks and caught up with that in the control group. Thus, EK is suggested to delay skin tumor development. These findings indicate that EK is effective not only for inhibiting TPA-induced acute inflammation, but also for reducing the tumor development induced by TPA.

Conclusions

EK strongly inhibited lipid peroxidation both in vitro and in vivo in mouse skin. The significant suppression of TPA-induced oxidative stress and tumor promotion by EK is suggested to be mediated by scavenging of the free radicals produced by leukocytes and by interfering with leukocyte infiltration to the inflamed region. The effects of EK on the generation of inflammatory cytokines and prostaglandins should be examined in future studies. The suppression of oxidative stress by dietary radical scavengers is an effective strategy for prevention of several lifestyle-related diseases. Investigations on the antioxidative effects of EK by oral administration in vivo as well as the mechanism of action of Kurosu constituents are now in process in our laboratory.

Aknowledgments

This study was supported partly by grants-in-aid for Scientific Research on Priority Areas—Cancer—(H.O.) and JSPS Research Fellow (Y.N.) from the Ministry of Education, Science, Sports and Culture of Japan.

References


