Effects of Nonylphenol on Mammary Carcinogenesis and Bone Mineral Density of the Tibia in SHN Virgin Mice

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Abstract

Nonylphenol has been shown to act as an estrogenic compound and endocrine disruptor. Nonylphenol is the final degradation product of alkylphenol polyethoxylates, which are widely used for preparing lubricating oil additives, resins, plasticizers, surface-active agents, toiletries, herbicides, paints, and cosmetics. The incidence of mammary tumors, the genesis of which needs a hormonal environment associated with prolactin and ovarian sex steroids, was reported to be higher in virgin mice of the SHN strain. Thus, we investigated the effects of nonylphenol on mammary carcinogenesis and bone mineral density of the tibia in SHN virgin mice. Nonylphenol did not affect body growth, liver function, or renal function in mice, the incidence and number of mammary tumors increased, and bone mineral density of the tibia decreased in mice given nonylphenol. These results indicate that nonylphenol plays the role of an estrogenic compound as an endocrine disruptor.

Key words —— Nonylphenol, endocrine disruptor, SHN mice, mammary tumor, bone mineral density.

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Introduction

Nonylphenol（NP）has been shown to act as an estrogenic compound and endocrine disruptor. NP is the final degradation product of alkylphenol polyethoxylates, which are widely used for preparing lubricating oil additives, resins, plasticizers, surface-active agents, toiletries, herbicides, paints, cosmetics, and many other products[1-3]. Studies on this environmental chemical have focused on its endocrine-disrupting and potentially adverse effects on the developing reproductive system. However, this endocrine disruptor was shown to cause tissue injury in the liver, kidney, brain, and other organs by promoting the production of reactive oxygen species[4, 5].

The incidence of mammary tumors, the genesis of which needs a hormonal environment associated with prolactin and ovarian sex steroids[6, 7], was shown to be higher in virgin mice of the SHN strain.

In the present study, we investigated the effects of NP on mammary carcinogenesis and bone mineral density in tibia of the SHN virgin mice.

Materials and Methods

Virgin mice of the SHN strain were established as mice with a higher incidence and development of mammary tumors by Nagasawa[6], Experimental Animal Research Laboratory, Meiji University, Japan and were maintained by Mori[7], Department of Biological Sciences, Graduate School of Science, University of Tokyo, Japan.

This strain used in the present study was presented by Nagasawa and Mori, and has been maintained in the Animal Research Center of Tokyo Medical and Dental University (Tokyo, Japan). Mice were housed in plastic cages with wood shavings under controlled temperature (24 ± 0.5℃) and lighting (12 h of light from 0600 to 1800 h), and were permitted free access to a commercial diet (CE-2, CLEA Japan, Tokyo, Japan) and tap water *ad libitum*. All procedures used were described in detail in a protocol that was approved by the Animal Care and Use Committee of the Graduate School of Medicine, Tokyo Medical and Dental University, and all experiments conformed to the regulations described in the U.S. National Institute of Health (NIH) Guide to the Care and Use of Laboratory Animals.

Animals were divided into 2 experimental groups of 10 and 12 mice, respectively, at 71 days old. Twenty-four mice were prepared for the control group. The commercial diet CE-2 (CLEA Japan, Tokyo, Japan) containing nonylphenol (NP: p-nonylphenol, $C_9H_{19}C_6H_4OH$, MW 220.35, Kanto Chemical Co., Tokyo, Japan: 2 dosages of 60 mg and 0.6 g in 1 kg of diet for 10 and 12 mice, respectively) was given to the two experimental groups for 210 days. CE-2 alone was given to the control group for 210 days. If daily intake dose of the diet was assumed to be 3.0 g per 30 g body weight, the intake amounts of NP in the 2 experimental groups were 0.18 mg and 1.8 mg per day, respectively.

When a palpable mammary tumor appeared in a mouse, the tumor was removed under anesthesized conditions and fixed in a 10 % formaldehyde buffer solution (pH 7.2) each time. Mice were anesthesized with ether, bled by cardiac puncture, sacrificed by cervical dislocation, and the organs were removed and weighed just before death or at 280 days of age. Plasma samples were stored at -80℃ for the later determination of plasma levels of biochemical markers. Removed mammary tumors were fixed in a 10 % formaldehyde buffer solution (pH 7.2), embedded in paraffin, and prepared as 5 μm serial sections. They were then stained with Mayer' s hematoxylin and eosin for histological examination. Each removed tibia was fixed in 99.5 % ethanol and stored for the later determination of bone mineral density (BMD).

BMD was determined by dual energy absorptiometry (DXA). Total BMD (Ca mg/cm^2) of the entire tibia was measured by DXA (Aloka, DCS-600, Tokyo, Japan) as bone mineral content (BMC) /bone area. The BMD of the distal metaphysis of the tibia was also measured by DXA as BMC/bone width (Ca mg/cm^2), i.e. part of the trabecular bone.

Data were expressed as the mean ± S.E.M.(standard error of the mean). Statistical analyses were carried out using the Student's *t*-test for plasma levels of biochemical markers, body growth, and the latent period of mammary tumor incidences, and the χ^2-test with the Yates' correction was conducted for the incidence and number of mammary tumors and the survival rates of mice at 40 weeks old. Differences between the groups were considered significant at the $p < 0.05$ level.

Results

Few significant differences were observed in plasma levels of blood urea nitrogen (BUN), creatinine, and triglyceride (TG), or the activities of aspartate aminotransferase (AST) and lactate dehydrogenase (LD) among the groups (**Table 1**). However, plasma levels of total cholesterol (TCh) ($p < 0.05$) and the activities of alkaline phosphatase (ALP) ($p < 0.01$) were significantly higher in mice fed the diet containing NP than in mice fed the control diet.

No significant differences were observed in body growth among the groups (**Table 2**). The incidence and number of mammary tumors were significantly higher in mice given high dose NP ($p < 0.05$). Few significant differences were observed in the latent period of tumorigenesis (tumor-free period) or number of survivors at age of 40 weeks (**Table 2**).

Bone mineral density (BMD) was significantly reduced to 88.9 % ($p < 0.01$) and 85.4 % ($p < 0.01$) of levels in the control by the intake of low and high doses of NP in the whole tibia, respectively (**Figure 1**). BMDs in the proximal metaphysis of the tibia were significantly reduced to 85.7 % ($p < 0.01$) and 84.1 % ($p < 0.01$) of levels in the control by the low and high doses of NP, respectively.

Table 1 Plasma biochemical markers

Groups	Control	Nonylphenol 60 mg/kg diet	Nonylphenol 0.6 g/kg diet
(n)	(24)	(10)	(12)
AST (IU/l)	71.8 ± 14.1	100.7 ± 14.5	119.5 ± 17.1
LD (IU/l)	10.3 ± 2.2	14.9 ± 7.0	13.9 ± 3.5
ALP (IU/l)	61.1 ± 4.2	90.8 ± 9.8**	99.4 ± 8.5**
BUN (mg/dl)	24.5 ± 1.3	26.6 ± 0.9	29.6 ± 2.1
Creatinine (mg/dl)	0.16 ± 0.01	0.11 ± 0.01	0.19 ± 0.03
TCh (mg/dl)	44.3 ± 4.3	61.4 ± 4.5*	68.5 ± 8.1*
TG (mg/dl)	20.3 ± 3.1	28.1 ± 2.8	32.7 ± 9.0

Data are means ± SEM.

**and *Significantly different from that of the Control: $p < 0.01$ and 0.05, respectively.

Table 2 Body growth and mammary tumors

Groups	Control	Nonylphenol 60 mg/kg diet	Nonylphenol 0.6 g/kg diet
(n)	(24)	(10)	(12)
Body growth			
Final body weight (g)	30.9 ± 0.4	29.7 ± 0.9	30.4 ± 1.2
% growth	110.5 ± 5.6	103.2 ± 1.2	105.4 ± 1.9
Mammary tumors			
Incidence	11/24	3/10	8/12*
Number	14/24	3/10	14/12*
Latent periods (w)	33.4 ± 1.1	32.3 ± 4.4	36.4 ± 1.1
Survival (40W of age)	20/24	8/10	7/12

Data are means ± SEM.

* Significantly different from that of the Control: $p < 0.05$, respectively

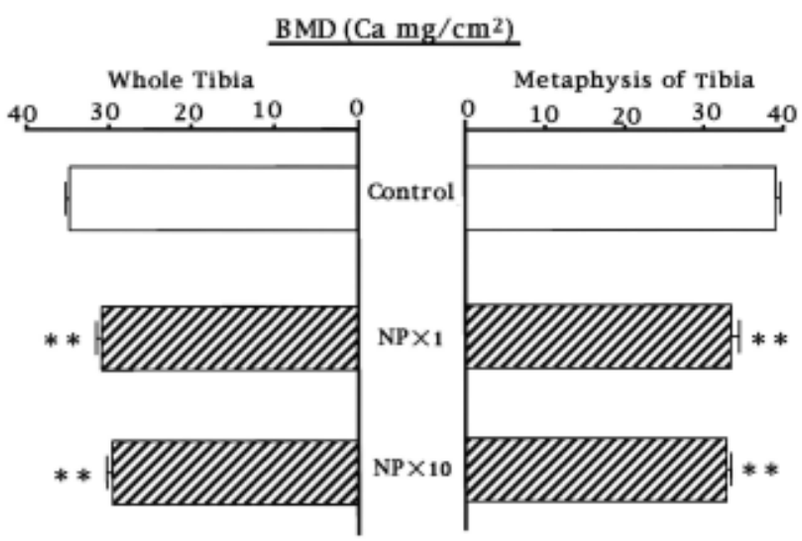

Figure 1 Bone mineral density (BMD: Ca mg/cm^2) of the tibia.
Data are the mean ± SEM in the whole tibia (left-sided) and proximal metaphysis of the tibia (right-sided). Upper right and left white bars for the control and bilateral middle and lower bars with the gray-stripe pattern for mice supplemented with 60 mg and 0.6 g of nonylphenol (NP) in 1 kg of the diet, respectively.
** Significantly different from each corresponding control at $p < 0.01$.

Discussion

NP is an estrogenic compound and endocrine disruptor. This environmental chemical has adverse effects on the developing reproductive system as an endocrine disruptor, and cause tissue injury in many organs by promoting the production of reactive oxygen species. SHN mice are recognized as a strain with a high incidence of mammary tumors. As previously reported, chronic oral administration of 1-(2-tetrahydrofuryl) -5-fluorouracil in combination with uracil as an anti-tumor drug suppressed not only *de novo*, but also salvage pathways for pyrimidine nucleotide synthesis, and reduced mammary tumorigenesis and tumor growth in SHN virgin mice[8]. In contrast, the combined administration of conjugated estrogens and medroxyprogesterone acetate shortened the latent period of mammary carcinogenesis and enhanced bone mineral density of the femur in SHN virgin mice[9]. Thus, in the present study, we investigated the effects of NP on mammary carcinogenesis and BMD of the tibia in SHN virgin mice.

NP did not affect body growth, liver function, or renal function in mice; therefore, it was suggested that NP exhibited fewer toxic characteristics in the present study than in other studies[4, 5], i.e. Aydogan and Korkmaz demonstrated that NP induce oxidative damages in brain, testes, kidneys and liver of male rats. Few significant differences were observed in plasma levels of AST, LD, BUN, and creatinine among groups. However, plasma levels of ALP and TCh were significantly higher in mice fed the diet containing NP than in mice fed the control diet. Belt *et al.* demonstrated that the *in vitro* observed estrogenic potencies of estrone and NP were of the same order of magnitude as their *in vivo* estrogenic potencies[10]. NP has been suggested to act *via* estrogen receptors in mice with an estrous cycle, followed by steroidal dysfunction, decreases in the circulating levels of endogenous estrogens, followed by enhanced bone absorption in the tibia resulting in low BMD, and increases in plasma levels of TCh in mice similar to postmenopausal women.

On the other hand, the incidence and number of mammary tumors were higher in mice given the higher dose of NP. Fukamachi *et al.* reported that NP at 10 ppm increased the incidence of adenocarcinoma and total mammary tumor multiplicity in rats[11]. Acevedo *et al.* showed that NP was more potent than initially predicted based on its affinity for the estrogen receptor and increased mammary cancer formation after chronic exposure to NP[12]. These findings indicate that NP may play a direct role as an estrogenic agent *via* estrogen receptors.

In conclusion, our results indicate that NP plays the role of an estrogenic compound as an endocrine disruptor.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SAK made substantial contributions to the study's conception, analyzed the data, carried out animal experiments, and was involved in drafting the manuscript. MOR and NAG provided the SHN strain mice and kind advice in the present study. SUZ and KUD participated in drafting the manuscript. All authors read and approved the final draft.

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マウス乳癌と脛骨骨密度に与えるノニルフェノールの影響

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要旨

Nonylphenol は，潤滑油添加物，樹脂，可塑剤，表面活性剤，洗面用品，除草剤，塗料，化粧品などの製造過程で広く使われており，近年，内分泌撹乱化学物質（環境ホルモン）としても知られている．今回，乳腺腫瘍自然発生系マウスである SHN 系雌マウスを用いて，乳腺癌化および脛骨骨密度に与える nonylphenol の影響について検討した．成長（体重増加），肝機能，腎機能には影響を与えなかった．Nonylphenol は，腫瘍発生までの平均潜伏期と 40 週齢における生存率に影響を与えなかったが，高濃度投与群において，乳腺腫瘍発生率，腫瘍数ともに有意な増加が認められた．一方，nonylphenol の投与濃度に影響される事なく，血中アルカリフォスファターゼ活性の上昇を伴いながら，脛骨骨密度を有意に減少させた．以上の結果，nonylphenol は，乳腺腫瘍発生には，エストロゲン作用により促進的に働き，一方，性周期を有する動物においては，エストロゲン受容体を介して抗エストロゲン作用により抑制的に働き，骨密度を低下させる事が示唆された．

キーワード

Nonylphenol，内分泌撹乱化学物質，SHN マウス，乳腺腫瘍，骨密度