

## RESEARCH COMMUNICATION

# Inhibitory Effects of High Temperature- and Pressure-Treated Garlic on Formation of 1,2-Dimethylhydrazine-Induced Mucin-Depleted Foci and *O*<sup>6</sup>-Methylguanine DNA Adducts in the Rat Colorectum

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### Abstract

High temperature- and pressure-treated garlic (HTPG) has been reported to have enhanced antioxidative and cytotoxic activities. However, there have been no reports on chemopreventive effects using animal cancer models. This study first examined the modifying effects of HTPG on 1,2-dimethylhydrazine (DMH)-induced mucin-depleted foci (MDF) and aberrant crypt foci (ACF), preneoplastic lesions in the rat colorectum. Male F344 rats (5 weeks old) were fed basal diet, or experimental diets containing 1% or 3% HTPG for 5 weeks. One week later, all rats were injected s.c. with DMH (40 mg/kg, once weekly for 2 weeks). At 10 weeks of age, all the rats were sacrificed, and the colorectum was evaluated for MDF and ACF. In rats given DMH and 3% HTPG, the numbers of MDF were decreased significantly as compared with those of rats given DMH alone ( $p < 0.01$ ), and the numbers of ACF showed a tendency to decrease, although not significantly. Next, the effects of HTPG on the formation of DMH-induced *O*<sup>6</sup>-methylguanine (*O*<sup>6</sup>-MeG) DNA adducts in rats were studied. Male F344 rats (5 weeks old) were fed the basal diet or 10% HTPG diet for 5 weeks. All rats were injected i.p. once with 40 mg/kg DMH at the end of week 5. The animals were sacrificed 6 hours after DMH injection to analyze the *O*<sup>6</sup>-MeG DNA adducts in the colorectal mucosa and liver. Dietary administration of HTPG significantly reduced the adduct levels in the colorectal mucosa and liver, compared with the controls (both  $p < 0.01$ ). The activities of some detoxification enzymes in the liver of DMH-treated rats were also measured. HTPG significantly reduced the activity of cytochrome P450 (CYP) 2E1, known to be responsible for activation of DMH in rat liver ( $p < 0.05$ ). In contrast, HTPG significantly enhanced the activities of phase 2 enzymes, quinone reductase (QR) and glutathione *S*-transferase (GST), in rat liver (both  $p < 0.05$ ). These results suggested that HTPG might have chemopreventive effects against colon carcinogenesis, at least in the initiation stage.

**Key Words:** High temperature- and pressure-treated garlic (HTPG) - mucin-depleted foci (MDF) - *O*<sup>6</sup>-methylguanine DNA adducts - colon carcinogenesis

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### Introduction

Garlic has been used as food and medicine worldwide. Epidemiological and laboratory studies have suggested that garlic possesses anticancer activity (see reviews; Milner, 2001; Khanum et al., 2004; Shukla and Kalra, 2007; Iciek et al., 2009). The important chemical constituents of garlic are organosulfur compounds, and the sulfur chemical profiles of garlic are reflected by the processing procedure (Lawson, 1996; Matsuura, 2000). Garlic has a well-known odour. Furthermore, garlic is also the cause of the unpleasant smell after ingestion. Thus, numerous attempts to hide or mask the typical garlic odour have been reported (Pentz and Siegers, 1996; Koch, 1996).

It was demonstrated previously that heated garlic powder made from unpeeled garlic bulbs blanched in boiling water to minimize the odour significantly inhibited duodenal and jejunal tumorigenesis in mice (Shimpo et al., 2002). Recently, Jeong et al (2006) reported that the antioxidant activity, total polyphenol, and total flavonoid contents of garlic juice increased with high temperature- and pressure-treatment (130°C, 2 hours), and the garlic juice had anti-cancer effects in some human cancer cell lines.

It was attempted to make high temperature- and pressure-treated garlic (HTPG) rapidly and easily using an autoclave (130°C, 2.5 hours), and then the antioxidative activities and total polyphenol content were determined. The results showed that the antioxidative activity was

enhanced and the total polyphenol content was increased 25-fold (Tomatsu et al., 2007).

The present study describes the inhibitory effects of dietary HTPG on 1,2-dimethylhydrazine (DMH)-induced preneoplastic lesions, including mucin-depleted foci (MDF) and aberrant crypt foci (ACF), in the rat colorectum. In addition, it was shown that HTPG inhibition on DMH-induced *O*<sup>6</sup>-methylguanine (*O*<sup>6</sup>-MeG) DNA adduct formation in the colorectal mucosa and liver of rats.

## Materials and Methods

### Animals

Male F344 rats (4 weeks old) were purchased from Japan SLC Inc. (Hamamatsu, Japan). The rats were kept in groups of two or three in plastic cages on woodchip bedding and fed on a basal diet, Oriental MF diet (Oriental Yeast Co. Ltd., Tokyo, Japan), in an animal facility controlled at a temperature of 23±5°C, 60±5% humidity, and in a 12-h light/dark cycle. The care and use of animals was in accordance with the 'Guidelines for the Management of Laboratory Animals in Fujita Health University, Fujita Memorial Nanakuri Institute', and the experimental protocols were approved by the Institutional Animal Care and Use Committee of Fujita Health University.

### HTPG preparation

HTPG preparation was described previously by Jeong et al. (2006) and Tomatsu et al. (2007). Briefly, garlic slices (Amari Spice Foods Co., Ltd., Kyoto, Japan) were pulverized. Then 100 g of the pulverized garlic was mixed with 250 ml of hot water to inactivate alliinase. After that, the mixture was heated at 130°C and pressured in an autoclave (Tomy SX-500, Tomy Seiko Co., Ltd., Tokyo, Japan) for 2.5 hours and then freeze-dried. The powder was finely pulverized using an Oster Power Blender (Osterizer, Osaka Chemical Co., Ltd., Osaka, Japan).

### Chemicals

DMH was purchased from Tokyo Chemical Industry CO., Ltd. (Tokyo, Japan) and prepared in 1 mM EDTA solution (pH 6.5). Alcian blue was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals were of the highest grade available commercially.

### Experimental protocols

Experiment 1 was performed as follows. The HTPG powder added to the basal diet at a concentration of 1% or 3% and mixed thoroughly using a ball mill. After acclimatization for 1 week, the animals were divided into 3 groups, and basal diet, 1% HTPG in the diet, or 3% HTPG was administered to each group for 35 successive days. On days 7 and 14 all rats were given s.c. injections of 40 mg/kg DMH. At 4 weeks after the first DMH treatment, all rats were anaesthetized with diethyl ether, sacrificed by exsanguination and carefully autopsied.

MDF and ACF were identified according to the method described by Yoshimi et al (2004). Briefly, the colorectum

was removed, flushed with saline, cut open along the longitudinal axis, attached flat on a filter paper, and fixed in 10% buffered formalin. After fixation, the specimens were cut into 2 cm pieces. Each piece was rinsed in 3% acetic acid and then stained with a 1% solution of Alcian blue (pH 2.5). Each piece was placed on a slide glass with the mucosal surface facing upward and the numbers of MDF and ACF were counted using a microscope at a magnification of x 40.

Experiment 2 was performed as described by Herron and Shank (1981) with some modifications (Shimpo et al., 2003). Briefly, after acclimatization for 1 week, the rats were fed the basal diet or 10% HTPG in the diet for 5 weeks. At the end of week 5, all rats were given one i.p. injection 40 mg/kg DMH. Six hours after injection, the rats were anesthetized with diethyl ether and sacrificed by exsanguination. The colorectal mucosa and liver were kept at -80°C until analysis of *O*<sup>6</sup>-MeG DNA adducts. Tissue DNA from the colorectal mucosa and liver was isolated by phenol extraction and precipitated with ethanol. The pellet was suspended in 0.1 M HCl (5 mg/ml), and hydrolyzed at 70°C for 30 min to release the purines as free bases. The DNA hydrolysates were analyzed by high-performance liquid chromatography equipped with a fluorescence detector. These results are expressed as the ratio of *O*<sup>6</sup>-MeG to guanine (G) in nmol/μmol.

In addition, liver detoxification enzyme activities were measured using the following methods: (a) Cytochrome P450 (CYP) 2E1 activity: Liver microsomes were prepared as described by Hanioka et al. (1999). CYP2E1 activity in liver microsomes was determined by monitoring *p*-nitrophenol (PNP) hydroxylation as described by Reinke and Moyer (1985). The PNP hydroxylase activity was determined by measuring *p*-nitrocatechol formation by HPLC with electrochemical detection according to the method of Mishin et al (1996). (b) Quinone reductase (QR) activity: The preparation of liver cytosolic fractions and the measurement of QR activity were performed using the method described by Benson et al (1980). The QR activity was determined with specific activities obtained from the spectrophotometric measurement of dicumarol-sensitive reduction of 2,6-dichloroindophenol by NAD(P)H at 600 nm. (c) Glutathione *S*-transferase (GST) activity: The cytosolic GST activity was determined spectrophotometrically using the substrate 1-chloro-2,4-dinitrobenzene, by measuring the amount of glutathione conjugated product at 340 nm according to the method of Habig et al (1974).

### Statistical Analysis

Statistical analysis of MDF and ACF formation were compared by Dunnett's multiple comparisons test. The *O*<sup>6</sup>-MeG DNA adducts and detoxification enzyme activities were compared by unpaired *t*-test. These procedures were performed using InStat version 3.0 for Windows (GraphPad Software, Inc., San Diego, CA, USA).

## Results

None of rats died during the experimental period. The

**Table 1. Effects of HTPG on the Formation of DMH-induced O<sup>6</sup>-MeG DNA Adducts in the Rat Colorectal Mucosa and Liver**

Treatment	No. of rats examined	Adducts (nmol/μmol G)	
		Colorectum	Liver
Basal diet	6	0.11±0.005 <sup>a</sup>	0.47±0.04
10% HTPG diet	7	0.08±0.005 <sup>b</sup>	0.25±0.04 <sup>b</sup>
% of control		72.7	53.2

<sup>a</sup>Values represent the means ±SE; <sup>b</sup>Significantly different from basal diet group : <sup>b</sup>p<0.01 (unpaired *t*-test).

**Table 2. Effects of HTPG on liver CYP2E1, QR and GST Activities**

Treatment	No. of rats	CYP2E1 (pmol*)	QR (nmol*)	GST (nmol*)
Basal diet	6	1272.9±57.0 <sup>a</sup>	53.5±3.8	511.2±32.1
10% HTPG diet	7	1031.8±54.7 <sup>b</sup>	69.4±5.1 <sup>b</sup>	616.9±25.6 <sup>b</sup>
% of control		81.1	129.7	120.7

\*, /min/mg protein; <sup>a</sup>Values are means ±SE; <sup>b</sup>Significantly different from basal diet group : <sup>b</sup>p<0.05 (unpaired *t*-test).

body weight gain, food consumption, and HTPG intake were not different among the 3 groups (data not shown).

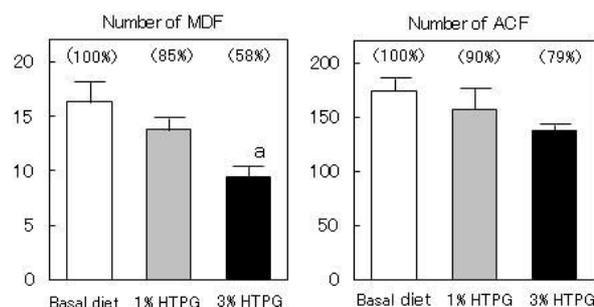
As shown in Figure 1, the total number of MDF/colorectum in the 3% HTPG group was significantly reduced compared with that in the basal diet group (p<0.01; percent of control, 58%). The number of MDF in the 1% HTPG group tended to be decreased compared with that in the basal diet group (percent of control, 85%). On the other hand, ACF/colorectum in the 3% HTPG group tended to be lower than that in the basal diet group (percent of control, 79%), although not significant.

As a second step (Experiment 2), the effects of HTPG on the formation of DMH-induced O<sup>6</sup>-MeG DNA adducts was examined in the rat colorectum and liver. Furthermore, the influence of HTPG on liver detoxification enzyme activities (CYP2E1, QR and GST) were examined to clarify the possible mechanisms involved in its inhibition of MDF formation. As shown in Table 1, the level of O<sup>6</sup>-MeG DNA adducts in the colorectal mucosa of the 10% HTPG group was decreased significantly to 72.7% compared with that of the basal diet group (p<0.01). That level in liver of the 10% HTPG group also was decreased to 53.2% compared with that of the basal diet group (p<0.01).

As shown in Table 2, the liver CYP2E1 activity in the 10% HTPG group was significantly reduced to 81.1% compared with that in the basal diet group (p<0.05). The QR activity in the 10% HTPG group increased significantly to 29.7% compared with that in the basal diet group (p<0.05). The GST activity in the 10% HTPG group increased significantly to 20.7% compared with that in the basal diet group (p<0.05).

## Discussion

To examine the chemopreventive effects on colorectal carcinogenesis in rats by HTPG, 2 putative preneoplastic endpoints were used: the ACF discovered by Bird (1987) and MDF firstly described by Caderni et al (2003). As a



**Figure 1. Effects of HTPG on DMH-induced MDF and ACF Formation.** Each bar represents the mean ± SE. Data in parentheses indicate % of control. <sup>a</sup>Significantly different from control (p<0.01; Dunnett's multiple comparisons test).

result, it was demonstrated that HTPG significantly inhibited DMH-induced MDF formation in the rat colorectum, whereas HTPG non-significantly reduced ACF. Yoshimi et al. (2004) examined the relationship among ACF, MDF, and β-catenin accumulated crypts (BCAC; Yamada et al., 2000, 2001), which have been considered as early preneoplastic lesions on the colorectal mucosa of DMH-treated rats. Their results indicated that MDF had more frequent dysplastic changes and overexpression of β-catenin than ACF, and MDF with more than 4 crypts or MDF with the appearance of ACF corresponded well to BCAC.

Next, it was examined whether HTPG inhibited the formation of DMH-induced O<sup>6</sup>-MeG DNA adducts in rat colorectum and liver. The results showed that HTPG significantly inhibited O<sup>6</sup>-MeG DNA adduct levels in the colorectal mucosa and liver compared with the controls.

Therefore, the present results suggest that HTPG might have a chemopreventive effect against colorectal carcinogenesis, although long-term carcinogenesis experiments to test the inhibitory effects of HTPG on the development of colorectal cancer in rodents are required. The mechanisms by which HTPG inhibited DMH-induced preneoplastic lesions (especially MDF formation) in the rat colorectum have not been clearly elucidated.

However, numerous reports suggested that the possible mechanisms of anticarcinogenic effects of garlic are involved in its capacity to decrease the activation and increase the detoxification of carcinogens (Milner, 2001; Le Bon et al., 2003; Khanum et al., 2004; Singh et al., 2006; Shukla and Kalra, 2007; Iciek et al., 2009). Thus, it is possible that HTPG inhibits activation enzymes (phase 1 enzymes), activates detoxifying enzymes (phase 2 enzymes), or reduces DNA adduct formation. Sohn et al (1991) showed that methylazoxymethanol (MAM), and its chemical and metabolic precursor, azoxymethane (AOM), both strong colon carcinogens in rodents, were metabolically activated by CYP2E1 *in vitro* and *in vivo*.

In the present study, it was indicated that dietary HTPG significantly decreased CYP2E1 (a phase 1 enzyme)-mediated PNP hydroxylase activity in the liver of rats following DMH treatment. It was also found that oral feeding with HTPG for 5 weeks significantly increased the activities of hepatic QR and GST (both phase 2 enzymes), although the present study did not determine QR and GST activities in the colorectum as a target tissue.

The characteristic of HTPG is its lack of odor due to the treatment at high temperature and pressure using an autoclave. Furthermore, the color of the powder is brown, as the process seems to involve the Maillard reaction. HTPG might be similar to aged garlic extract, because it has a low content of volatile sulfur compounds and new physiologically active substances are generated by various kinds of chemical reactions to including the Maillard reaction.

It is well known that water-soluble organosulfur compounds, such as *S*-allylcysteine (SAC), *S*-allylmercaptocysteine (SAMC), and  $\gamma$ -glutamyl-*S*-allylcysteine (GSAC), are present at high levels in aged garlic extract (Kyo, 2003). In particular, SAC has been reported to have not only antioxidant activities but also demonstrated inhibition of DMH-induced colon cancer in mice (Sumiyoshi and Wargovich, 1990) and the growth of both cultured human neuroblastoma cells (Welch et al., 1992) and breast carcinoma cells (Li et al., 1995).

The present study analyzed SAC concentration in HTPG, and found that the concentration was increased 4.7-fold compared with that in garlic slices (data not shown). Inhibition of the formation of putative neoplastic lesions in the rat colorectum may strongly relate to the enhancement of antioxidative activity. Furthermore, an increasing in SAC concentration is also associated with this inhibition. As yet, SAMC in HTPG has not been analyzed, but HTPG is assumed to contain this compound. SAMC has been reported to decrease CYP2E1 activity in the mouse liver (Sumioka et al., 1998) and to inhibit the growth of erythroleukemia cell lines by inducing apoptosis (Sigounas et al., 1997).

Furthermore, Ryu et al (2001) reported that fructosyl arginine in Maillard reaction products in aged garlic extract had potent scavenging activity against hydrogen peroxide. In addition, Sato et al (2006a) reported that the superoxide dismutase (SOD)-like activity in garlic extract that was fermented for a short period of time (40 days at 60~70°C, 85~95% relative humidity) was about 13 times higher than that of a control garlic extract. Sato et al (2006b) also reported that compounds in garlic consequently increased tetrahydro- $\beta$ -carboline derivatives. It was assumed that these are contained in HTPG, and contributed to the enhancement of antioxidative activity.

In conclusion, these results indicated that HTPG inhibited DMH-induced MDF and *O*<sup>6</sup>-MeG DNA adduct formation in the rat colorectum and reduced CYP2E1 enzymatic activity and increased QR and GST activities in the rat liver. Long-term carcinogenesis experiments to test the inhibitory effects of HTPG on the development of colorectal cancer in rodents are now underway in our laboratory.

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