

Lack of Modification by Environmental Estrogenic Compounds of Thyroid Carcinogenesis in Ovariectomized Rats Pretreated with *N*-bis(2-hydroxypropyl)nitrosamine (DHPN)

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The effects of environmental estrogenic compounds, soy isoflavone mixture (SI), genistein (GEN), and nonylphenol (NP), and the possible goitrogen 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX), on thyroid carcinogenesis were investigated in ovariectomized (OVX) female rats. Five-week-old OVX F344 rats were given a single subcutaneous injection of *N*-bis(2-hydroxypropyl)nitrosamine (DHPN; 2400 mg/kg, body weight) or vehicle alone. Starting 1 week later, 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), sulfadimethoxine (SDM), a known thyroid tumor-promoter (1000 ppm in drinking water), or β -estradiol 3-benzoate (EB), a synthetic estrogen (0.5 mg in cholesterol pellet, s.c.) were administered for 12 weeks. SDM and EB were included as positive controls. At sacrifice the major organs including the thyroid, pituitary, liver, kidney, uterus, vagina, brain and pancreas were collected and histopathological observation was performed. Thyroid weights were significantly increased ($P < 0.001$) only in the SDM treatment group and pituitary weights were elevated with SDM ($P < 0.05$) and EB ($P < 0.001$). Kidney and uterus weights were also significantly increased ($P < 0.05$) by EB. Histopathologically, proliferative lesions of the thyroid were only observed in the SDM treatment group and of the pituitary in the SDM or EB treatment groups. Renal tubule lesions, uterine squamous metaplasia, vaginal keratinization and telangiectasia of pancreatic islets were also observed with EB. There were no organ weight changes or histopathological lesions in the major organs, including the thyroid, in the GEN, SI, MX or NP treatment groups. Our results thus indicated a lack of modifying effects on thyroid carcinogenesis in female OVX rats, in agreement with our previous finding in males.

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

Considerable attention has recently been focused on environmental estrogenic chemicals (EECs) that can disrupt the endocrine system. The Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) under the US Environmental Protection Agency (EPA) has recommended screening of compounds for their potential to act as estrogen receptor (ER) or androgen receptor (AR) agonists or antagonists, or steroid biosynthesis inhibitors, or for their ability to alter thyroid function.¹⁾ Endocrine disrupting chemicals (EDCs) may interfere with thyroid hormone function and homeostasis by inhibiting synthesis, altering serum transport proteins, or increasing catabolism of thyroid hormones. There are two basic mechanisms whereby chemicals induce thyroid gland tumors in rodents. One involves direct carcinogenic effects at the DNA level in follicular cells. The other is associated with hypersecretion of thyroid stimulating hormone (TSH) that

stimulates growth of follicular epithelium, resulting in thyroid neoplasias.²⁾ Recently, receptor binding activity has been noted for EDCs like the ER agonists bisphenol A and methoxychlor and the AR antagonists linuron and *p,p'*-DDE.³⁾ However, the actual impact of such compounds on the thyroid gland has not been well defined.

Castration markedly reduces radiation-induced thyroid carcinomas⁴⁾ and testosterone causes elevation of both baseline and TSH-releasing hormone (TRH)-stimulated TSH levels,⁵⁾ and increase of methylthiouracil or radiation-induced rat thyroid tumors.^{4,6)} Furthermore, ovariectomy (OVX) was found to lower the incidence of thyroid carcinomas in female Long-Evans rats,⁷⁾ pointing to roles for both sex hormones in the development of thyroid cancer.⁵⁾ For accessing the impact of estrogenicity of estrogenic chemicals, immature or OVX females are necessary, since they are much more sensitive to estrogenic chemicals than intact mature females.

In the present study, the modifying effects of the EECs, soy isoflavone mixture (SI), genistein (GEN) and non-

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ylphenol (NP) on thyroid carcinogenesis were investigated in OVX rats pretreated with *N*-bis(2-hydroxypropyl)nitrosamine (DHPN). For comparison, sulfadimethoxine (SDM), a known thyroid tumor promoter, 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5*H*)-furanone (MX), a possible thyroid tumor promoter and β -estradiol 3-benzoate (EB), a synthetic estrogen, were also investigated.

MATERIALS AND METHODS

Animals Specific-pathogen-free OVX F344 rats, 4 weeks old, were obtained from Japan SLC Inc. (Shizuoka) and housed five to a polycarbonate cage with wood chips as bedding in an air-conditioned animal room (room temperature, $23 \pm 2^\circ\text{C}$; relative humidity, $60 \pm 5\%$; a 12h 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 *ad libitum* were used throughout the study.

Chemicals DHPN was obtained from Nacalai Tesque Inc. (Kyoto) and SDM from Sigma Chemicals (St. Louis, MO). MX was synthesized from tetrachloroacetone and (carbomethoxy)triphenylphosphorane according to the method of Padmapria *et al.*⁸⁾ Its purity was determined as 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 (genistein 12–18%, diadzein 12–18% and glycitein 2–4%). GEN was synthesized as described previously⁹⁾ and its purity proved to be higher than 97%. EB and cholesterol were obtained from Wako Pure Chemical Industries Ltd. (Osaka). EB-containing pellets were prepared in medical grade Silastic tubes (Kaneka Medix, Co., Osaka), filled with 33 mg of EB mixed with 1 g of cholesterol and 1 ml of olive oil. The content of EB in each pellet (1 cm) was approx. 0.5 mg.

Dose selection for each compound was based on previous reports of thyrotropic, carcinogenic or endocrine-modulating effects. Because 1000 ppm of SDM induced thyroid proliferative lesions within 12 weeks,¹⁰⁾ we selected the SDM treatment group as a positive control. The dose (400 ppm) of SI used in this study is as high as that contained in 20% defatted soybeans, which induced thyroid proliferative lesions.¹¹⁾ Further, 250 ppm of GEN is approximately the concentration present in ~20% soybean protein diet, and exhibits antioxidant activities.¹²⁾ The dose of 250 ppm of NP administered in the diet corresponds to approximately 11–44 mg/kg/day in rats and approximately 1/10 of the maximum tolerated dose (MTD). The minimum detection level for estrogenic response to NP in rodents, following oral administration,

was ca. 40 mg/kg/day.¹³⁾ The dose (30 ppm) of MX selected in this study induced cell proliferation as well as lipid peroxidation in the gastric mucosa¹⁴⁾ and exerted promoting effects in two-stage glandular stomach carcinogenesis in rats.¹⁵⁾

Experimental procedures Rats were divided into 10 groups, each consisting of 10 animals, so that initial mean body weights of each group were similar. Rats of 9 groups received a single s.c. injection of 2400 mg/kg DHPN. From 1 week thereafter, they were given no further treatment, SDM (1000 ppm in drinking water), SI (400 ppm in diet), GEN (250 or 25 ppm in diet), NP (250 or 25 ppm in diet), MX (30 ppm in drinking water) or EB (0.5 mg) for 12 weeks. EB pellets were implanted in the subcutis of the interscapular area under light anesthesia by ethyl ether and replaced every 4 weeks. Non-treatment control rats were fed basal diet without DHPN pretreatment for up to 12 weeks. The experimental diets were prepared in Oriental Yeast Co. (Tokyo) by adding test chemicals to the soy protein-free CRF-1 diet. At autopsy, major organs including the thyroid, pituitary, liver, kidney and uterus were carefully examined macroscopically, and then weighed. In addition to the organs mentioned above, the brain, vagina and pancreas were fixed in 10% phosphate-buffered formalin, and 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. The intakes of test chemicals were well correlated with the doses applied.

Body and organ weights Data for mean body weight gain are illustrated in Fig. 1. There were no significant differences among the final body weights in the MX, NP, GEN and SI treatment groups. SDM and EB treatments significantly reduced ($P < 0.05$) the body weight gain (Table III). Relative organ weights are given in Table III. Thyroid ($P < 0.001$) and pituitary ($P < 0.05$) weights in the SDM treatment group were significantly greater than those of the basal diet group. Pituitary ($P < 0.001$), uterus ($P < 0.001$) liver ($P < 0.05$) and kidney weights ($P < 0.05$) in the EB treatment group were significantly greater than those of the basal diet group. Thyroid weights were also increased in the EB group albeit without statistical significance. MX, NP, GEN and SI-supplemented groups did not exhibit changes in any organ weights, including the thyroid. **Histopathology** (Table IV) Histopathologically, proliferative lesions of the thyroid can be classified into follicular cell hypertrophy, hyperplasia, adenoma, and carcinoma.¹⁶⁾

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

Group	Daily intake		Total intake
	Food (mg/day/rat)	Test chemical (mg/day/rat)	Test chemical (mg)
DHPN+SDM (1000 ppm)	9.4±1.3	—	—
DHPN+MX (30 ppm)	12.0±1.4	—	—
DHPN+Genistein (250 ppm)	11.4±1.7	2.85	239.4
DHPN+Genistein (25 ppm)	11.8±2.1	0.30	24.8
DHPN+Nonylphenol (250 ppm)	11.9±2.1	2.98	250
DHPN+Nonylphenol (25 ppm)	11.4±1.7	0.29	23.9
DHPN+Isoflavone (400 ppm)	12.0±1.5	4.80	403.2
DHPN+β-Estradiol 3-benzoate (0.5 mg)	11.6±1.4	—	—
DHPN	11.7±2.2	—	—
Control	11.6±1.3	—	—

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

Group	Daily intake		Total intake
	Water (mg/day/rat)	Test chemical (mg/day/rat)	Test chemical (mg)
DHPN+SDM (1000 ppm)	14.8±1.2	14.8	1243.2
DHPN+MX (30 ppm)	16.5±1.3	0.5	41.6

The SDM treatment significantly increased the occurrence of all four, as well as pituitary hyperplasias. On the other hand, MX, NP, GEN, SI or EB did not exert any significant influence. Histopathological changes were seen in the pars distalis of the pituitary following OVX. The affected cells were large with abundant, pale and eosinophilic cytoplasm and round vesicular nuclei. Uterus atrophy was also observed in the OVX rats. In addition, cytoplasmic vacuolation in the liver, hyaline droplet nephropathy in the kidney, squamous metaplasia of uterine epithelium, telangiectasia of pancreatic islets, keratinization of the vaginal epithelium and adenomas of the pituitary/pars distalis were observed in the EB treatment group. There were no organ weight changes or histopathological lesions in the major organs, including the thyroid, in the SI, GEN, NP or MX treatment group.

DISCUSSION

In the present study, SI, GEN, NP or MX did not influence thyroid carcinogenesis in a two-stage tumor model using OVX rats initiated with DHPN. These results are consistent with our previous finding for male F344 rats,¹⁷⁾ in which there were no organ weight changes or histopathological lesions in the major organs including the thyroid after GEN, SI, NP or MX treatment under the same

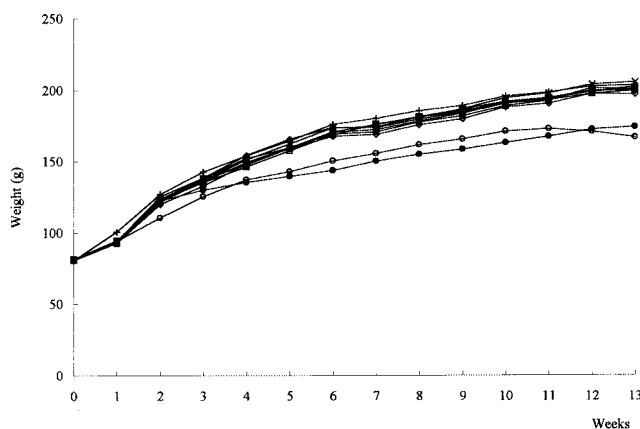


Fig. 1. Body weight curves. ● DHPN+SDM (1000 ppm), □ DHPN+MX (30 ppm), ▲ DHPN+Genistein (250 ppm), △ DHPN+Genistein (25 ppm), ◆ DHPN+Nonylphenol (250 ppm), ◇ DHPN+Nonylphenol (25 ppm), × DHPN+Isoflavone (400 ppm), ○ DHPN+β-Estradiol 3-benzoate (0.5 mg), ■ DHPN, + Control.

experimental conditions as in the present study. Since 1000 ppm of SDM was earlier found to induce thyroid proliferative lesions within 12 weeks,¹⁰⁾ it was used as a positive control. The obvious increase in lesion development validated the experimental model applied.

Synthetic and natural estrogens have been shown to induce tumors in various organs such as the kidney,¹⁸⁾ liver,¹⁹⁾ pituitary,²⁰⁾ and mammary gland.²¹⁾ In addition, estrogen is known to enhance extra-thyroidal conversion of thyroxine (T₄) to triiodothyroxine (T₃) and to affect the pituitary-thyroid axis indirectly.^{22, 23)} 17β-Estradiol can remarkably increase secretion of TSH in a dose-dependent fashion^{24, 25)} and ovarian hyperstimulation in women is associated with elevated estradiol and T₃ although decreased free T₄, TSH and T₄-binding globulin.²⁶⁾ Fur-

Table III. Final Body and Relative Organ Weights

Group	Body weight (g)	Pituitary (mg)	Thyroid (mg)	Liver (g)	Kidneys (g)	Uterus (g)
DHPN+SDM (1000 ppm)	174.0±7.9*#	7.25±0.9*	89.5±28.7***#	2.77±0.11*#	0.9±0.04	0.023±0.005
DHPN+MX (30 ppm)	199.3±10.9	5.57±0.6	7.1±0.9	2.67±0.11	1.1±0.07	0.023±0.005
DHPN+Genistein (250 ppm)	200.8±7.5	5.53±0.2	6.3±0.9	2.62±0.07	1.0±0.03	0.024±0.005
DHPN+Genistein (25 ppm)	200.1±4.9	5.50±0.6	7.6±1.1	2.57±0.28	1.1±0.09	0.028±0.005
DHPN+Nonylphenol (250 ppm)	202.0±9.0	5.60±0.4	7.5±0.7	2.35±0.07	1.0±0.02	0.024±0.004
DHPN+Nonylphenol (25 ppm)	196.8±9.4	5.19±0.4	7.7±0.8	2.36±0.08	1.0±0.06	0.022±0.005
DHPN+Isoflavone (400 ppm)	205.2±5.8	5.36±0.4	7.6±0.6	2.42±0.11	1.1±0.04	0.022±0.006
DHPN+β-Estradiol 3-benzoate (0.5 mg)	166.7±18.3*#	179.56±95.9***#	10.1±1.2	4.00±0.32*#	1.4±0.09*#	0.290±0.070***#
DHPN	199.5±6.3	5.47±0.4	8.1±1.1	2.37±0.10	1.0±0.05	0.026±0.007
Control	203.1±13.8	5.38±0.4	7.0±0.9	2.26±0.11	1.1±0.08	0.025±0.006

* Significantly different from the control value 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 $P<0.001$.

Table IV. Incidence of Histopathological Lesions (%)

Organ	No. of rats :	SDM	MX	GEN (H)	GEN (L)	NP (H)	NP (L)	SI	EB	DHPN	Control
		10	10	10	10	10	10	10	10	10	10
Thyroid											
Hyperplasia/hypertrophy		100	—	—	—	—	—	—	—	—	—
Adenoma		100	—	—	—	—	—	—	—	—	—
Carcinoma		20	—	—	—	—	—	—	—	—	—
Pituitary/pars distalis											
Cellular changes/diffuse		100	100	100	100	100	100	100	100	100	100
Hyperplasia/diffuse		100	—	—	—	—	—	—	—	—	—
Adenoma		—	—	—	—	—	—	—	100	—	—
Liver											
Cytoplasmic vacuolation/single cell		—	—	—	—	—	—	—	100	—	—
Hypertrophy/diffuse		—	—	—	—	—	—	—	100	—	—
Kidney											
Hyaline droplet nephropathy		—	—	—	—	—	—	—	90	—	—
Pancreas/islets											
Telangiectasia		—	—	—	—	—	—	—	100	—	—
Uterus											
Squamous metaplasia/epithelium		—	—	—	—	—	—	—	50	—	—
Atrophy		100	100	100	100	100	100	100	—	100	100
Vagina											
Keratinization/epithelium		—	—	—	—	—	—	—	100	—	—
Brain											
		—	—	—	—	—	—	—	—	—	—

SDM, DHPN+Sulfadimethoxine (1000 ppm); MX, DHPN+3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone (30 ppm); GEN (H), DHPN+Genistein (250 ppm); GEN (L), DHPN+Genistein (25 ppm); NP (H), DHPN+Nonylphenol (250 ppm); NP (L), DHPN+Nonylphenol (25 ppm); SI, DHPN+Isoflavone (400 ppm); EB, DHPN+β-Estradiol 3-benzoate (0.5 mg in cholesterol pellet); DHPN, DHPN+Basal diet; Control, Basal diet; —, No lesions.

thermore, administration of estradiol to rats was found to enhance the development of thyroid proliferative lesions under the influence of a low iodine diet.^{7,27,28} Thyroid proliferative lesions were not promoted by EB in the present study, although thyroid weight was somewhat

increased, suggesting a marginal effect. ER has been identified as a principal cellular target for estrogenic compounds²⁹ and there is also some evidence for ER-regulated gene transcription *in vitro*³⁰ and *in vivo*.³¹ It has been reported that normal and tumor thyroid cells have

ERs,^{32,33}) and estradiol might promote thyroid tumorigenesis through receptor binding.^{7,28}) However, studies on ER expression have failed to provide clear insight into its potential role in thyroid tumor progression.^{34,35}) Direct mitogenic action of estrogenic hormones,³⁶) and catechol estrogens such as 4-hydroxyestradiol^{17,37}) may also play a role. In the present study, 0.5 mg of EB in cholesterol pellets induced pituitary adenomas and increased uterine weight. Thus, the dose of EB was estrogenic in the uterus and in the hypothalamic/pituitary axis, but was not sufficient to exert a major effect on the thyroid.

Pituitary adenomas, kidney tubule lesions, telangiectasia of pancreatic islets, uterus squamous metaplasia and keratinization of the vagina were also observed in the EB treatment group in this study. The kidney hyaline droplet nephropathy and liver cytoplasmic vacuolation lesions might be in line with the carcinogenicity of estrogen in these organs.^{18,19}) The lesions observed in the pituitary, uterus and vagina are well known to be due to estrogenic effects.³⁸) Telangiectasia of the pancreatic islets was also observed previously in hamsters treated with ethynyl estradiol or levonorgestrel (Furukawa, unpublished data) and seems to be related to the influence of estradiol³⁹) and 2-hydroxyoestradiol, a metabolite of estrogen, on insulin secretion.⁴⁰)

The soybean has been implicated in diet-induced goiter⁴¹) and several investigators have reported induction of goiter^{42,43}) and carcinoma in rats maintained on a soybean diet with an iodine deficiency.⁴⁴) GEN (4,5,7-trihydroxyisoflavone), a major isoflavone, inhibits thyroid peroxidase (TPO), a key enzyme in the biosynthesis of thyroid hormone.⁴⁵) Nevertheless, in the present study, SI and GEN did not induce thyroid proliferative lesions after DHPN pre-treatment.

EECs have long been recognized to have uterotrophic activity in a variety of animal species. Isoflavones are among the most important phytoestrogens, which may have estrogenic and antiestrogenic effects. In the present study, SI did not induce uterine changes, in line with the earlier observation that 148 mg/day isoflavone-rich soy protein isolate did not induce proliferation in endometrial and mammary tissues in postmenopausal female macaques (*Macaca fascicularis*).⁴⁶) On the other hand, dietary GEN (375 and 750 µg/g) increased uterine wet and dry weights ($P < 0.05$) in OVX, but not in immature rats.⁴⁷) While 210 ppm GEN produced a large uterotrophic effect in immature OVX rats,⁴⁸) this was not observed in the study by Casanova *et al.*⁴⁹) using pregnant Sprague-Dawley females fed 200 or 160 ppm GEN. In the present case, the administration of 250 ppm GEN did not affect uterine weight. However, the experimental protocols and dosages differed among reports, and this could provide an explanation for the apparent anomalies.⁴⁹) The nonlinear dose-response of

the uterotrophic effect of dietary GEN clearly warrants further study.

NP, a weak estrogenic compound, has been shown to have dose-dependent uterotrophic effects in immature female rats when given i.p. at levels of 20 and 50 mg/kg⁵⁰) or p.o. at a level of 50 mg/kg,⁵¹) although 2000 ppm of NP did not induce significant uterine changes in a subchronic study using rats⁵²) and in a multigeneration reproductive study.⁵³) In the present study, the dose of 250 ppm NP administered in the diet corresponds to approximately 11–44 mg/kg/day in rats. It did not induce uterine or thyroid lesions. In addition to the fact that the dosing regimen of NP, exposure route, or experimental method in our studies was different from those in previous studies, species as well as strain variability in response to compounds with estrogenic activity is well documented.⁵⁴) Furthermore, OVX females are less susceptible to estrogenic chemicals than immature or prepubertal females.^{51,55}) Indeed, 50 mg/kg of NP induced a significant increase in uterine weight in prepubertal rats, but was ineffective for stimulating a similar response in OVX adult rats in a 3-day uterotrophic assay.⁵¹) Based upon the dose-response data for NP, the dose of 250 ppm does not appear to pose any problem regarding uterotrophic or thyroid carcinogenic effects.

MX formed during chlorination of drinking water is a very potent bacterial mutagen⁵⁶) and a mammalian cell clastogen,⁵⁷) and tumor-promoting effects have been reported.^{14,15,58}) A recent carcinogenicity study using rats showed MX to be definitely carcinogenic, in a dose-dependent manner, when given in drinking water at doses of 5.9, 18.7 and 70.0 ppm for 104 weeks.⁵⁹) The most prominent tumors were follicular adenomas and carcinomas of the thyroid glands in both sexes, the mechanism apparently being a direct carcinogenic effect rather than promotion through increasing the TSH level.⁵⁹) However, MX did not induce thyroid proliferative lesions in this study.

In conclusion, the EECs, SI, GEN and NP, and a possible goitrogenic chemical, MX, did not exert any promotional effect on thyroid carcinogenesis in OVX rats initiated with DHPN under the present experimental conditions. The results are also in good agreement with our findings in our recent study using male F344 rats.¹⁷)

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