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Lack of Modifying Effects of Environmental Estrogenic Compounds on the Development of Thyroid Proliferative Lesions in Male Rats Pretreated with *N*-Bis(2-hydroxypropyl)nitrosamine (DHPN)

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The modifying effects of various environmental estrogenic compounds on thyroid carcinogenesis were investigated in a rodent two-stage carcinogenesis model. The compounds examined were a soy isoflavone mixture (SI) and genistein (GEN) as phytoestrogens, nonylphenol (NP) as a xenoestrogen, 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX) as a thyroid carcinogen and sulfadimethoxine (SDM) as a known thyroid tumor promoter. Five-week-old male F344 rats were given a single subcutaneous injection of *N*-bis(2-hydroxypropyl)nitrosamine (DHPN; 2800 mg/kg, body weight) or the vehicle alone. Starting one week thereafter, GEN (250 or 25 ppm in diet), SI (400 ppm in diet), NP (250 or 25 ppm in diet), MX (30 ppm, in drinking water) or SDM (1000 ppm in drinking water) was administered for 12 weeks. Major organs including the thyroid, pituitary, liver, kidney, testis, brain and pancreas were weighed and histopathological observation was performed. Thyroid weights were significantly increased ($P<0.001$) only in the SDM treatment groups, especially with DHPN pretreatment. Kidney weights were slightly increased in the NP or MX treatment groups, albeit without statistical significance. Histopathologically, thyroid proliferative lesions were only observed in the SDM alone or DHPN+SDM group with significant focal hyperplasias, adenomas and adenocarcinomas limited to the combined treatment case. There were no organ weight changes or histopathological lesions in the major organs including the thyroid in the GEN, SI, NP, and MX treatment groups regardless of DHPN pretreatment. Our results thus indicate that the weakly estrogenic compounds GEN, SI and NP and the environmental rat thyroid carcinogen MX do not exert any modifying effects on thyroid carcinogenesis in rats under the present experimental conditions.

Key words: Estrogenic compounds — Thyroid carcinogenesis — Genistein — Nonylphenol — 3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone

In general, thyroid diseases are more prevalent in women than men,[1)] and the use of several estrogen-containing preparations is associated with an increased risk of thyroid cancer,[2)] suggesting possible roles for female sex hormones in the carcinogenic process. Male rats generally develop more thyroid tumors after radiation or application of goitrogens compared with female rats, but this may be because they have higher thyroid stimulating hormone (TSH) levels.[3)] Furthermore, the incidence of thyroid carcinomas induced by *N*-methyl-*N*-nitrosourea (MNU) in female rats is higher than in males[4, 5)] and administration of estradiol enhances the development of rat thyroid proliferative lesions.[4–6)] Recently, estradiol was found to increase growth of FRTL-5 cells (Fischer rat thyroid epithelial cells) in a time- and concentration-dependent manner, in either the absence or the presence of TSH.[7)] This demonstration of a direct effect of estradiol on thyroid follicular cells raises the possibility that estrogenic activity may play a role in the sexually dimorphic prevalence of goiter.

There is a general consensus that endocrine disrupting chemicals (EDCs) interfere with thyroid hormone function and homeostasis either by inhibiting synthesis, altering serum transport proteins, or increasing catabolism of thyroid hormones. There are three major types of environmental estrogen-mimicking chemicals (EECs), phytoestrogens, mycoestrogens, and xenoestrogens. Phytoestrogens are constituents contained in plants, mycoestrogens are products of fungi, and xenoestrogens are man-made chemicals synthesized for commercial use or formed as byproducts of manufacturing processes and combustion of wastes, for example.[8)] Recently, interest has been focused on the potential anticancer role of phytoestrogens, plant constituents with weak estrogenic activity. These compounds, including several flavonoids, isoflavones, lignans, phytosterols and coumestans, exert antiestrogenic effects by competitive binding to estrogen receptors.[9)] Several

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epidemiological studies have suggested that soy products and isoflavones may be protective against breast and other hormone-related cancers in humans.[10–12] However, dietary soybean has been implicated as a goitrogenic factor in many studies[13–17] and the possible role of isoflavones, its active constituents, in thyroid carcinogenesis remains unclear. Also some xenoestrogens such as organochlorine,[18] TCDD,[19, 20] metho-xychlor[21] and alachlor[22] have been shown to exert thyroid effects in man and experimental animals. However, little is known at present about the effects of weakly estrogenic chemicals on thyroid carcinogenesis.

Development of the rat thyroid two-stage carcinogenesis model applying DHPN as an initiator has made a great contribution to the investigation of thyroid tumorigenesis as well as detection of thyroid tumor modulators.[23, 24] The present study was therefore performed to examine the modifying effects of a soy isoflavone mixture (SI) and genistein (GEN) as well as the xenoestrogen nonylphenol (NP) and an environmental thyroid carcinogen 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX), on post-initiation development of thyroid proliferative lesions in rats pretreated with *N*-bis(2-hydroxypropyl)nitrosamine (DHPN).

MATERIALS AND METHODS

Animals Specific-pathogen-free male F344 rats, 4 weeks old, were obtained from Charles River Japan Inc. (Kanagawa) and housed five to a polycarbonate cage with wood chips as bedding in an air-conditioned animal room (room temperature; 23±2°C, relative humidity; 60±5%, 12 h light/dark cycle). Animals without any abnormal findings after a 1-week acclimation period were selected for the present study. Powdered basal diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo), from which soy constituents were eliminated, and ion exchange water as the drinking water were available *ad libitum* throughout the study.

Chemicals DHPN was purchased from Nacalai Tesque Inc. (Kyoto) and sulfadimethoxine (SDM), a strong thyroid tumor-promoter, from Sigma Chemicals (St. Louis, MO). MX was synthesized from tetrachloroacetone and (carbomethoxylene)triphenylphosphorane according to the method of Padmapria *et al*.[25] Its purity was determined to be 97% by high-performance liquid chromatography (R-ODS-5 column, YNS, Osaka). NP and SI were respectively obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo) and Kikkoman Co., Ltd. (Tokyo). SI contained more than 30% isoflavone aglycone (GEN 12–18%, diadzein 12–18% and glycitein 2–4%). GEN was synthesized as described previously[26] and its purity was proven to be higher than 97%.

Experimental procedure Rats were divided into 16 groups, each consisting of 10 or 5 animals, with comparable initial mean body weights. Rats of 8 groups, 10 rats in each group, received a single s.c. injection of 2800 mg/kg DHPN. From one week after the DHPN initiation, they were given no supplement, MX (30 ppm in drinking water), GEN (250 or 25 ppm in diet), NP (250 or 25 ppm in diet), SI (400 ppm in diet) or SDM (1000 ppm in drinking water) for 12 weeks. The 5 rats each in the other 8 groups used as matched controls were similarly treated without DHPN pretreatment. The experimental diets were prepared by Oriental Yeast Co., by adding test chemicals to soy protein free CRF-1.

At autopsy, major organs including the thyroid, pituitary, liver, kidney, and testis were carefully examined macroscopically, weighed and fixed together with the

Table I. Intake of Food and Test Chemicals over 12 Weeks

Group	Daily intake		Total intake
	Food (mg/day/rat)	Test chemicals (mg/day/rat)	Test chemicals (mg)
DHPN+genistein (250 ppm)	15.9±0.9	3.97	333.9
DHPN+genistein (25 ppm)	15.1±0.9	0.38	31.7
DHPN+nonylphenol (250 ppm)	14.7±1.4	3.68	308.7
DHPN+nonylphenol (25 ppm)	15.3±0.9	0.38	32.1
DHPN+isoflavone (400 ppm)	14.8±0.8	5.90	497.3
DHPN (basal diet)	14.9±0.7	—	—
Genistein (250 ppm)	15.8±1.1	3.95	331.8
Genistein (25 ppm)	14.8±1.0	0.37	31.1
Nonylphenol (250 ppm)	14.4±0.9	3.60	302.4
Nonylphenol (25 ppm)	14.7±1.0	0.37	30.9
Isoflavone (400 ppm)	15.8±0.8	6.30	530.9
Saline (basal diet)	15.1±0.5	—	—

Values are mean±SD.

brain and pancreas in 10% phosphate-buffered formalin. After routine processing, sections stained with hematoxylin and eosin (H-E) were observed under a microscope. The results were statistically analyzed using ANOVA followed by Student's *t* test. Differences were considered statistically significant at $P<0.05$.

RESULTS

Test chemical intakes Daily intakes of test chemicals estimated from food and water consumption data are shown in Tables I and II. They were well correlated with the doses applied.

Table II. Intake of Water and MX or SDM over 12 Weeks

Group	Daily intake		Total intake
	Food (mg/day/rat)	Test chemicals (mg/day/rat)	Test chemicals (mg)
DHPN+MX (30 ppm)	19.7±3.6	0.59	49.6
MX (30 ppm)	21.5±4.2	0.65	54.2
DHPN+SDM (1000 ppm)	16.4±1.3	16.4	1377.6
SDM (1000 ppm)	15.8±1.5	15.8	1327.2

Values are mean±SD.

Body and organ weights Mean final body weights are shown in Table III. Body weights of rats given DHPN were lower than those of rats without DHPN treatment throughout the experiment. However, there were no significant differences among final body weights in the MX, NP, GEN and SI treatment groups. Only the SDM treatment significantly ($P<0.001$ or 0.05) decreased the body weight gain.

Relative organ weights are shown in Table III. Mean thyroid weights in the DHPN treatment groups did not show any statistically significant variation from those in the non-DHPN treatment groups. Thyroid ($P<0.001$) and pituitary ($P<0.05$) weights were significantly increased by SDM treatment (Fig. 1). The MX, NP, GEN and SI supplemented groups, regardless of pretreatment with DHPN, did not show any changes in thyroid weight. Kidney weights in the MX and NP treatment groups were greater than those in the basal diet group although this was not statistically significant.

Histopathology Histopathologically, proliferative lesions of the thyroid were classified into follicular cell hypertrophy, hyperplasia, adenoma, and carcinoma categories.[27] No thyroid proliferative lesions were found in the DHPN alone group. The SDM treatment significantly increased the occurrence of thyroid follicular hypertrophy, hyperplasias, adenomas, and adenocarcinomas after DHPN pre-

Table III. Final Body and Relative Organ Weights

Group	No. of rats	Body weight	Thyroid	Pituitary	Kidney	Liver	Testes
DHPN+SDM (1000 ppm)	10	267.7±4.45$^{**,\#}$	78.87±13.0$^{**,\#\#}$	4.41±0.89*	0.54±0.03$^{\#}$	2.89±0.18	1.02±0.03
DHPN+MX (30 ppm)	10	287.5±13.63*	6.62±1.73	3.71±0.94	0.58±0.03*	3.10±0.13	0.96±0.06
DHPN+GEN (250 ppm)	10	291.2±14.76	5.93±0.51	3.59±0.55	0.57±0.02	3.07±0.13	0.86±0.31
DHPN+GEN (25 ppm)	10	289.7±13.01*	6.22±0.95	3.11±0.28	0.56±0.01	3.05±0.13	0.92±0.12
DHPN+NP (250 ppm)	10	284.7±12.96*	5.57±0.60	3.45±0.54	0.59±0.02*	2.99±0.15	0.89±0.09
DHPN+NP (25 ppm)	10	302.7±8.14	5.49±0.55	3.30±0.26	0.57±0.02	3.00±0.10	0.90±0.05
DHPN+SI (400 ppm)	10	291.4±12.29	5.80±0.37	3.46±0.37	0.58±0.02	2.88±0.08	0.90±0.07
DHPN	10	294.6±17.04	5.34±0.47	3.30±0.42	0.57±0.02	2.97±0.09	0.89±0.09
SDM (1000 ppm)	5	272.6±8.14*	68.02±6.45**	3.75±0.21*	0.56±0.02	2.81±0.13	1.06±0.05*
MX (30 ppm)	5	302.6±15.41	6.30±0.66	3.24±0.12	0.60±0.02*	3.02±0.06	0.95±0.04
GEN (250 ppm)	5	309.1±13.83	5.77±0.48	3.24±0.24	0.59±0.03	3.04±0.08	0.93±0.04
GEN (25 ppm)	5	301.9±22.24	5.65±0.41	3.31±0.09	0.56±0.02	2.91±0.13	1.01±0.07
NP (250 ppm)	5	298.4±13.13	5.98±0.41	3.36±0.36	0.57±0.02	2.99±0.09	0.99±0.02
NP (25 ppm)	5	311.3±21.94	5.92±0.20	3.11±0.65	0.55±0.02	2.99±0.20	0.94±0.15
SI (400 ppm)	5	313.4±15.10	5.50±0.43	2.81±0.07	0.56±0.02	2.93±0.08	0.93±0.03
Saline	5	311.6±11.12	5.33±0.32	3.02±0.39	0.55±0.03	2.93±0.19	0.95±0.09

Values are mean±SD.
∗ Significantly different from the control value (saline) at $P<0.05$.
∗∗ Significantly different from the control value at $P<0.001$.
Significantly different from the DHPN treatment group value at $P<0.05$.
Significantly different from the DHPN treatment group value at $P<0.001$.
Thyroid and pituitary: mg/100 g body weight.
Liver, kidney and testes: g/100 g body weight.

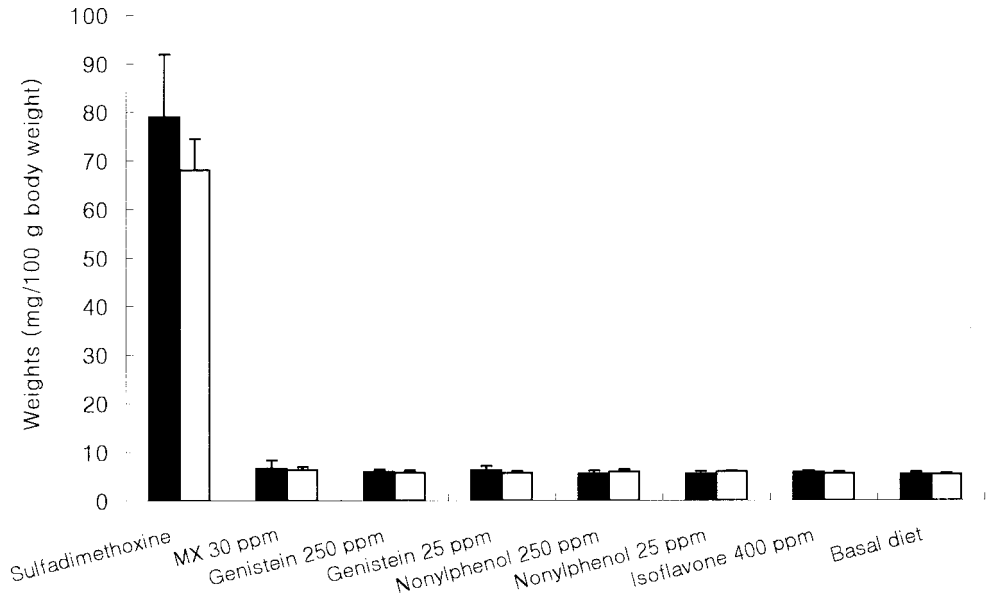

Fig. 1. Relative thyroid gland weights. ■ DHPN treatment, □ saline treatment.

Table IV. Data for Incidence and Multiplicity of Proliferative Lesions in the Thyroids of Rats Treated with DHPN and SDM

Lesion	SDM+DHPN		SDM	
	Incidence (%)	Multiplicity[a)]	Incidence (%)	Multiplicity
Hypertrophy	100	—	100	—
Hyperplasia/diffuse	100	—	100	—
Hyperplasia/focal	100	7.7±2.5	—	—
Adenoma	100	6.8±2.8	—	—
Adenocarcinoma	70	1.1±0.9	—	—

a) Mean±SD, No. of lesions/animal.

treatment (Table IV). However, MX, NP, GEN or SI did not influence the post-initiation development of such thyroid proliferative lesions. Hyperplasia and cellular changes were seen in the pars distalis in SDM treated rats. The affected cells are large with abundant, pale eosinophilic cytoplasm and round vesicular nuclei. Except for thyroid and pituitary proliferative lesions found in SDM-treated rats, specific pathological lesions related to test chemicals were not observed in other tissues in this study.

DISCUSSION

In the present study, MX, GEN, NP, and SI did not influence thyroid carcinogenesis in the two-stage tumor model initiated with DHPN. Because 1000 ppm of SDM induces thyroid proliferative lesions within 12 weeks,[24] we selected SDM treatment as a positive control. The fact that development of thyroid proliferative lesions initiated by pretreatment with DHPN was obviously increased by SDM validates the experimental model used in the present study. It is well documented that sulfonamides including SDM exert a goitrogenic effect by inhibition of thyroperoxidase, resulting in marked decreases in circulating T_3 and T_4 and consequent compensatory increases in TSH and thyroid weight in rats.[3]

Epidemiological studies suggest that a diet rich in soy products reduces the risk of several cancers, notably those of the prostate,[10] breast,[11] uterus[12] and stomach.[28] However, goiter and hypothyroidism have been reported in infants receiving a formula containing soy.[14, 15] Several investigators have reported induction of goiter[13, 17] and thyroid carcinomas[16] in iodine-deficient rats maintained on a soybean diet. Divi *et al*.[29] observed that the soy isoflavones genistein and daidzein inhibit thyroid peroxidase (TPO), a key enzyme in the biosynthesis of thyroid hormone. Thus, it might be predicted that soy isoflavone would inhibit TPO-mediated thyroid hormone synthesis. However, in the present study, SI and GEN did not cause thyroid proliferative lesions with or without DHPN pretreatment. The dose of 400 ppm of SI used in this study is as high as that contained in 20% defatted soybean, which earlier induced thyroid proliferative lesions.[17] Furthermore, 250 ppm of GEN is approximately the concentration present in ~20% soybean protein diet, which exhibits antioxidant activities.[30] Thus, our results suggest that SI and

GEN may not be involved in the thyroid goitrogenic effects of soybean.

NP has been shown to possess estrogen-like properties,[31] being positive in a uterotropic bioassay in mice[32] and rats[29] and stimulating the growth of estrogen-dependent MCF-7 cells.[32] NP was also found to increase the proliferative activity of epithelial cells of the Noble rat mammary gland[33] and to stimulate vitellogenin production,[34] a process that is normally estrogen-dependent.[35] For estrogenic responses, the minimum levels of NP in rodents, following oral, dietary and implant routes of exposure, were around 40 mg/kg/day.[36] In the present study, the dose of 250 ppm of NP administered in the diet corresponds to approximately 13–40 mg/kg/day in rats, and did not induce thyroid lesions. From these data, it would appear that NP has no marked effect on the thyroid glands. In addition, NP increased relative kidney weight, but did not cause tubular lesions in the present study. This is in line with the earlier observation that NP increased kidney weight in a subchronic toxicity study, but this was not considered toxicologically significant.[37]

MX formed during chlorination of drinking water is a very potent bacterial mutagen[38] and mammalian cell clastogen.[39] Several results pointing to carcinogenicity of MX have been reported.[40–43] It induces DNA single strand scissions, enhances replicative DNA synthesis and increases ornithine decarboxylase activity in the glandular stomach of rats.[40] Also, MX induces cell proliferation as well as lipid peroxidation in the gastric mucosa[41] and exerted promoting effects in two-stage glandular stomach carcinogenesis in rats.[43] In a recent carcinogenicity study, it was shown to be definitely carcinogenic when given in drinking water at doses of 5.9, 18.7 and 70.0 ppm for 104 weeks, in a dose-dependent manner. Most tumors were follicular adenomas and carcinomas of the thyroid gland, and cholangiomas in the liver, but mean plasma levels of T_3, T_4 and TSH in MX-treated animals were not significantly different from those in control animals at the end of the study, suggesting that the thyroid cancers were due to a direct carcinogenic effect rather than being indirectly caused by excessive hormonal stimulation.[42] The dose of 30 ppm of MX selected in the present study can induce cell proliferation as well as lipid peroxidation in the gastric mucosa[41] and exert promoting effects on two-stage glandular stomach carcinogenesis in rats.[43] In spite of the high frequency of follicular adenomas and carcinomas in MX-treated animals in the long-term study,[42] MX did not induce proliferative lesions in the present study. Thus, its biological impact on the thyroid gland remains unclear. Previously, increased kidney weight were observed in a subchronic toxicity study of MX,[44] together with kidney tubular damage.[45] In the present study, MX increased relative kidney weight but did not cause tubular lesions. Differences in experimental period and dosage may account for the differences from the results reported by Komulainen *et al*.[45]

Estrogen receptor (ER) has been identified as a principal cellular target for estrogenic compounds[46] and there is also some evidence for ER-regulated gene transcription *in vitro*[47] and *in vivo*.[48] It has been reported that normal and tumor thyroid cells have ERs,[49, 50] and estradiol might promote thyroid tumorigenesis through receptor binding.[4, 5] However, studies on ER expression have failed to provide clear insight into its potential role in thyroid tumor progression.[51, 52] Recently, receptor binding activity has been noted for EDCs such as the ER agonists bisphenol A and methoxychlor and the androgen receptor antagonists linuron and p, p′-DDE.[53] In addition, Masuyama *et al*.[54] suggested that EDCs may affect endocrine functions by altering steroid hormone metabolism through Pregnane X receptor (PXR). Further investigation may explain the possible role of receptor binding activity of EDCs in thyroid carcinogenesis. Although no positive control for either ER- or other non-TSH systems was used in the present study, our recent study in ovariectomized rats using a positive control for the ER system (Son, manuscript submitted) strongly supports the results found in the present study. In that study, no organ weight changes or histopathological lesions in the major organs including the thyroid were induced by the GEN, SI, NP, MX and β-estradiol 3-benzoate treatments under the same experimental conditions as in the present study.

In conclusion, various estrogenic compounds, such as a SI, GEN and NP, as well as an environmental chemical, MX, did not exert any promoting effect on thyroid carcinogenesis in rats initiated with DHPN under the present experimental conditions, although further studies on DNA synthesis, hormone dynamics and receptor binding activity may provide more information.

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