Original Article

Effect of S-allylcysteine on oxidant-antioxidant status during N-methyl-N'-nitro-N-nitrosoguanidine and saturated sodium chloride-induced gastric carcinogenesis in Wistar rats

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We investigated the chemopreventive effect of S-allylcysteine (SAC), a water-soluble garlic constituent against gastric carcinogenesis induced in male Wistar rats by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) and saturated sodium chloride (S-NaCl). The animals were divided into four groups of six animals. Rats in groups 1 and 2 were administered MNNG (200 mg/kg body weight) on days 0 and 14 as well as S-NaCl (1mL/rat) three days during weeks 0 to 3, and thereafter placed on basal diet until the end of the experiment. Rats in group 2 in addition received SAC (200 mg/kg body weight) three times per week starting on the day following the first exposure to MNNG and continued until the end of the experimental period. Group 3 animals were given SAC alone as in group 2. Group 4 animals received basal diet and tap water throughout the experiment and served as the untreated control. The animals were sacrificed after an experimental period of 21 weeks. Measurement of lipid peroxidation and antioxidants of the glutathione redox cycle in the stomach tissue, liver and venous blood was used to monitor the chemopreventive potential of SAC. All animals that received MNNG and S-NaCl alone, developed tumours, identified histologically as squamous cell carcinomas. In the tumour tissue, diminished lipid peroxidation was accompanied by increase in reduced glutathione (GSH) and GSH-dependent enzymes, whereas in the liver and circulation, enhanced lipid peroxidation was associated with antioxidant depletion. Administration of SAC suppressed the incidence of MNNG+S-NaCl-induced gastric tumours as revealed by the absence of carcinomas. SAC ameliorated MNNG-induced decreased susceptibility of the gastric mucosa to lipid peroxidation, whilst simultaneously increasing the antioxidant status. In the liver and blood, SAC reduced the extent of lipid peroxidation and significantly enhanced antioxidant activities. We suggest that SAC exerts its chemopreventive effects by modulating lipid peroxidation and enhancing GSH-dependent antioxidants in the target organ as well as in the liver and blood.

Key Words: gastric carcinogenesis, N-methyl-N'-nitro-N-nitrosoguanidine, chemoprevention, garlic, S-allylcysteine, lipid peroxidation, antioxidants.

Introduction

Chemoprevention by dietary constituents has emerged as a cost-effective approach to control the incidence of gastric cancer, the second most common malignancy worldwide, and a major cause of mortality in Chennai, India.[1,2] Gastric cancer induced by the administration of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) in Wistar rats which shows similarities to human gastric tumours is an ideal model for investigating the development of stomach cancer and the effects of intervention by chemopreventive agents.[3] In previous reports from this laboratory, we demonstrated the protective effects of neem leaf, garlic and lycopene in the MNNG model.[4,5]

Garlic (*Allium sativum Linn*) has been extensively used as an effective chemopreventive agent in a wide range of malignancies. Previously, we reported the antioxidant, hepatoprotective and anticarcinogenic effects of garlic in 7,12-dimethylbenz[a]anthracene (DMBA)-induced hamster buccal pouch (HBP) carcinogenesis as well as in MNNG-induced rat stomach carcinogenesis.[6,7] Recently, we demonstrated induction of cellular differentiation by garlic in the HBP model.[8] S-Allylcysteine (SAC), a naturally occurring, non-toxic, water-soluble, organosulfur compound is considered as one of the important biologically active constituents of garlic.[9] SAC is recognized to possess anti-inflammatory and antioxidant properties.[10,11]

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SAC has come under extensive study in the light of its anticancer effects both in vitro and in vivo.[12] Recently, we demonstrated a positive correlation between the antioxidant properties of SAC and its chemopreventive efficacy against DMBA-induced HBP carcinogenesis.[13] We have also documented the apoptosis-inducing effects of SAC in the HBP model.[14] Many phytochemicals are known to exert their anticarcinogenic effects by scavenging oxygen free radicals (OFR) and modulating host antioxidant defence mechanisms.[15] Previously, we demonstrated that assay of OFR-induced lipid peroxiation and antioxidant enzymes in the liver and blood in addition to the target organ is a reliable method for screening putative chemopreventive agents. [6,13,16,17] In the present study, we have evaluated the chemopreventive potential of SAC by measurement of lipid peroxidation and the antioxidant status with respect to the glutathione redox cycle in the stomach tissue, liver and venous blood during MNNG+S-NaCl-induced gastric carcinogenesis.

Materials and methods

Animals

All the experiments were carried out with male Wistar rats aged 6-8 weeks obtained from the Central Animal House, Annamalai University, India. They were housed six in a polypropylene cage and provided food and water ad libitum. The animals were maintained under standard conditions of temperature and humidity with an alternating 12 hours light/dark cycle. All animals were fed standard pellet diet (Mysore Snack Feed Ltd, Mysore, India) and maintained in accordance with the guidelines of the National Institute of Nutrition, Indian Council of Medical Research, Hyderabad, India, and approved by the ethical committee of Annamalai University.

Chemicals

Bovine serum albumin, 2-thiobarbituric acid, trichloroacetic acid, 2,4-dinitrophenylhydrazine (DNPH), reduced glutathione (GSH), 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), 1-chloro-2,4-dinitrobenzene (CDNB) and flavine adenine dinucleotide (FAD) were purchased from Sigma Chemical Company, St. Louis, USA. MNNG of purity ≥97% was obtained from Fluka-Chemika-Biochemika, Buchs, Switzerland. SAC of purity 99.9% kindly provided by Wakunaga Pharmaceutical Co. Ltd. (Hiroshima, Japan), was dissolved in distilled water before use. All other reagents used were of analytical grade.

Treatment schedule

Animals were randomized into experimental and control groups and divided into four groups of six. The experimental protocol for the present study is shown in Fig. 1. Rats in group 1 were given MNNG (200 mg/kg body weight) by intragastric intubation on days 0 and 14 as well as S-NaCl (1mL/rat) every 3 days during weeks 0 to 3 (six times; on days 3,7,10,14,17,21).[18] Rats in group 2 administered MNNG+S-NaCl as in group 1, in addition received intragastric intubation of SAC (200 mg/kg body weight) three times per week starting on the day following the first exposure to MNNG (day 1) and continued until the end of the experimental period.[19] Group 3 animals were given SAC alone as in group 2 but

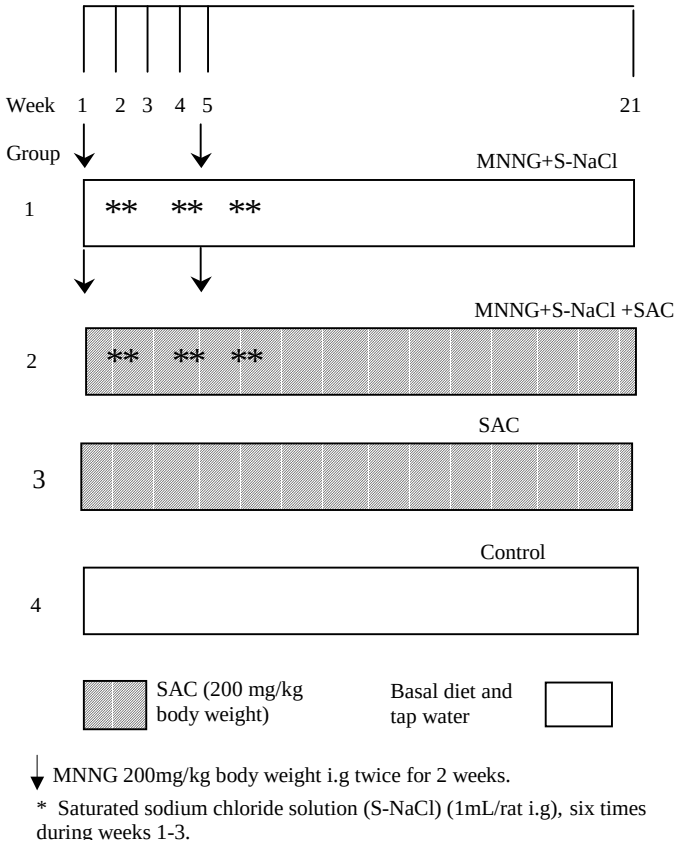

Figure 1. Experimental protocol

without MNNG and S-NaCl. Group 4 received basal diet and tap water throughout the experiment and served as the untreated control. All animals had free access to food and water.

The experiment was terminated at 21 weeks and all animals were killed by cervical dislocation after an overnight fast. Fresh tissues were used for estimations. Biochemical estimations were carried out in stomach tissue, liver and blood samples of experimental and control animals. For histopathological examination, tissues were fixed in 10% formalin, embedded in paraffin and 2-3 μm sections were cut on a rotary microtome and stained with haematoxylin and eosin.

Preparation of hemolysate

Blood samples were collected in heparinised tubes and plasma was separated by centrifugation at 1000 g for 15 minutes. After centrifugation, the buffy coat was removed and the packed cells washed thrice with physiological saline. 0.5 mL of erythrocytes was lysed with 4.5 mL of hypotonic phosphate buffer, pH 7.4. The hemolysate was separated by centrifuging at 2500 g for 15 min at 2°C.

Biochemical methodology

Lipid peroxidation as evidenced by the formation of thiobarbituric acid reactive substances (TBARS) was assayed in tissues by the method described by Ohkawa *et al.*,[20] in the plasma by the method of Yagi[21] and in erythrocytes by the method of Buege and Aust.[22] The pink coloured chromogen formed by the reaction of 2-thiobarbituric acid with the breakdown products of lipid peroxidation was read at 535 nm. Reduced glutathione (GSH) was determined by the method of Anderson[23] based on the development of a yellow colour when DTNB

is added to compounds containing sulfhydryl groups. Glutathione peroxidase (GPx) activity was assayed by the method of Rotruck *et al*.,[24] with modifications. A known amount of enzyme preparation was incubated with hydrogen peroxide in the presence of GSH for 10 minutes. The amount of hydrogen peroxide utilized was determined by estimating GSH content by the method of Anderson.[23] The activity of glutathione S-transferase (GST) was determined as described by Habig *et al.,*[25] by following the increase in absorbance at 340 nm using CDNB as substrate. Glutathione reductase (GR) activity was determined by the method of Carlberg and Mannervik[26] using oxidized glutathione as substrate and FAD as cofactor.

The protein content was estimated by the method of Lowry *et al*.[27] Plasma ascorbic acid was estimated by the method of Omaye *et al*.[28] This involves oxidation of ascorbic acid by copper followed by treatment with DNPH to form the derivative bis 2,4-dinitrophenyl-hydrazone that undergoes rearrangement to form a product with an absorption maximum at 520 nm. Plasma vitamin E was measured by the method of Baker *et al.,*[29] on the basis of the reduction of ferric ions to ferrous ions by α-tocopherol and the formation of a red coloured complex with 2,2'-dipyridyl at 520 nm. Haemoglobin in erythrocytes and hemolysate was measured according to the method of Drabkin and Austin.[30] Blood was diluted in an alkaline medium containing potassium cyanide and potassium ferricyanide. Haemoglobin oxidized to methe-moglobin combines with cyanide to form cyan-methemoglobin, which was measured at 540 nm.

Statistical analysis

Statistical analysis on the incidence of lesions was performed using Fisher's exact probability test. The body weight and biochemical parameters were analysed using ANOVA and the group means were compared by Duncan's multiple range test (DMRT). The results were considered statistically significant if the $P<0.05$.

Gross observations

Table 1 shows the mean body weights and the incidence of gastric cancer in control and experimental animals in each group. Rats in group 1 (MNNG+S-NaCl) showed a tendency to be lower in body weight gain during the experiment and the mean final body weights were decreased compared with all other groups. Multiple friable chalky white nodules with small cauliflower-like growth were seen in the forestomach of group 1 animals. The forestomach of group 2 animals revealed fewer and smaller nodules without cauliflower like growth. No obvious changes were observed in groups 3 and 4.

Histopathological observations

The incidence of gastric tumours in group 1 was 100 per cent. No malignant neoplasms were observed in the stomach in groups 2 through 4. Forestomach tumours induced by MNNG+S-NaCl were squamous cell carcinomas with a number of epithelial and keratin pearls. The cells showed increased nuclear/cytoplasmic ratio, nuclear pleomorphism and hyperchromatism (Fig. 2). One of the six animals treated

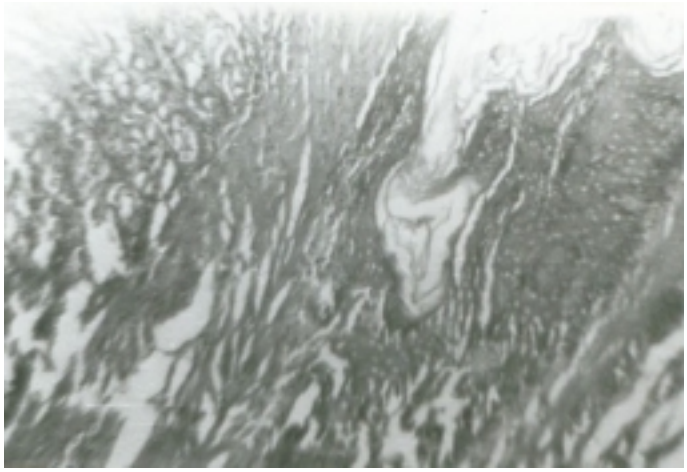

Figure 2. Photomicrograph showing squamous cell carcinoma with extensive infiltration: Group 1 (H and E X 50)

Table 1. Body weight, tumour incidence and histopathological changes in each group during MNNG + S-NaCl-induced gastric carcinogenesis (mean ± SD; $N = 6$)

Group	Treatment	Body weight		Hyperplasia	Dysplasia	Carcinoma
		Initial	Final			
1.	MNNG + S-NaCl	126.3 ± 4.2	249.7 ± 9.2[a]	6 (100)	6(100)	6 (100)
2.	MNNG + S-NaCl + SAC	125.1 ± 3.8	270.5 ± 13.6[b]	3(50)	1(16.6)	0
3.	SAC	123.7 ± 4.7	290.4 ± 10.3	-	-	0
4.	Control	121.4 ± 4.6	284.1 ± 10.4	-	-	0

[a] Significantly different from group 4 $P<0.05$; [b] Significantly different from group 1 $P<0.05$ (Duncan's multiple range test); Parantheses – percentage of lesions

Table 2. TBARS in stomach, liver and blood in each group during MNNG+S-NaCl-induced gastric carcinogenesis (mean ± SD; $N = 6$)

Group	Treatment	Stomach TBARS (nmols/100mg protein)	Liver TBARS (nmols/100mg protein)	Plasma TBARS (nmols/ml)	Erythrocyte TBARS (pmol/mg Hb)
1.	MNNG + S-NaCl	89.2 ± 6.20^{d}	202.8 ± 17.49^{a}	5.01 ± 0.31^{a}	3.78 ± 0.30^{a}
2.	MNNG + S-NaCl + SAC	110.0 ± 9.41^{c}	170.2 ± 12.82^{b}	3.97 ± 0.14^{b}	2.63 ± 0.15^{b}
3.	SAC	123.1 ± 9.20^{b}	125.9 ± 10.57^{d}	2.51 ± 0.13^{d}	0.83 ± 0.08^{d}
4.	Control	134.6 ± 8.02^{a}	139.9 ± 11.64^{c}	2.96 ± 0.13^{c}	1.41 ± 0.13^{c}

Values not sharing a common superscript letter differ significantly at $P<0.05$ (Duncan's multiple range test)

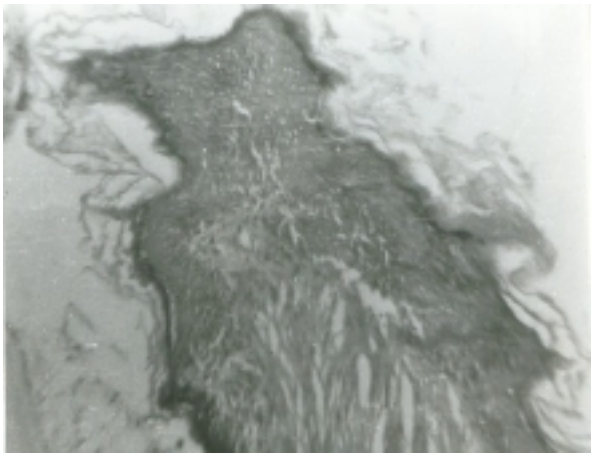

Figure 3. Photomicrograph showing stratified squamous epithelial layer with mild dysplasia and invasion: Group 2 (H and E X 50)

with MNNG and SAC (group 2) showed severe dysplasia while the remaining exhibited normal keratinised stratified squamous epithelium with mild dysplasia and hyperplasia of lining epithelium (Fig. 3). The forestomach of rats in groups 3 and 4 showed normal lining of keratinised stratified squamous epithelium with a limiting ridge demarcating the forestomach from the glandular stomach lined by mucous glands (Fig. 4,5).

Biochemical findings

Table 2 shows TBARS in the stomach tumour tissue in group 1 to be the lowest among all groups. TBARS in groups 2 and 3 were significantly higher than those in group 1. In the liver and blood, TBARS were significantly increased in group 1 compared with group 4. In groups 2 and 3, the values were significantly lower than in group 1.

Table 3 shows markedly higher levels of GSH and activities of GPx, GST and GR in stomach tissues in group 1 animals than in group 4. The levels were raised in group 2 compared with groups 1 and 4. In group 3, the levels were significantly higher compared with group 4. The levels of antioxidants in the livers of control and experimental animals in each group are presented in Table 4. The levels of GSH and the activities of GSH-dependent enzymes (GPx, GST and GR) in liver tissues were significantly lower in group 1 compared to group 4. The antioxidant levels in groups 2 and 3 were higher than those of group 1.

Table 5 shows levels of GSH, vitamin C and vitamin E in plasma and activities of GSH-dependent enzymes in erythrocyte lysate of control and experimental animals in each group. In group 1, the levels of GSH and the activities of GSH-dependent enzymes as well as vitamin C and vitamin E were found to be depleted compared to group 4. The concentrations of GSH, vitamin C and vitamin E and the activities of GPx, GST and GR in groups 2 and 3 were significantly higher compared to group 1.

Discussion

Effect of MNNG + S-NaCl on target tissue

Cell proliferation, which plays a key role in cancer development, is associated with changes in OFR-induced lipid peroxidation and the status of antioxidants that use GSH as a substrate. Lipid peroxidation involved in the regulation of cell division, is reported to be inversely

Table 3. Glutathione concentration and glutathione-dependent enzyme activities in stomach tissues of rats in each group during MNNG and S-NaCl-induced gastric carcinogenesis (mean ± SD; $N = 6$)

Group	Treatment	GSH (U^{A})	GPx (U^{B})	GST (U^{C})	GR (U^{D})
1.	MNNG + S-NaCl	0.73 ± 0.07^{b}	21.38 ± 1.50^{b}	1.63 ± 0.17^{b}	46.20 ± 4.11^{b}
2.	MNNG + S-NaCl + SAC	0.94 ± 0.08^{a}	30.07 ± 2.04^{a}	2.20 ± 0.18^{a}	58.38 ± 4.02^{a}
3.	SAC	0.38 ± 0.03^{c}	19.31 ± 1.35^{c}	1.36 ± 0.15^{c}	34.36 ± 2.31^{c}
4.	Control	0.30 ± 0.03^{d}	13.59 ± 1.06^{d}	0.93 ± 0.08^{d}	26.18 ± 1.90^{d}

Values not sharing a common superscript letter differ significantly at $P<0.05$ (Duncan's multiple range test); ^{A}mg/g tissue; ^{B}µmols of GSH utilized/min/g protein; ^{C}µmols of CDNB-GSH conjugate formed/min/mg protein; ^{D}µmols of NADPH oxidized/h/mg protein.

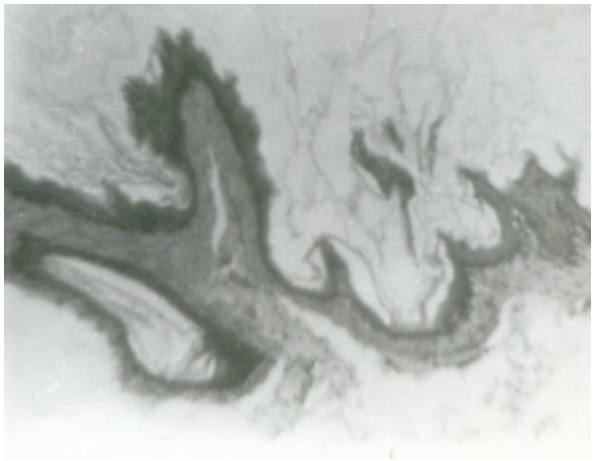

Figure 4. Photomicrograph of the stomach showing hyperkeratosis and papillomatosis: Group 3 (H and E X 50)

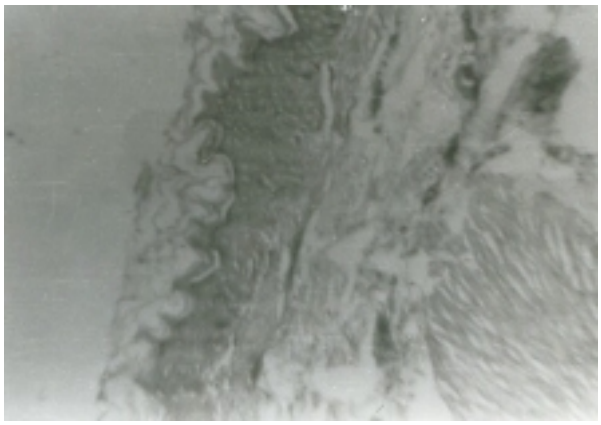

Figure 5. Photomicrograph of the stomach showing normal keratinised stratified squamous epithelium: Group 4 (H and E X 50)

related to cell proliferation with highly proliferating tumours showing low levels of lipid peroxidation compared to their normal counterparts.[31] GSH, an important non-protein thiol in conjunction with GPx, and GR, plays a regulatory role in cell proliferation.[32] Obrador *et al.*,[33] observed a positive correlation between enhanced synthesis of GSH and high rates of cell proliferation in tumours. We postulate that diminished lipid peroxidation combined with enhanced GSH-dependent antioxidant capacity of MNNG+S-NaCl induced gastric tumours facilitates cell proliferation offering a selective growth advantage to tumour cells over their surrounding normal cells.

Effect of MNNG + S-NaCl on liver and blood

In contrast to decreased lipid peroxidation in tumour tissues, we found enhanced lipid peroxidation associated with antioxidant depletion in the liver and blood of tumour bearing animals. We observed similar results during 4-nitroquinoline-1-oxide-induced rat tongue carcinogenesis and DMBA-induced HBP carcinogenesis in addition to experimental rat stomach carcinogenesis.[6,17,34,35] These findings are also consistent with the observation that lipid peroxidation is decreased in rapidly proliferating tumours compared to their normal counterparts.[36,37] Thus the tumour and host tissue appear to comprise two separate metabolic compartments with respect to their susceptibility to lipid peroxidation.

MNNG is known to produce toxic and highly diffusible OFR capable of producing deleterious effects in the liver and blood.[38] Free radical-mediated oxidative stress has been implicated in the hepatotoxic effects of MNNG.[39] The erythrocytes are also highly susceptible to oxidative damage due to the high content of iron and polyunsaturated fatty acids and their role as oxygen transporters.[40] Thus enhanced lipid peroxidation in the liver and blood of tumour-bearing rats reflects overproduction of OFR in these tissues exacerbated by a compromised GSH redox cycle.

The liver, which contains high amounts of GSH supplies it to various extrahepatic tissues including the erythrocytes and plasma. Tumour cells have been reported to sequester essential antioxidants such as GSH to meet the demands of a growing tumour.[5] The deficiency of antioxidants in the liver and blood of tumour-bearing animals may be ascribed to increased utilization to scavenge lipid peroxides as well as sequestration by tumour cells.

Effect of SAC on MNNG + S-NaCl-induced oxidant-antioxidant changes

Administration of SAC significantly suppressed the incidence of MNNG and S-NaCl-induced gastric cancer as revealed by the absence of carcinomas. The results of the present study substantiate the anticarcinogenic activity of SAC reported by us as well as by other workers. SAC

Table 4. Glutathione concentration and glutathione-dependent enzyme activities in liver tissues of rats in each group during MNNG and S-NaCl-induced gastric carcinogenesis (mean ± SD; *N*= 6)

Group	Treatment	GSH (U^A)	GPx (U^B)	GST (U^C)	GR (U^D)
1.	MNNG + S-NaCl	0.35 ± 0.02^d	7.56 ± 0.52^d	0.93 ± 0.05^d	20.55 ± 1.49^d
2.	MNNG + S-NaCl + SAC	0.42 ± 0.02^c	10.39 ± 1.09^c	1.67 ± 0.13^c	27.51 ± 1.58^c
3.	SAC	0.58 ± 0.03^a	18.04 ± 1.51^a	2.26 ± 0.14^a	37.34 ± 1.41^a
4.	Control	0.53 ± 0.04^b	14.28 ± 1.50^b	1.91 ± 0.13^b	31.57 ± 1.65^b

Values not sharing a common superscript letter differ significantly at $P<0.05$ (Duncan's multiple range test)
[A] mg/g tissue; B- µmoles of GSH utilized/min/g protein; [C] µmoles of CDNB-GSH conjugate formed/min/mg protein.
[d] µmoles of NADPH oxidized/h/mg protein.

Table 5. Glutathione concentration and glutathione-dependent enzyme activities in blood of rats in each group during MNNG+S-NaCl-induced gastric carcinogenesis (mean ± SD; $N = 6$)

Parameter	MNNG	MNNG + SAC	SAC	Control
Plasma				
GSH (mg/dL)	20.88 ± 1.54^{d}	33.12 ± 2.39^{c}	53.58 ± 3.45^{a}	43.06 ± 3.47^{b}
Vitamin C (mg/dL)	0.77 ± 0.04^{d}	1.17 ± 0.08^{c}	1.99 ± 0.17^{a}	1.48 ± 0.05^{b}
Vitamin E (mg/dL)	0.35 ± 0.03^{d}	0.89 ± 0.04^{c}	1.68 ± 0.03^{a}	1.31 ± 0.06^{b}
Erythrocytes				
GSH (U^{A})	19.56 ± 1.36^{d}	28.35 ± 2.16^{c}	56.28 ± 2.51^{a}	45.65 ± 3.61^{b}
GPx (U^{B})	12.45 ± 1.07^{d}	18.51 ± 1.86^{c}	31.41 ± 1.85^{a}	26.23 ± 2.92^{b}
GST (U^{C})	1.23 ± 0.13^{d}	1.78 ± 0.14^{c}	2.53 ± 0.19^{a}	2.05 ± 0.18^{b}
GR (U^{D})	30.69 ± 2.32^{d}	38.96 ± 3.23^{c}	56.40 ± 4.05^{a}	49.59 ± 3.14^{b}

Values not sharing a common superscript letter differ significantly at $P<0.05$ (Duncan's multiple range test); [A] mg/dL; [B] μmoles of GSH utilized/min/g Hb; [C] μmoles of CDNB-GSH conjugate formed/min/mg Hb. [D] μmoles of NADPH oxidized/h/ml/red cells.

is known to inhibit DMBA and N-methylnitrosourea-induced mammary carcinogenesis and 1,2-dimethyl-hydrazine-induced colon cancer.[13,19,41,42] The antiproliferative effects of SAC against human melanoma and neuroblastoma cancer cell lines have been documented.[43,44]

The different effects of SAC on lipid peroxidation in the target organ and host tissues reflect its modulatory role on cell proliferation. SAC reversed the susceptibility of gastric tumours to lipid peroxidation whilst simultaneously increasing the antioxidant status with consequent suppression of cell proliferation in the target organ. These findings potentiate reports by us as well as other workers that chemopreventive agents exert an 'electrophilic counterattack response' characterized by the elevation of phase II enzymes that utilize GSH as substrate.[4,5,45] In the liver and blood, SAC reduced the extent of lipid peroxidation and enhanced the antioxidant status. Chemopreventive agents are known to upregulate the ability of the liver to metabolise carcinogens and induce antioxidant enzymes altering tumour development at extrahepatic sites.[6,15]

SAC has been demonstrated to possess high radical scavenging activity and protect hepatocytes against oxidative damage.[11,46] SAC has been reported to inhibit OFR-induced formation of 8-oxodeoxyguanosine in DNA as well as activation of transcription nuclear factor kappa B in human T cells.[47,48] Hatano *et al.*,[49] showed a positive correlation between the chemopreventive efficacy of SAC and its enhancing effects on GST activity. Our results support the working hypothesis that the chemopreventive potential of antioxidants such as SAC is related to specific effects on GSH-dependent carcinogen detoxification systems.

Although multiple mechanisms may be involved in the cancer chemopreventive action of SAC, the results of the present study taken together with our previous reports validate the hypothesis that diet-derived agents with antioxidant properties such as SAC, are effective chemopreventive agents. Further studies are underway in our laboratory to elucidate the molecular mechanisms of the anticarcinogenic activity of SAC.

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