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Excess Taurine Induced Placental Glutathione *S*-transferase Positive Foci Formation in Rat

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The purpose of this study was to examine the chemopreventive potential of taurine at various levels on the diethylnitrosamine (DEN)-induced hepatocarcinogenesis. Male Sprague-Dawley rats were fed on diets containing 0, 1, 2, 3% taurine or 5% β-alanine for taurine depletion. Then they were treated with DEN and 2/3 partial hepatectomy. The number of placental glutathione *S*-transferase positive (GST-P$^+$) foci, as a preneoplastic marker in the 1% taurine group was lower than the control diet group. However the difference was insignificant. Although taurine diets reduced the thiobarbituric acid reactive substance (TBARS) level, the number of GST-P$^+$ foci was increased in 3% taurine diet group. The 1% taurine diet increased the glutathione (GSH) level and GST activity, however they unfortunately did not suppress the foci formation. In the 3% taurine group, the GSH level and GSH peroxidase (GPx) activity were significantly decreased. Excess taurine supplementation of the pharmaceutical dose worked against hepatic chemoprevention, which might result from modulation of GPx activity and GSH utility. On the contrary, taurine might work as an antioxidant against TBARS production as the 1% taurine diet increased GSH level. The potency of the cancer preventive effect of taurine still remains and further studies should investigate the effect of taurine with less than 1% levels on the prevention of hepatic cancer.

Keywords: Glutathione peroxidase, Hepatocarcinogenesis, Pharmaceutical dose, Placental glutathione S-transferase positive foci, Taurine

Introduction

Taurine (2-aminoethane sulfonic acid) is a sulfur-containing β-amino acid that is the most abundant free amino acid in most mammal tissue. It has many physiological functions, such as membrane protection (Redmond *et al.*, 1998), conjugation with bile acids (Monte *et al.*, 1997), antioxidation (Raschke *et al.*, 1995; Cunningham *et al.*, 1998), anti-atherogenicity (Yokogoshi *et al.*, 1999; Zhang *et al.*, 1999), enhancement of the host defense system (Redmond *et al.*, 1998), and many others.

In some manner, taurine ameliorates toxicity induced by a variety of chemicals has a potent effect on cancer prevention. In various studies (Reddy *et al.*, 1993; Kerai *et al.*, 1998), it has been reported that taurine suppressed microsomal cytochrome P450 monooxygenases that are induced by alcohol (Lee *et al.*, 1998) and increased phase II detoxifying enzymes. The most well-known characteristic of taurine related to cancer prevention is antioxidation. Both endogenous and exogenous taurine are effective (Raschke *et al.*, 1995; Cunningham *et al.*, 1998). The pro-oxidant condition induced in carcinogenesis may result in damage to the cellular antioxidant defense enzymes as well as the membranes (Koh *et al.*, 1999). Moreover, taurine inhibits the production of nitric oxide and prostaglandin E_2 (Liu *et al.*, 1998). In addition, taurine has effected the cell proliferation according to some studies (Chen *et al.*, 1998; Lima & Cubillos, 1998; Zhang *et al.*, 1999). Taurine, as low as 0.3 mM, inhibited DNA synthesis and proliferation in rat aorta vascular smooth muscle cells (Zhang *et al.*, 1999). On the contrary, taurine affected an outgrowth of retina and DNA synthesis in neurons (Chen *et al.*, 1998; Lima & Cubillos, 1998). Recently, there were reports that taurine showed chemoprevention against colon and hepatic cancers. Reddy *et al.* (1993) examined chemopreventive effects of several organosulfur compounds against azoxymethane-induced colon cancer and the induction of GSH *S*-transferase (GST) activity in the liver of rats exposed to taurine. The protection of hepatocytes by taurine showed correlation with the inhibition of lipid peroxidation (You & Chang, 1998) and ornithine decarboxylase activity (Okamoto *et al.*, 1996). However, there were insignificant results in the chemopreventive effect of taurine (Lubec *et al.*,

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1996). Therefore, the cancer prevention and the antiproliferation by taurine are disputable.

Although many studies have investigated the biological function of taurine, there is a little research based on the role of taurine in hepatic carcinogenesis. In particular, the biological variation, according to the levels of dietary taurine, has seldom been reported. The present study was conducted in order to examine the effect of taurine at various levels on diethylnitrosamine (DEN)-induced carcinogenesis and on GSH-dependent detoxifying enzymes in rat liver. Supplementation of 5% β-alanine diet was used to induce taurine depletion in rat hepatic carcinogenesis. This study should contribute to the discussion of the chemopreventive effectiveness of taurine supplementation at pharmaceutical doses.

Materials and Methods

Animals and diet Weanling male Sprague-Dawley rats weighing 40-60 g were supplied by the Animal Care Facility of Seoul National University (Seoul, Korea) They were kept in polycarbonate cages in a room with controlled temperature (23±2°C), humidity (55±5%), and light-dark-cycle (0700-1900). According to the experimental diets and treatment, they were divided into 6 groups (Fig. 1), and fed one of the experimental diets for 11 weeks. All of them, except the control group, were given intraperitoneal injection of DEN (200 mg/kg body weight; Sigma Chemical Co., St. Louis, USA) at 6-week old, and 2/3 partial hepatectomy were performed after 3 weeks (Ito *et al.*, 1989). The control group was treated with saline and sham operation. They were sacrificed by decapitation at the age of 11 week, and their livers were used for assay. Liver sections were fixed in cold acetone and residual liver was homogenized in a Tris-HCl buffered solution (pH 7.4). Microsomal and cytosolic fractions were prepared by differential centrifugation and stored in small aliquots at -70°C until used.

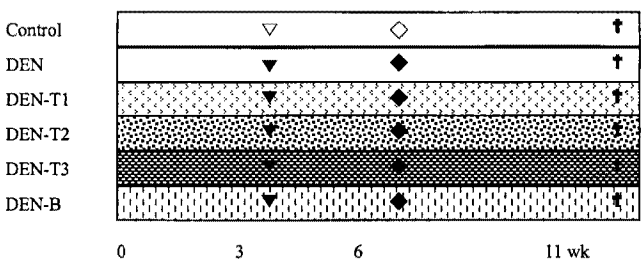

Fig. 1. Experimental protocol. Hepatocarcinogenesis was induced by i.p. injection of DEN (▼), and 2/3 partial hepatectomy (◆). Instead of DEN and hepatectomy, control group was treated with saline (▽) and sham operation (◇). All animals were fed with experimental diet (basal diet, ❑; 1, 2, or 3% taurine supplemented diet, ❑, ❑, ❑; 5% β-alanine diet, ❑) for 11 weeks, and sacrificed (†).

Table 1. Experimental diet composition

Component	(g/100g diet)
Corn starch	54.7
Casein	20.0
Corn oil	15.0
α-Cellulose	5.0
Mineral Mixture	4.0
Vitamin Mixture	1.0
DL-Methionine	0.3

Taurine was added to 100 g diet at the levels of 1, 2, or 3 g. β-Alanine was added with 5 g.

Taurine or β-alanine was added to the basal diet (Table 1). The levels of taurine supplemented were 1, 2, and 3% (w/w), and that of β-alanine was 5%. The basal diet was composed of 20% casein protein (Sigma Chemical Co., St. Louis, USA), 54.7% corn starch, 15% corn oil, 5% α-cellulose, 3.5% AIN-76 Mineral mixture (ICN Biochemicals, Cleveland, USA), 1% AIN-76 fortified Vitamin mixture (ICN Biochemicals, Costa Mesa, USA), and 0.3% DL-methionine (calorie density 433.8 kcal per 100 g diet). The control group and DEN-C group (DEN control group) were fed the basal diet; the DEN-T1, DEN-T2, and DEN-T3 groups were fed the 1, 2, and 3% taurine diet, respectively; DEN-B group 5% β-alanine diet.

Immunohistochemical staining for placental GST positive foci (GST-P$^+$ foci) The liver sections from acetone-fixed tissues were stained for GST-P$^+$ foci. It was carried out using the avidin-biotin-peroxidase complex method (Vectastain ABC kit, Vector Lab. Inc., Burlingame, USA; GST-P antibody, MBL Co., Japan; Hsu *et al.*, 1981). The area and number of foci were measured by means of an image analyzer with a microscope (Image-Pro Plus v.4, Media Cybernetics, L.P., Silver Spring, USA). The number of GST-P$^+$ foci was counted as foci >0.2 mm in diameter.

Determination of lipid peroxidation and glucose 6-phosphatase (G6Pase) activity Microsomal lipid peroxidation was determined by measuring the formation of thiobarbituric acid reactive substances (TBARS) according to the method of Buege and Aust (1978). The absorbance of the resulting chromophore was determined at 535 nm, and the extinction coefficient for TBARS was taken to be 1.56×10^5 $M^{-1}cm^{-1}$. G6Pase activity was determined by the method of Baginski *et al.* (1983), in which inorganic phosphate liberated was determined with ammonium molybdate and the absorbance was measured at 840 nm.

Determination of total GSH content Total GSH content was measured by a modification of Griffith (Anderson, 1985), using the 5,5'-dithiobis(2-nitrobenzoic acid)-GSH reductase (GR) recycling procedure. The rate of 5-thio-2-nitrobenzoic acid formation was followed at 412 nm and the total GSH content was expressed as μmole of reduced GSH equivalents per g liver.

GSH-dependent enzyme assay and protein concentration determination GST activity was assayed in the hepatic cytosolic fraction using the method of Habig *et al.* (1974). The conjugate of GSH with 1-chloro-2,4-dinitrobenzene (CDNB) was measured at 340 nm using a dual beam spectrophotometer. Calculation was made using a molar extinction coefficient of

Table 2. Effect of taurine supplementation on final body weight, liver weight, and relative liver weight

Group	No. of rats	Body weight (g)	Liver weight (g)	Relative liver weight (g/100g body weight)
Control	7	441±16[a]	12.7±0.53[a]	2.89±0.11
DEN-C	7	418±11[a]	12.0±0.59[a]	2.88±0.08
DEN-T1	7	428±12[a]	11.4±0.63[a]	2.65±0.12
DEN-T2	7	439±06[a]	12.4±0.31[a]	2.83±0.06
DEN-T3	7	435±07[a]	12.2±0.53[a]	2.81±0.12
DEN-B	7	366±07[b]	9.7±0.19[b]	2.66±0.02

Rats, except control group, were treated with DEN and partial hepatectomy to induce hepatic carcinogenesis. Rats were fed with an experimental diet containing taurine; Control and DEN-C group, basal diet; DEN-T1, -T2, -T3 group, 1, 2, and 3% taurine diet, respectively; DEN-B group, 5% β-alanine diet. Values are mean±SE from final weight of body and liver. Relative liver weight was calculated by % of liver weight to body weight. Means with the same superscripts are not significantly different at $p<0.05$ by Duncan's multiple range test.

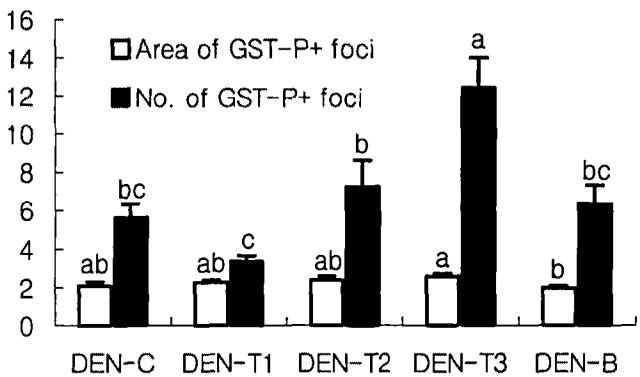

Fig. 2. Effect of taurine supplementation on the area and number of GST-P$^+$ foci in the rat liver. Rats were treated with DEN and partial hepatectomy to induce hepatic carcinogenesis. Rats were fed with an experimental diet containing taurine; DEN-C group, basal diet; DEN-T1, -T2, -T3 group, 1, 2, and 3% taurine diet, respectively; DEN-B group, 5% β-alanine diet. Values are mean±SE from image reading of stained GST-P$^+$ foci. The unit of areas is mm^2/cm^2, and that of number is No./cm^2. Means with the same letters are not significantly different at $p<0.05$ by Duncan's multiple range test.

9.6 $mM^{-1}cm^{-1}$. Activities of GSH peroxidase (GPx) and GR in the hepatic cytosolic fraction were measured by monitoring the oxidation of NADPH at 340 nm, according to the method of Tappel (1978), Calberg and Mannervik (1985), respectively. Protein concentration was determined by modified Lowry method (Bollag *et al.*, 1996).

Statistical analysis All statistical analyses were carried out using SAS program with Duncan's multiple range test. Differences were considered statistically significant at $p<0.05$.

Results

There was no energy difference in the intake among groups, except in the β-alanine group. The energy intake of the β-alanine group significantly decreased compared to those of other groups (approximately 81% of DEN control group, data not shown). This resulted in decreases in the body and liver weights (Table 2). Relative liver weight, however, showed no difference.

Table 3. Effects of taurine supplementation on thiobarbituric acid reactive substance (TBARS) content

Group	TBARS (nmole/mg protein)
Control	0.2280±0.012[ab]
DEN-C	0.2388±0.006[a]
DEN-T1	0.2141±0.010[abc]
DEN-T2	0.2096±0.007[bc]
DEN-T3	0.2114±0.009[bc]
DEN-B	0.1967±0.006[c]

Rats, except control group, were treated with DEN and partial hepatectomy to induce hepatic carcinogenesis. Rats were fed with an experimental diet containing taurine; Control and DEN-C group, basal diet; DEN-T1, -T2, -T3 group, 1, 2, and 3% taurine diet, respectively; DEN-B group, 5% β-alanine diet. Values are mean±SE from absorbance readings of chromophore. Means with the same superscripts are not significantly different at $p<0.05$ by Duncan's multiple range test.

GST-P$^+$ foci, the preneoplastic lesions, appeared in all rats exposed to DEN (Fig. 2). Although the number of GST-P$^+$ foci in the 3% taurine group were higher than that of the DEN control group, the areas of GST-P$^+$ foci were not different. The number of GST-P$^+$ foci in the 1% taurine group was slightly decreased compared to the DEN control group, however the difference was significant.

The lipid peroxidation products were frequenly regarded to have an effect on cancer promotion (Burk *et al.*, 1980). Also, the TBARS contents, the index of lipid peroxidation product level, in 2 and 3% taurine diet groups were significantly lower than that of the DEN control group (Table 3). Beta-alanine supplementation also significantly decreased the TBARS

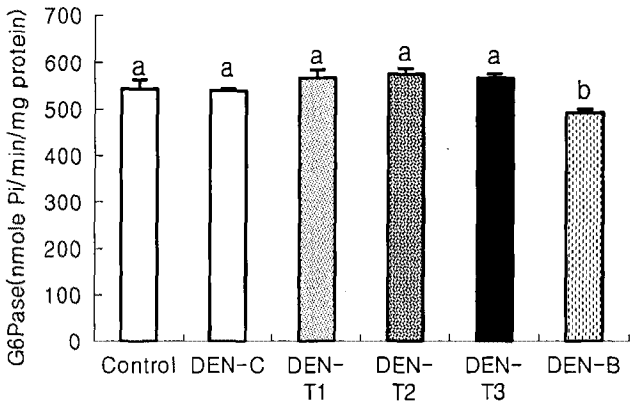

Fig. 3. Effects of taurine supplementation on glucose 6-phosphatase (G6Pase) activity. Rats, except control group, were treated with DEN and partial hepatectomy to induce hepatic carcinogenesis. Rats were fed with an experimental diet containing taurine; Control and DEN-C group, basal diet; DEN-T1, -T2, -T3 group, 1, 2, and 3% taurine diet, respectively; DEN-B group, 5% β-alanine diet. Values are mean±SE from absorbance readings of inorganic phosphate liberated. Means with the same letters are not significantly different at $p<0.05$ by Duncan's multiple range test.

Table 4. Effect of taurine supplementation on the activities of glutathione *S*-transferase (GST) and glutathione reductase (GR)

Group	GST (nmole CDNB conjugated /min/mg protein)	GR (nmole NADPH oxidized /min/mg protein)
Control	807.9±22^{b}	81.2±1.4^{bc}
DEN-C	787.1±23^{b}	85.4±0.9^{a}
DEN-T1	929.2±34^{a}	79.4±0.5^{c}
DEN-T2	842.1±38^{ab}	82.6±1.1^{abc}
DEN-T3	838.4±37^{ab}	85.1±1.1^{a}
DEN-B	876.1±24^{ab}	83.6±1.6^{ab}

Rats, except control group, were treated with DEN and partial hepatectomy to induce hepatic carcinogenesis. Rats were fed with an experimental diet containing taurine; Control and DEN-C group, basal diet; DEN-T1, -T2, -T3 group, 1, 2, and 3% taurine diet, respectively; DEN-B group, 5% *b*-alanine diet. Values are mean ± SE from change rate of conjugated CDNB or oxidized NADPH. Means with the same superscripts are not significantly different at $p<0.05$ by Duncan's multiple range test.

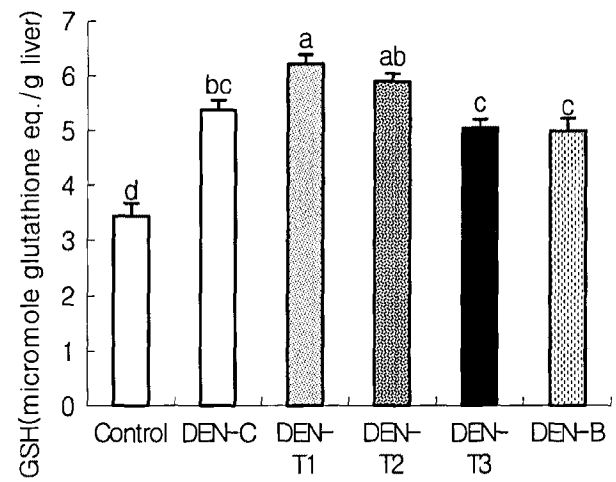

Fig. 4. Effect of taurine supplementation on the glutathione (GSH) levels. Rats, except control group, were treated with DEN and partial hepatectomy to induce hepatic carcinogenesis. Rats were fed with experimental diet containing taurine; Control and DEN-C group, basal diet; DEN-T1, -T2, -T3 group, 1, 2, and 3% taurine diet, respectively; DEN-B group, 5% β-alanine diet. Values are mean±SE from the rate of 5-thio-2-nitrobenzoic acid formation. Means with the same letters are not significantly different at $p<0.05$ by Duncan's multiple range test.

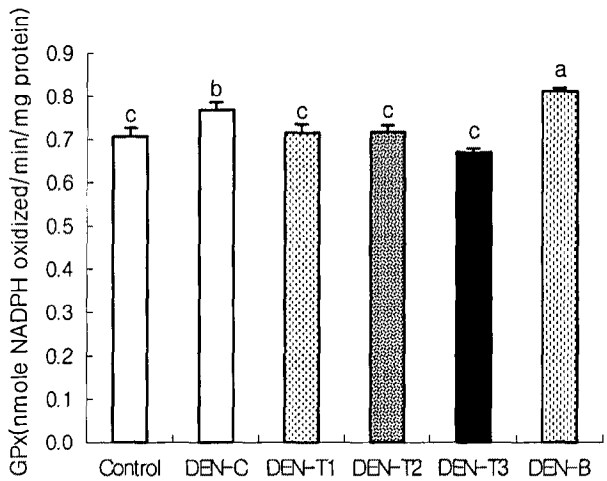

Fig. 5. Effect of taurine supplementation on glutathione peroxidase (GPx) activity. Rats, except control group, were treated with DEN and partial hepatectomy to induce hepatic carcinogenesis. Rats were fed with an experimental diet containing taurine; Control and DEN-C group, basal diet; DEN-T1, -T2, -T3 group, 1, 2, and 3% taurine diet, respectively; DEN-B group, 5% β-alanine diet. Values are mean±SE from change rate of oxidized NADPH. Means with the same letters are not significantly different at $p<0.05$ by Duncan's multiple range test.

content compared to the control diet. The activity of G6Pase in the β-alanine diet group was significantly lower than that in the other groups (Fig. 3).

GSH has been known to be a potent antioxidant and substrate of GPx that results in the protection of lipid peroxidation and the elimination of peroxide (Stout and Becker, 1986). The GSH level was significantly increased by the treatment of DEN and partial hepatectomy (Fig. 4). In comparison to the DEN control group, only the 1% taurine diet significantly increased the GSH level. GST activity was significantly increased by the 1% taurine diet (Table 4). GPx activity was increased in the DEN treatment groups, but it was

significantly decreased by taurine supplementation (Fig. 5). Beta-alanine increased GPx activity. It was correlated with G6Pase activity ($r = -0.2715$, $p<0.05$) and the area and number of GST-P$^+$ foci ($r = -0.2571$, $p<0.05$; $r = -0.3861$, $p<0.05$, respectively). GR activity also increased in the DEN treatment groups, and the elevation was suppressed by the 1% taurine diet.

Discussion

In experimentally induced cancer models, organosulfur compounds showed cancer preventive effects, by blocking enzyme activities that are responsible for the biotransformation of a particular procarcinogen to carcinogen form (Brady *et al.*, 1991), or by enhancing detoxification enzyme activities (Reddy *et al.*, 1993; Seo *et al.*, 1999). Moreover, some organosulfur compounds act as antioxidants scavenging free radicals (Cunningham *et al.*, 1998). Taurine is one of organosulfur compounds with antioxidant activity, and its biological functions are closely related with chemopreventive potential.

Our study showed that taurine supplementation could affect the formation of preneoplastic lesion, GST-P$^+$ foci, which was not the expected result of the chemoprevention of taurine. Both taurine supplementation and depletion seemed to have an effect on GST-P$^+$ foci growth, but the mechanisms are expected to be different. Energy restriction affects tumor incidence and growth (Albanes, 1987). The reduction of energy intake was examined in the β-alanine group. Although energy reduction could effect the prevention of carcinogenesis, the foci formation in the β-alanine group was not less than that of DEN control group. The stimulation of the GST-P$^+$ foci growth by the depletion of taurine might offset the preventive effect of dietary restriction.

No report has shown the stimulatory effects of taurine on tumor incidence or the development of preneoplastic lesions. Several researches reported the suppression of preneoplastic lesions by taurine supplementation (Reddy *et al.*, 1993; Okamoto *et al.*, 1996), but their levels of taurine supplementation were less than 2000 ppm in the diet. In some short-term taurine supplementation studies that targeted atherogenesis or diabetes mellitus, researchers even used 5% taurine in the diet or in drinking water, and reported its usefulness (Obrosova and Stevens, 1999; Yokogoshi *et al.*, 1999). Our study, however, showed that more than 2% taurine could stimulate the formation of GST-P$^+$ foci in hepatocarcinogenesis. Yokogoshi *et al.* (1999) examined the dose effect of taurine at the levels 0.025 to 5.0%. They reported that a 1% taurine diet for 2 weeks was sufficient to reduce cholesterol levels of plasma or major tissues in rats fed on a high cholesterol diet. Their works was based on treatment against experimentally induced pathology, not focused on prevention. Taurine supplementation levels to prevent chronic diseases could be much lower than that for treating diseases. Recently, Wargovich *et al.* (2000) reported the chemopreventive effect of taurine on rat colon aberrant crypt formation. The rats were given 0.06% and 0.12% taurine before the initiation of colon carcinogenesis. You and Chang (1998), also suggested that 1% taurine in drinking water appears to protect the liver against lipid peroxidation and membrane disintegration in rat hepatocarcinogenesis. Therefore, the optimal dose of taurine supplementation against hepatocarcinogenesis in the preventive stage should be determined through studies with taurine at less than 1%. Long term use of excess taurine supplementation could adversely effect the host in hepatocarcinogenesis. When we supposed that the average amount of diet comsumed is 1 kg, then the level of 3% taurine means more than 30 g taurine per daily human diet. That is a very high amount comsumed by pill or mixed diet. It seems impractical to achieve this amount.

Antioxidation is known as an important role of taurine. Taurine protected the heart against lipid peroxidation (Rascheke *et al.*, 1995), and it could be converted into various aldehyde in response to oxidative stress (Cunningham *et al.*, 1998). In our study, TBARS contents were significantly reduced by more than 2% levels of taurine supplementation, although a 3% taurine diet affected the GST-P$^+$ foci formation. The GSH level was known to be increased accordingly to the demand of detoxification (Stout & Becker, 1986), which was supported by our study. GSH levels were increased by taurine supplementation; however these increases were not dose-dependent in taurine supplementation groups. The GSH level in the 2% and 3% taurine group was not different than that of the DEN control group. The sparing effect of GSH was shown in the 1% taurine diet group only, and its level by taurine depletion was significantly decreased. In our study, antioxidation by taurine could be sufficient to reduce the demand of endogenous taurine or GSH synthesis. Exogenous taurine is displaced with endogenous taurine, and antioxidation by taurine precursors is more effective than that by taurine itself (Aruoma *et al.*, 1998). Therefore, over-dose of exogenous taurine, 2 or 3% taurine, could reduce the biosynthesis of taurine and its precursors, such as cysteamine and hypotaurine, or GSH. In a rhesus monkey study (Sturman *et al.*, 1991), taurine supplementation did not modulate the key enzyme activities of taurine biosynthesis, but there are few reports with rats. Moreover, most researches on taurine as an antioxidant were based on *in vitro* studies or used of a few week short-term models (Raschke *et al.*, 1995; Cunningham *et al.*, 1998). This is differently from our long-term *in vivo* study. Therefore, many long-term *in vivo* studies should be needed to discuss the effect of taurine on antioxidation.

G6Pase activity in β-alanine group showed a decrease of membrane stability compared to other groups. Many researches have supported the theory that taurine could affect membrane stability, but this was not evidenced in our study. The reduction of G6Pase activity in the β-alanine group, however, partially showed the relationship between other β-amino acid depletion and membrane stability. Reddy *et al.* (1993) reported the marginal inhibition of colon tumor

multiplicity and GST induction in rat liver that was exposed to 1200 ppm taurine and other organosulfur compounds. In our study, 1% taurine diet might work as an activator of GST, and it slightly suppressed preneoplastic foci formation. However, a significant reduction of foci formation was not shown. GPx activity had a negative correlation with both the area and number of GST-P$^+$ foci, which showed that the decrease of GPx activity by taurine was related to the development of GST-P$^+$ foci. GPx contributed to the elimination of peroxides and protection of cells from oxidative stress (Burk *et al.*, 1980). Also, the elevation of GPx activity that is induced by hepatocarcinogenesis might be one of host defense mechanism synchronized by the increase of GSH levels. Habitual excess taurine supplementation could weaken the host defense system against oxidation. The decrease of both the GSH level and GPx activity by excess taurine could be contrary to hepatic carcinogenesis that is induced by DEN. It is reported that GR activity in the tumor tissue of the mouse liver was higher than that in normal tissue (Stout and Becker, 1986), and we also examined the GR activity increase in the DEN control group compared to the control group. Only a 1% taurine diet lowered the GR activity, which can contribute to the GSH level sustenance and detoxification through converting oxidized GSH to a reduced form. The high level of GSH in a 1% taurine group might not demand the induction of the GR activity, because GSH level was enough to induce GST activity (Manson *et al.*, 1997).

Therefore, we concluded that more than 2% taurine affected the development of GST-P$^+$ foci, which might result from the modulation of GSH utilization and GPx activity. In the case of hepatic cancer prevention, the taurine supplementation of a pharmaceutical dose (as much as 2 or 3% of total dietary intake) might induce proliferation of cancer cells initiated by DEN. Nevertheless the potency of chemopreventive property (owing to taurine at less than 1%) still remains, which could be suggested from the fact that 1% taurine diet worked as an activator of GST and increased GSH level. Further studies should investigate the effect of taurine with lower than 1% levels on the prevention of hepatic cancer, as well as the role of taurine in proliferation and growth of cancer cells.

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