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Goitrogenic Action of Manganese on Female Mouse Thyroid through Three Generations

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ABSTRACT—The effect of manganese on mouse thyroids was examined through three generations. The continuous supply of drinking water containing 200 mg/l $MnCl_2 \cdot 4H_2O$ induced mild goiter only in female mice.

The manganese treatment did not significantly affect the serum thyroxine (T_4) levels in dams or the plasma levels in neonates, suggesting that the effect was not severe. The syntheses of T_4 and triiodothyronine (T_3) were examined in hydrolyzed thyroids. The results showed that ratios of radioiodide activity in hormonal fractions of the manganese-given groups to those of control groups were greater than 1 for all three generations of female mice. These results indicated that newly synthesized hormones were retained in thyroids of the manganese-treated female mice. On the other hand, in males, the ratio was reduced to less than 1 as the numbers of generations increased.

"Dwarf" neonates born in the third generation of the manganese-treated group showed an ataxial motion in their gait of walk. However, histological examination of cerebral and cerebellar regions of the dwarfs revealed no severe changes.

INTRODUCTION

It has been shown that excess manganese induces an endemic goiter when iodide intake is low [1, 2]. In laboratory experiments, acute and subacute administrations of manganese suppressed thyroidal iodide uptake and affected iodide metabolism [3, 4]. The authors also demonstrated that the radioactive manganese accumulated to a high level in mouse thyroid as well as in other endocrine organs, such as the pituitary, pancreas and adrenal [5]. Furthermore, it was found that under ordinary iodide intake, a 7 week administration of manganese in the drinking water resulted in thyroid enlargement in female mice, but not in male mice [5].

For goiter formation, a prolonged manganese treatment through an oral route was necessary because a single [3, 6] or a sustained [4] parenteral injection of manganese caused very severe reductions of iodide uptake and synthesis of thyroid hormones, but did not induce thyroid enlargement. Although prolonged treatment increases thyroid hormone levels in the gland and reduced the hormones in the blood [5], the mechanism of goiter formation by manganese is not well understood. Thus, we were interested in determining whether oral administration of manganese for several generations caused larger goiters, and also whether hereditary factors were involved in the manganese-induced goiter. In this study, we examined the chronic effects of manganese on growth, thyroid function and brains of mice in three consecutive generations.

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MATERIALS AND METHODS

Chemicals The chemical reagents used in this study were purchased from Wako Pure Chemical Industries (Osaka, Japan) and oxytocin was from Sigma Chemical Co. (St. Louis, MO., USA). [^{125}I]Na was purchased from New England Nu-

clear Co. (Boston, MA., USA). The radioimmunoassay kit, SPAK T_4 was purchased from Daiichi Isotope Institute (Tokyo, Japan). Pronase was a product of Kaken Pharmaceutical Co. (Tokyo, Japan), and filter paper No. 51 for chromatography was obtained from Toyo Roshi Co. (Tokyo, Japan).

Animals Male and female ddY mice weighing 18–20 g (4 weeks old) were obtained from a local supplier and maintained on a 12 hr light-dark cycle in an air conditioned room at 23–24°C and 50% moisture. Animals were allowed free access to solid food (Type MF, Oriental Yeast Co. Tokyo, Japan) and tap water for the control. The iodide and manganese contents in the food pellets were 1.04 and 64.5 μg/g, respectively, as reported previously [5]. Mice were given 200 mg/l of $MnCl_2 \cdot 4H_2O$ in the drinking water for 7 weeks. These animals were designated as the first generation of manganese-treated mice (parents). Some mice were sacrificed at 11 weeks of age to examine thyroid enlargement. The remaining mice were mated to provide the second generation of manganese-treated mice. During mating and subsequent pregnancy, manganese administration was continued. The offsprings from these parents with manganese treatment were designated as the second generation. The third generation of manganese-treated mice was obtained by a brother-sister mating of the second generation with manganese treatment.

On day 1 when neonates were born, they were anesthetized with ether and blood was collected into a heparinized test tube by cutting the carotid artery. The blood from a litter was pooled and centrifuged to obtain plasma for hormone analysis. Another group of neonates was sacrificed on day 5 to collect plasma. Dams were also sacrificed on day 1 and day 5 after delivery. Blood samples were taken from the dams. The thyroids were excised and their weights were recorded.

Intrathyroidal iodine metabolism Mice treated with manganese and the control were intraperitoneally given 3–4 μCi [^{125}I]Na per animal 24 hr prior to sacrifice. At autopsy, the thyroids were excised, weighed and pooled in each group because an individual thyroid was insufficient for analysis. The thyroids were added to a test tube containing 50 mM phosphate buffer (pH 7.4), 1 mM methylmercaptoimidazole and two drops of ethanol. The mixture was subjected to proteolytic hydrolysis with Pronase (20 : 1 weight ratio). For this purpose, the test tube was bubbled with N_2 gas, sealed with a stopper and incubated at 37°C for 24 hr. Digestion was terminated by immersing the tube in boiling water. Samples of the hydrolysates were spotted on paper and subjected to paper chromatography using two different solvent systems (*n*-butanol : acetic acid : H_2O=4 : 1 : 2, v/v and *n*-butanol : ethanol : 2N ammonium hydroxide =5 : 1 : 2, v/v). Radioiodinated compounds were analyzed by autoradiography. Areas on the paper showing dark spots were cut and their radioactivities were measured by an auto gamma scintillation counter.

Hormone measurement Serum was prepared from adult animals, but pooled plasma was prepared from neonates because of an insufficient volume of blood from an individual neonate. Circulating T_4 was assayed by SPAK T_4 RIA kit. In this assay system, 25 μl of specimen were used in a single measurement and at least duplicate measurements were carried out for each specimen. A cross reaction with T_3 was not detectable with this kit.

Histological study Thyroids were excised and fixed in Bouin's fluid. Using ordinary processes, samples were embedded in a paraffin wax, sliced and sections were stained in hematoxylin-periodate. Whole brain regions were fixed in 10% formaldehyde solution and embedded in an epon. The specimens were stained in luxol fast blue-cresylechtviolet solution for simultanous staining of Nissl bodies and the myelin sheath, and in hematoxylin-eosin solution for ordinary staining. Stainability and histological changes were examined under a light microscope.

RESULTS

Thyroid enlargement by manganese

A morphological study of thyroids of female mice treated with 200 mg/l manganese chloride for 7 weeks revealed moderate size goiters with colloid filled lumens and flattened epithelial cells. Table 1

TABLE 1. Manganese effect on body and thyroid weights in three generations of mice

	1st generation		2nd generation		3rd generation	
	C	Mn	C	Mn	C	Mn
Female						
No. of animal	14	12	11	10	9	9
Body weight (g)	31.0±3.9	32.5±2.5	32.0±3.0	32.3±2.4	32.0±3.9	31.2±2.5
Thyroid weight (mg)	2.7±0.3	3.3±0.3	3.1±0.3	4.3±0.4	2.8±0.5	3.7±0.4
	$p<0.001$		$p<0.001$		$p<0.001$	
Male						
No. of animal	9	11	16	11	11	11
Body weight (g)	40.6±3.4	40.9±3.0	42.2±4.1	43.2±3.8	41.5±3.4	44.3±3.4
Thyroid weight (mg)	3.3±0.4	3.8±1.0	3.5±0.4	3.8±0.3	3.6±0.3	3.6±0.3
	NS		NS		NS	

C: Control, Mn: Mn treated, NS: Not significant.

shows that, in the first generation, thyroids of female mice were enlarged by manganese chloride, but thyroids of male mice were unchanged by the same treatment. When the mice with manganese treatment were mated, 9 out of 15 females became pregnant (60%), whereas in the control group 8 out of 12 became pregnant (67%). In the second generation, the frequencies of pregnancy of the manganese-treated female mice and the control were 7/14 and 10/15, respectively. The body weight gain and other external appearances were not changed by manganese treatment. At delivery time, the average number of neonates bred and their mean body weight were also similar to those of control group. When animals of the second generation were 11-12 weeks old, some were sacrificed to examine their thyroids. The thyroid weight of the females of the second generation was slightly greater than that of the first generation. In the third generation, the size of glands was similar to that of the first generation but smaller than that of the second generation.

Manganese effect on circulating T_4 level

The results of the circulating T_4 levels of non-pregnant adult female mice, dams after delivery (day 1 and day 5) and neonates (day 1 and day 5) are summarized in Table 2. Just after delivery, serum T_4 levels of dams were within normal ranges and not influenced by the birthing process. Dams with manganese treatment maintained normal T_4 levels at day 1. However, the T_4 levels of dams in both groups fell to subnormal levels during the term of lactation (day 5). T_4 levels of neonates on day 1 were very low but increased slightly during the first five days. This increase in T_4 level was also observed in the manganese-treated group. Thus, there was no significant effect of manganese on circulating T_4 levels.

Manganese effect on radioiodide uptake into hormonal fraction of thyroid

In order to determine whether manganese interferes with hormone synthesis, the radioiodide uptake into intrathyroidal hormonal fractions was examined in control and manganese-treated mice through three generations. Table 3 shows the radioiodide distribution in T_3+T_4 fractions of each group. ^{125}I activity in the females treated with manganese was much higher (10–20%) than that of the control group, resulting in the ratios of Mn/C being greater than one or at least equal to one. On the other hand, in males, the ratios of Mn/C were always less than one. Furthermore, as

TABLE 2. Manganese effect on thyroxine level in blood

Group		1st generation	2nd generation	3rd generation
		ng/ml	ng/ml	ng/ml
Non-pregnant adult female	Control	38.5±5.0 (n=4)	38.9±7.9 (n=4)	
	Mn-treated	33.5±2.5 (n=4)	40.8±8.5 (n=5)	
Dam	Control	38.0±4.7 (n=6)	28.1±1.9 (n=4)	
Day 1	Mn-treated	37.5±14.2 (n=5)	30.8±7.6 (n=6)	
Dam	Control	17.6±4.2 (n=6)	16.8±1.0 (n=4)	
Day 5	Mn-treated	21.8±8.7 (n=6)	16.5±1.6 (n=6)	
Neonate	Control		6.4±6.3 (n=4)	9.4±3.9 (n=4)
Day 1	Mn-treated		5.1±2.9 (n=5)	14.1±5.9 (n=6)
Neonate	Control		10.3±0.6 (n=3)	14.9±1.9 (n=4)
Day 5	Mn-treated		18.1±0.1 (n=2)	17.9±1.6 (n=6)

Data show serum hormone levels for adults and plasma hormone levels for neonates.
Each value is the mean±SD.
The numbers within the parentheses for the adult females and dams represent the numbers of mice used in the study. However, the numbers within the parentheses for neonates represent the numbers of litters in each group.

the number of generations increased, the ratios became smaller in male mice.

"Dwarfs" and the histological examination of their brains

Continuous manganese administration did not affect the growth of parents and the second generation of mice when their body weights were monitored. However, in the third generation of the manganese-treated group, some smaller neonates were observed, although the incidence was not high: 0/11 for the control and 3/17 for the manganese-treated group (The numerator and denominator represents numbers of dams which bred a "dwarf" and total numbers of dams examined, respectively. In this experiment, the term "dwarf" is defined for neonates weighing less than 70% of the average body weight of siblings in the same litter on day 1). After a half number of normal siblings were isolated from their mother, dwarfs were still unable to grow at the rate of normal mice, suggesting that the dwarfs were not the results of interrupted lactation but of some other endogenous dysfunctions.

Figures 1 and 2 illustrate the growth curves and the photograph of normal and dwarf mice, respectively. Dwarfs grew slowly and never reached the level of body weight of normal animals. Since

TABLE 3. ^{125}I incorporation into iodothyronine fraction in thyroid

1st generation			2nd generation			3rd generation		
C	Mn	Mn/C	C	Mn	Mn/C	C	Mn	Mn/C
%	%		%	%		%	%	
Female								
8.92 (11.56)	11.05 (10.73)	1.24 (0.93)	6.18 (10.60)	7.05 (10.10)	1.14 (0.95)	5.85 (6.09)	7.09 (7.68)	1.21 (1.26)
Male								
5.18 (5.88)	3.82 (4.95)	0.74 (0.84)	9.08 (11.50)	6.12 (9.57)	0.67 (0.83)	7.45 (9.87)	3.42 (5.32)	0.46 (0.54)

C: Control, Mn: Mn treated.
The values represent the percentages of ^{125}I activity in T_3+T_4 fractions to total radioactivity on chromatograms. Values were obtained by using developing solvent system of *n*-butanol:ethanol:2N ammonium hydroxide=5:1:2 (v/v) and the values in parentheses were obtained by using a system of *n*-butanol : acetic acid : H_2O=4:1:2 (v/v).

some dwarfs showed ataxial motion, the brains of dwarfs were subjected to histological examination. Although the photographs are not shown, both control and dwarf neonates displayed regular lamination of nerve cells in the motor area of the cerebral cortex, and the dwarfs exhibited nerve cells which were stained lighter than the control. There was no critical difference in lamination of nerve cells of cerebellar cortex between the two groups. In the dwarfs, however, many swollen and lightly stained Purkinje cells in the cerebellar cortex were seen as well as an increased number of nerve fibers in the cerebellar medulla, there was no difference in stainability of the Nissl bodies of the nuclei cerebri in normal and dwarf mice.

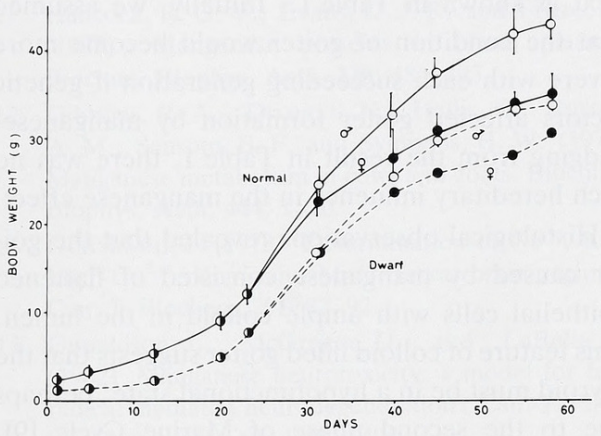

FIG. 1. Growth curve of normal and dwarf neonates. The normal group consisted of 13 male and 9 female mice from 2 litters and for the dwarfs, one male and one female. The vertical bar represents the standard deviation.

FIG. 2. A photograph of normal and dwarf neonates. The photograph shows a dwarf (upper) and a normal (lower) neonate on day 9 after birth.

DISCUSSION

Although symptoms caused by manganese deficiency [7] and neurological dysfunctions by excess manganese [8] have been well documented, knowledge of neonatal endocrinology related to excess manganese ingestion is scant. Previously, we stated that even with sufficient iodide supply, excess manganese can be goitrogenic in the female mouse thyroid, but not in the male mouse thyroid [5]. The present study provided additional confirming evidence to support the observation mentioned above. Moreover, goiters were always produced in three generations by continuous administration of manganese. However, the size of goiters did not increase from one generation to the next as shown in Table 1. Initially, we assumed that the condition of goiter would become more severe with each succeeding generation if genetic factors affected goiter formation by manganese. Judging from the result in Table 1, there was no such hereditary influence in the manganese effect.

Histological observations revealed that the goiter caused by manganese consisted of flattened epithelial cells with ample colloid in the lumen. This feature of colloid filled goiter suggests that the thyroid must be in a hypofunctional state, perhaps due to the second phase of Marine Cycle [9]. Therefore, it is likely that the use of colloid by epithelial cells was blocked by manganese in female mice. This speculation is supported by the fact that radioiodide activity in the intrathyroidal T_3+T_4 fraction was slightly but steadily higher in female mice with manganese treatment than in control mice (Table 3). This result suggests that in females, the *de novo* synthesized hormones remain in the lumen, and in males, the hormones are promptly released from the lumen.

From the present study, it is not known whether TSH levels were altered by manganese. We attempted to use a rat-TSH antibody to measure mouse TSH level by radioimmunoassay, but the rat-TSH antibody did not react with the mouse TSH. Buthieau and Autissier reported that the serum TSH level was reduced in manganese injected rats [4]. If one could extend this evidence to mice, excess manganese may depress the thyroid and the pituitary functions. Na^+, K^+-ATPase, which suplies energy to epithelia for colloid endocytosis, was not significantly inhibited by manganese *in vitro* (authors, unpublished). This evidence was compatible with the fact that the prolonged oral administration of manganese only slightly affected the ratio of ^{125}I activity in tissue to that in serum [5]. Therefore, this feature was entirely different from the acute parenteral treatment [3]. At present, the cause of the sex difference in the manganese effect on colloid accumulation is not clear, but in castrated male mice, goiter was induced by manganese, suggesting levels of male and female hormones with manganese may somehow bring about the formation of goiter [5].

Serum T_4 levels in non-pregnant females, dams and the plasma of neonates were not significantly affected by manganese administration. Perhaps the manganese effect was mild so that regulatory processes to maintain homeostasis of the hormone level could operate properly in all ages of animals examined. Since the presence of manganese transferrin has been demonstrated in sera from several species [10–12], a similar carrier protein may exist in the mouse to reduce the toxic effects of an overload of manganese.

In the third generation of manganese treatment, dwarfs were born, but there is no information suggesting that this was specifically due to manganese treatment. When the manganese content in milk of the treated dams was measured, it was not significantly different from the control milk (data not shown). The course of dwarfism may occur during the period of pregnancy. Dwarfs could grow, but showed ataxial motion. We do not know whether the ataxia in dwarfs mimics Parkinsonism in humans caused by manganese intoxication [8]. When organ distribution of manganese was examined, the level of manganese content in the entire brain was not high compared with other organs [5, 16]. However, high amounts of endogenous manganese was found in the hypothalamus region of rats [13], indicating that manganese could be localized in narrow regions to cause aberrations in the nervous system.

Donaldson *et al.* [14] have shown that manganese can exist *in vivo* as either an oxidant or an antioxidant, depending on its valency state; Mn^{2+} may reduce norepinephrine levels in brain regions

whereas Mn^{3+} enhances lipid peroxidation and formation of free radicals. Our recent study on partition of Mn^{2+} and total manganese in rat organs showed that only a very small fraction of the total manganese remained in the form of Mn^{2+} in most organs after manganese chloride administration [16]. These data suggest that transvalency of manganese by biological systems is very active. Histological study of the brain region of dwarfs exposed to manganese in early developmental stages showed no drastic alterations. It is likely that the effect of excess manganese on the perinatal nerve systems may not be drastic but mild and latent in nature.

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