Lack of enhancement of susceptibility to mammary and thyroid carcinogenesis in rats exposed to DMBA and DHPN following prepubertal iodine deficiency

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Epidemiologic and experimental studies suggest that iodine deficiency increases the risk of mammary as well as thyroid cancers, but susceptibility to tumor development when this occurs during the prepubertal stage is not completely understood. In the present study, we therefore evaluated this question in F344 rats. Dams during the lactation period and their weaned offspring until postnatal week 7 were fed an iodine-free diet. Female offspring were then given 7,12-dimethylbenz[a]anthracene (DMBA, 50 mg/kg body weight) by gavage for mammary tumor induction in week 7. Both the male and female rats were given free access to drinking water containing *N*-bis(2-hydroxypropyl)nitrosamine (DHPN), (0.1 and 0.2% for male and female rats, respectively) for wide spectrum tumor induction in organs, including the thyroid gland, from weeks 7–11. All offspring were killed at week 50 for histopathological examination. The iodine deficiency had no significant influence on incidences and/or multiplicities of mammary and thyroid tumors. Furthermore, tumor induction in the liver, kidney, lung, esophagus and urinary bladder was not affected in either sex. The present results thus indicate a lack of influence of iodine deficiency condition early in life on subsequent carcinogenic susceptibility. (*Cancer Sci* 2006; 97: 1031–1036)

Recently, environmental pollution with man-made chemicals that may disturb the endocrine system of wildlife and human beings has become an important social problem. Endocrine disrupting chemicals (EDCs) such as polychlorinated biphenyls and dioxin inhibit thyroid hormone binding to plasma transport proteins, resulting in more rapid clearance and reduced thyroid hormone levels.[1] In particular, effects of perinatal exposure to such EDCs are a focus of attention because of the potential for influence on the growth and differentiation of organs. One marked difference between exposure to endocrine disruptors during critical periods in development *versus* adulthood is the likely irreversibility of any effect with the former.[2] Thyroid hormones are critically involved in growth, development, and function of the central nervous system in animals and man. As demonstrated by Eayrs,[3] thyroid hormone deficiency requires early remediation for normal development in the rat, as in humans. Studies on the effects of iodine deficiency in animals have confirmed the morphological and biochemical modifications seen with congenital hypothyroidism and brain damage in humans.[4,5] Severe hypothyroidism has been demonstrated to affect growth overall,[6] bone growth[7] and sexual maturation,[8,9] as well as brain growth retardation.

Recognized risk factors for thyroid cancer are a history of benign thyroid disease, such as thyroiditis, goiter, hyperthyroidism, hypothyroidism and adenoma,[10] radiation[11] and long-term residence in regions with an iodine imbalance, including areas of high endemic goitre, such as iodine-deficient regions in the Alps.[10] Animal experiments have also shown an increased development of thyroid carcinomas in various animals such as mice[12] and golden hamsters[13] fed a low-iodine diet. Initially, the thyroids show hyperplasia and it has been proposed that this and the subsequent cancer development are due to overstimulation by thyrotropin (TSH), the main growth factor for the thyroid gland.[14] There is also evidence that epidermal growth factor and insulin-like growth factor I are required for TSH to induce its growth-stimulating effect.[15] The results of early studies have been verified in many animal experiments and a model for thyroid tumorigenesis has been established.[16,17]

Iodine has recently attracted interest regarding the epidemiology of other types of cancer, in particular breast cancer.[18] Specific evidence was provided by the demonstration of the sodium iodide symporter (NIS) and chloride iodide transporter, identified as pendrin (PEN), in several organs, including the thyroid and mammary gland.[19,20] In this context it should be mentioned that Japanese women have a relatively low incidence of breast cancer,[21] and Japan has one of the highest iodine intakes in the world due to ingestion of Japanese edible Wakame seaweed. Inorganic iodine has experimentally been shown to suppress DMBA-induced breast cancer in Sprague-Dawley rats,[22,23] while iodine deficiency causes breast dysplasia and cancer in rats and conceivably in humans.[24,25] Clearly, any modifying effects of iodine deficiency in different periods of life on mammary and thyroid carcinogenesis need to be clarified. The present investigation was therefore conducted focusing on the influence of a prepubertal iodine deficient status for 7 weeks after birth in F344 rats subsequently treated with 7,12-dimethylbenz (a)anthracene (DMBA), a genotoxic carcinogen targeting the mammary gland, and *N*-bis(2-hydroxypropyl)nitrosamine (DHPN), causing tumors in the thyroid.

Materials and Methods

Animals and treatment. In experiment 1, 10 pregnant (gestation day 13) Fischer rats were obtained from Charles River Japan Inc. (Kanagawa, Japan). They were individually housed in clear polycarbonate cages with heat-treated white wood chips for bedding in an air conditioned room (24 ± 1°C, 55 ± 5% relative humidity, 12 h light and dark cycle) and given basal diet (CRF-1, Oriental Yeast Co., Tokyo, Japan) and tap-water *ad libitum* until parturition. The experimental schedule is outlined in Fig. 1. Five dams each for the iodine sufficiency and deficiency groups were then given free access to AIN-93G or AIN-93G/Iodine free, respectively (Oriental Yeast Co., Tokyo, Japan) for 3 weeks after parturition. After the weaning, the dams were killed. Pups in the litters were selected randomly, resulting in five males and five females per group at 2 days after birth, to maximize the uniformity of growth rates of the offspring. After weaning the

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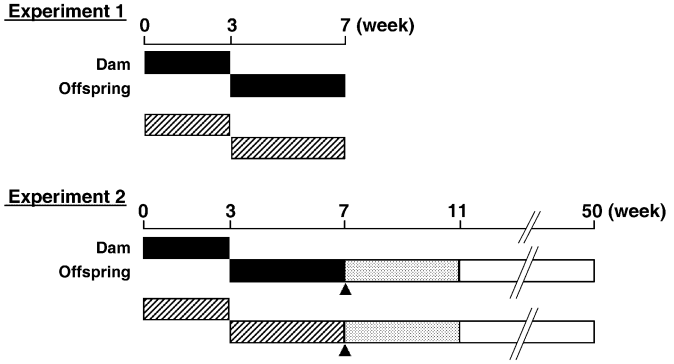

Fig. 1. Experimental schedule. Solid black, AIN-93G; hatched, AIN-93G/iodine free; light dots, *N*-bis(2-hydroxypropyl)nitrosamine (DHPN; 0.1% and 0.2% for males and females, respectively, in drinking water); arrowhead, 7,12-dimethylbenz[a]anthracene (DMBA; 50 mg/kg b.w. only for female, i.g); open, basal diet.

offspring were maintained on the same diets as the dams for a further 4 weeks. In this experiment, five males and five females of a total 20 of each sex were used for the increase of serum hormone levels, body weight and histopathology.

In experiment 2, 10 pregnant Fischer rats and their offspring were maintained for 7 weeks in the same manner with the diets as described above, and then a basal diet (Fig. 1). Totals of 22 female and 20 male rats each in basal and iodine-free diet groups were allocated and received drinking water containing 0.1 and 0.2% DHPN (Nacalai Tesque, Inc., Kyoto, Japan) for males and females, respectively, from weeks 7–11. At week 7, a single gavage dose of 50 mg/kg body weight of DMBA (Sigma Chemical Co., St. Louis, MO), dissolved in corn oil was given only to female rats.

Biological materials and tumors. In experiment 1, offspring were killed to confirm iodine deficiency at 7 weeks. Blood samples were collected from the abdominal aorta of all offspring under ether anesthesia and stored frozen until serum T3, T4, and TSH levels were radioimmunoassayed at SRL (Tokyo, Japan). Thyroids were dissected, fixed with buffered 10% formalin, weighed and routinely processed to paraffin-embedded and H&E-stained sections for histological examination.

In experiment 2, animals were checked for the presence of mammary tumors from week 0 after cessation of DHPN treatment, once a week. The first palpable nodules appeared at week 5 after cessation of DHPN treatment, at week 16 after birth, and the numbers and sizes of such lesions were recorded. The length of the longest axis, the width perpendicular to the longest axis and the height of the shortest axis, determined with calipers, were used to calculate tumor volumes as follows:

Tumor volume = length × width × height × 0.52

Except for single male and female rats that were severely cannibalized, all rats that died after the carcinogen treatment period were included in the effective numbers, the first dead animal was found to have mammary tumors. All surviving rats were killed under ether anesthesia and autopsied at the end of week 50. After careful macroscopic examination, skin with mammary tissue, any subcutaneous nodules, thyroids, liver, lungs, kidneys, esophagus, urinary bladder and macroscopic abnormalities were dissected, fixed in buffered 10% formalin and routinely processed to paraffin-embedded and H&E-stained sections for histological examination. The livers, kidneys and thyroids were weighed before processing. Animals that died or became moribund during the experiment were also autopsied, but organs or tissue were partially lost by cannibalism in some cases. The experiments were carried out in accordance with the Guide for Animal Experimentation of the National Institute of Health Sciences of Japan.

Statistical analysis. Statistical analysis of differences in means and incidences was carried out using analysis of variance (ANOVA), and Fisher's exact probability or χ^2 for independence tests, with or without Yate's correction, respectively. Data on survival were analyzed with the Kaplan-Meier test.

Results

Experiment 1. No dams showed any abnormalities in general condition during the lactation period. Body weights of iodine-deficient female offspring were significantly decreased ($P < 0.01$, Table 1). The same result was shown in all of the 20 female offspring (data not shown). Relative thyroid weights of the male offspring that were fed the iodine-free diet were significantly ($P < 0.01$) increased, and those of the female offspring showed a tendency to increase, but without statistical significance (Table 1). The serum T4 concentration was significantly ($P < 0.05$) decreased in the iodine deficiency male group, but there were no inter-group differences in the values for serum T3 or TSH concentrations (Table 1). Histopathologically, diffuse hypertrophy or hyperplasia of follicular cells was observed in all male and female offspring of the iodine deficiency group (Fig. 2B,D). The thyroids without exposure to iodine deficiency did not demonstrate apparent histological abnormality (Fig. 2A,C).

Experiment 2. Cumulative mortality curves for offspring are shown in Figure 3. There were no inter-group differences in the survival rates. Dead/moribund animals progressively increased from weeks 27 and 39 in females and males, respectively. The cause of death of males in most cases was confirmed to be renal insufficiency due to multiple kidney tumors on macroscopic and microscopic observation, and that of female rats was considered to be anemia due to hemorrhage from tumor masses. The growth curves of rats throughout the experimental period are shown in Figure 4. Slightly low values were maintained in the iodine deficiency male group until the end of the experiment with statistical significance ($P < 0.05$) only at weeks 27 and 28. Unlike in experiment 1, no significant decreases of body weights of female rats at week 7 were observed. Data for liver, kidney and thyroid weights relative to body weights are summarized in Table 2. The kidney weights in males and females showed a tendency for

Table 1. Body and thyroid weights and serum thyroid hormone levels for rats exposed to a prepubertal iodine deficiency condition for 7 weeks (Experiment 1)

Treatment	*n*	Body weight (g)	Thyroid weight (mg/100 g BW)	T3 (ng/mL)	T4 (μg/dL)	TSH (ng/mL)
Female						
Iodine deficient	5	90.1 ± 11.4**†	11.9 ± 1.2	1.18 ± 0.10	3.2 ± 0.3	5.1 ± 1.0
Iodine sufficient	5	114.8 ± 6.4	10.2 ± 1.4	1.26 ± 0.07	4.6 ± 1.1	5.6 ± 0.8
Male						
Iodine deficient	5	124.9 ± 10.8	13.5 ± 2.1**	1.28 ± 0.04	5.1 ± 0.4*	7.0 ± 1.7
Iodine sufficient	5	134.3 ± 16.2	9.0 ± 1.7	1.29 ± 0.08	6.2 ± 0.6	6.3 ± 0.5

†Mean ± SD; *$P < 0.05$; **$P < 0.01$ *versus* Iodine sufficient group of the respective sex.

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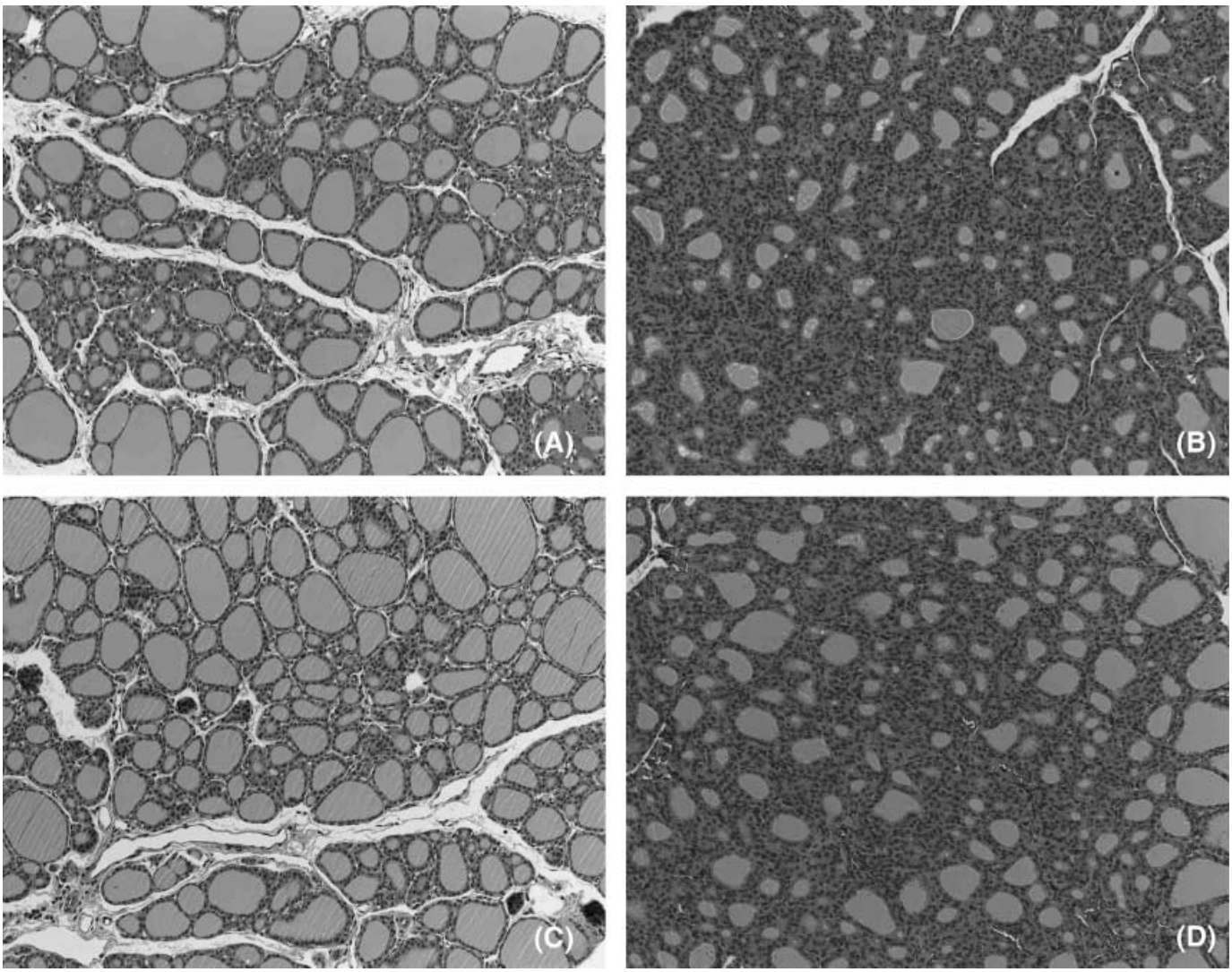

Fig. 2. Appearance of thyroids of female and male rats with and without exposure to iodine deficiency for 7 weeks after birth. (A,C) Intact thyroids in control female and male rats, respectively. (B,D) Note diffuse hyperplasia in female and male rats receiving the iodine deficiency diet. (Original magnification × 100)

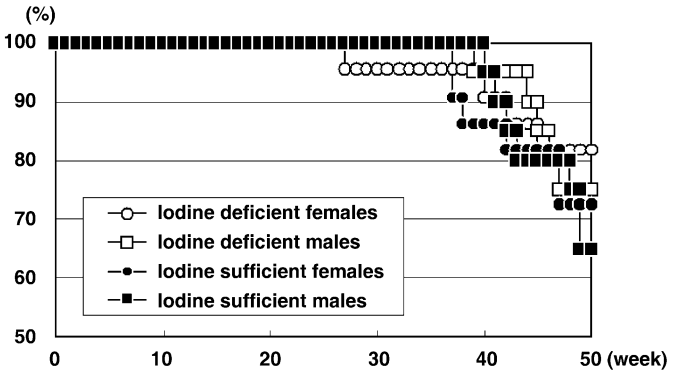

Fig. 3. Survival curves for rats treated with *N*-bis(2-hydroxypropyl) nitrosamine and 7,12-dimethylbenz[a]anthracene during adolescence following a prepubertal iodine deficiency condition.

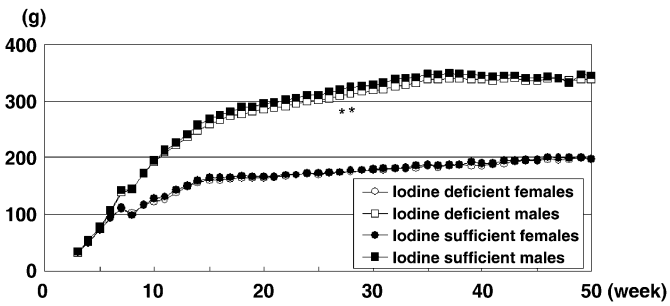

Fig. 4. Body weight curves for rats treated with *N*-bis(2-hydroxypropyl) nitrosamine and 7,12-dimethylbenz[a]anthracene during adolescence following a prepubertal iodine deficiency condition. $*P < 0.05$ *versus* the iodine sufficient male group.

decrease with iodine deficiency, but without statistical significance. Liver and thyroid relative weights were not influenced.

In the mammary glands, incidences of palpable mammary tumors gradually increased in all female groups after carcinogen treatment (Fig. 5A). Multiplicity values showed a tendency for lowering in the iodine deficiency group compared to the controls from week 7 after carcinogen treatment to the end of the experimental period without statistically significant difference (Fig. 5B). However, the lower value in the iodine deficiency group was mainly due to one rat in the control group bearing five mammary masses. Volume exhibited increased values from

Table 2. Body and organ weights of rats treated with DHPN and DMBA during adolescence following a prepubertal iodine deficiency condition (Experiment 2)

Treatment	*n*	Body weight (g)	Liver (g/100 g BW)	Kidney (g/100 g BW)	Thyroid (mg/100 g BW)
Female					
Iodine deficient	18	196.4 ± 12.2[†]	2.8 ± 0.3	0.7 ± 0.04	7.8 ± 1.5
Iodine sufficient	16	196.7 ± 15.9	2.6 ± 0.2	0.9 ± 0.80	7.5 ± 2.4
Male					
Iodine deficient	15	337.5 ± 28.5	2.9 ± 0.3	1.0 ± 0.80	36.4 ± 57.3
Iodine sufficient	13	343.9 ± 43.7	2.9 ± 0.4	1.4 ± 2.20	33.6 ± 25.5

[†]Mean ± SD.

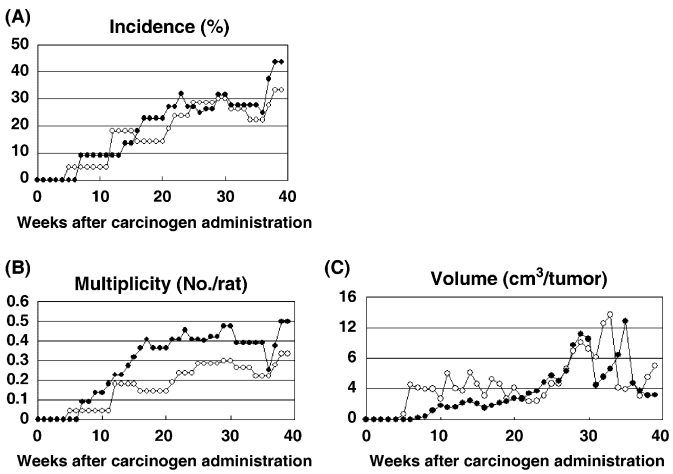

Fig. 5. Incidence (A), multiplicity (B) and volume (C) data for palpable mammary tumors in female rats treated with *N*-bis(2-hydroxypropyl) nitrosamine and 7,12-dimethylbenz[a]anthracene during adolescence following a prepubertal iodine deficiency condition. Open circles, iodine deficiency; closed circles, iodine sufficient.

weeks 5–20 after carcinogen treatment, but without statistical significance (Fig. 5C). Histopathologically, most subcutaneous nodules in females were diagnosed as mammary gland fibroadenomas or adenocarcinomas and their incidences and multiplicities did not show any alteration with iodine deficiency (Table 3). Fibromas were detected in two control males (Table 3).

In the thyroid glands, multiple focal follicular cell hyperplasias, and adenomas and adenocarcinomas were observed in appreciable percentages of female and male rats, but without any significant differences in their incidences or multiplicities between the two groups (Table 4). Neoplastic lesions in other organs were also variously observed, as summarized in Table 5. Tumors in the lung were found in almost all rats, while the incidences of tumors in liver, kidney, esophagus and urinary bladder were relatively high, but again without statistical significance for differences between the deficiency and sufficiency cases.

Discussion

Although there is evidence from animal experiments and epidemiological studies that iodine deficiency is involved in mammary[24,25] and thyroid [10,12–14,16,17,26] carcinogenesis, the present results pointed to a lack of influence, and also no effects were noted on tumor development in other organs, like the liver, kidneys, urinary bladder and lungs.

Recently Garcia-Solis *et al*. showed that iodine is a potent protective agent against N-methyl-N-nitroso urea (MNU)-induced mammary cancer,[27] in agreement with several reports showing that treatments with iodine-rich seaweeds or Lugol's solution (a mixture of iodine and iodide) have a protective effect against chemical-induced mammary carcinogenesis.[22,23] Their data also showed that iodine treatment increases PEN expression, suggesting a positive uptake mechanism for iodine, in line with the finding that virgin mammary glands are capable of capturing radiolabeled iodine in several forms even when the NIS-dependent iodide uptake has been blocked.[27] The occurrence of PEN expression and the presence of sulfate/iodine exchanger in mammary glands should be stressed in this context.[20,28] Venturi *et al*. have further suggested that iodide might have an ancestral antioxidant function in all iodide-concentrating thyroid cells, phylogenetically derived from primitive iodide-concentrating gastroenteric cells.[29] Mammary cells also are derived from primitive iodide-concentrating ectoderm which, during evolution, specialized in uptake and storage of iodide in order to adapt organisms from iodine-rich sea to iodine-deficient land.[29] In the present study, development of mammary tumors induced by DMBA following a period of iodine deficiency for 7 weeks from birth was not significantly modified. Since enhancement of both PEN[20] and NIS[30] expression

Table 3. Incidence, multiplicity and volume of mammary tumors in rats treated with DHPN and DMBA during adolescence following a prepubertal iodine deficiency condition (Experiment 2)

Treatment	*n*	Fibroma		Fibroadenoma		Adenocarcinoma		Tumor volume (cm³/tumor)
		Incidence (No./rat)	Multiplicity	Incidence (No./rat)	Multiplicity	Incidence (No./rat)	Multiplicity	
Female								
Iodine deficient	21	0	0	2 (10%)	0.1 ± 0.3	8 (38%)	0.4 ± 0.5	13.3 ± 16.9
Iodine sufficient	22	0	0	2 (9%)	0.1 ± 0.5	9 (41%)	0.6 ± 0.9	13.3 ± 18.3
Male								
Iodine deficient	20	0	0	0	0	0	0	0
Iodine sufficient	19	2 (11%)	0.1 ± 0.3[†]	0	0	0	0	3.1 ± 2.2

[†]Mean ± SD.

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Table 4. Incidences of proliferative lesions in the thyroids of rats treated with DHPN and DMBA during adolescence following a prepubertal iodine deficiency condition (Experiment 2)

Treatment	*n*	Follicular cell				C-cell		
		Hy (%)	Ad (%)	Ca (%)	Total (%)	Hy (%)	Ad (%)	Ca (%)
Female								
Iodine deficient	21	6 (29)	2 (10)	3 (14)	10 (48)	2 (10)	1 (5)	1 (5)
Iodine sufficient	22	9 (41)	6 (27)	4 (18)	14 (64)	0	0	0
Male								
Iodine deficient	16	12 (75)	12 (75)	12 (75)	16 (100)	0	2 (13)	1 (6)
Iodine sufficient	17	13 (76)	13 (76)	15 (88)	16 (94)	0	1 (6)	1 (6)

Ad, adenoma; Ca, carcinoma; Hy, focal hyperplasia.

Table 5. Incidences of neoplastic lesions in organs other than the thyroid of rats treated with DHPN and DMBA during adolescence following a prepubertal iodine deficiency condition (Experiment 2)

Treatment	Liver		Kidney					Lung			Esophagus			Urinary bladder		
	n	Ad (%)	*n*	Ad (%)	Ca (%)	TCC (%)	Nb (%)	*n*	Ad (%)	Ca (%)	*n*	Pa (%)	SCC (%)	*n*	Pa (%)	TCC (%)
Female																
Iodine deficient	21	1 (5)	21	1 (5)	1 (5)	3 (14)	0	21	21 (100)	20 (95)	21	2 (10)	0	21	1 (5)	0
Iodine sufficient	22	4 (18)	22	4 (18)	1 (5)	0	1 (5)	22	22 (100)	20 (91)	22	4 (18)	2 (9)	22	0	0
Male																
Iodine deficient	20	0	20	9 (45)	7 (35)	12 (60)	8 (40)	19	19 (100)	19 (100)	19	6 (32)	4 (21)	20	1 (5)	2 (10)
Iodine sufficient	19	0	19	6 (32)	2 (11)	9 (47)	13 (68)	19	19 (100)	17 (89)	19	2 (11)	3 (16)	18	0	4 (22)

Ad, adenoma; Ca, adenocarcinoma; Nb, nephroblastoma; Pa, papilloma; SCC, squamous cell carcinoma; TCC, transitional cell carcinoma.

in rat mammary tissue has been reported with prolactin stimulation, we speculate that little uptake of iodine and low sensitivity of iodine deficiency were exhibited in mammary cells during the prepubertal period because of the lack of any prolactin surge.

Generally, TSH from the pituitary gland plays an important role in the enhancement of thyroid tumor development. Its production and release are controlled by thyroid hormone levels in blood via the negative feedback of the pituitary-thyroid axis.[31,32] In rodents, especially rats, follicular hyperplasia can be caused by long-term administration of a low-iodine diet, which reduces the production of thyroid hormone, decreases serum T3 and T4, and increases serum TSH.[33] Moreover, chronic administration of a low-iodine diet to DHPN-initiated rats results in the development of thyroid follicular tumors.[34] In contrast to the chronic response to a low-iodine diet in rats, the acute response is not characterized by a significant rise in serum TSH up to about 15 days, despite a rapid decrease in T4 levels.[33] In the present study, the development of thyroid follicular tumors on treatment with DHPN and DMBA after a period of iodine deficiency for 7 weeks from birth was not significantly modified. At week 7 thyroid weights and histopathological appearance, as well as serum thyroid hormone concentrations in the iodine deficient male rats, were altered in line with our expectations, although histopathological findings were only altered in females (Fig. 2). Our findings in both males and females at least indicate that iodine content as an antioxidant in thyroid tissue was significantly low at the time of DHPN treatment. This might explain why deficiency did not influence the susceptibility to thyroid tumor induction.

Rats born to severely iodine-deficient mothers and subsequently maintained on a low-iodine diet from birth to 41 days, exhibit severe goiter, high plasma TSH and low plasma T4 in comparison to controls, although adaptation is apparently adequate to maintain a nearly euthyroid state.[35] Although the rodent thyroid is very sensitive to derangement by chemicals and physiologic perturbation due to the short plasma half-life of T4 (12–24 h),[36] studies using the fetus or newborn can be difficult to interpret because of their ability to use maternal iodine, T3 and T4 through transplacental transfer, but not TSH.[37] Maternal milk is a source of iodine during suckling,[38] and contains a high concentration of maternal thyroid hormone.[39] The milk of dams fed low-iodine diet during pregnancy and lactation was found to contain 22% of the amount of iodine found in milk from control dams, in contrast to an iodine intake only about 4% of that of control rats, with significantly high and low levels of T4 and TSH, respectively.[40] Ingestion of this hormone through the milk, in addition to synthesis by the offspring's own thyroid glands, may be sufficient to prevent hypothyroidism, suggesting that early postnatal development may not require a high concentration of thyroid hormone. However, iodine deficiency treatment for 7 weeks from birth can be considered appropriate for our study purposes, since the iodine content in thyroid and mammary cells of the 7-week-old rat were found to be significantly lowered. Longer exposure to iodine deficiency conditions from early gestation might impair the development of the fetus.[4–8]

In consideration of the fact that tap-water was provided to iodine deficiency groups, the possibility that included iodine might have interfered with the present experiment should be considered. However, the mean concentration of total iodine in drinking water in Japan is about 4 p.p.b., which is 4% of that in the control diet, AIN-93G. Therefore, any influence would be expected to be minor. Furthermore, iodine in drinking water is presumably iodide to a large extent and recently it was reported that while continuous iodine treatment has a potent antineoplastic effect on the progression of mammary cancer, this was not the case with iodide.[27] Iodine was found to be distinctly more effective than iodide at diminishing ductal hyperplasia and perilobular fibrosis in mammary glands, when the same total iodine doses were applied.[41]

In conclusion, the prepubertal iodine deficiency conditions generated under the present experimental conditions did not increase the susceptibility to subsequent tumor induction in the mammary gland and thyroid, or in the liver, kidney, lung, esophagus and urinary bladder.

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