NOTE *Anatomy*

Bisphenol–A (BPA) Affects Reproductive Formation across Generations in Mice

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ABSTRACT. To understand effects of Bisphenol-A (BPA) exposure on the reproductive organ across generations, we analyzed morphology of the uterus and ovary, and the methylation pattern of HOXA10 gene of the 2nd generation. Pregnant mice (F0) were treated with sc injection of BPA in sesame oil at various doses of 0–1,000 mg/kg Bwt on days 12–16 of gestation. Their offspring (F1) were bred by foster mice, and the offspring (F2) from F1 mice were prepared. That is, F1 mice experienced *in utero* BPA exposure during the developmental period of reproductive organs, while F2 mice did not at all. Using these F2 mice, the present study was carried out. Comparing to the control, the body weights in BPA exposure groups were significantly increased. Correlating with the increase of body weight, the relative weights of the ovary and uterus in each group were decreased. The histological analysis revealed expansion or emphraxis of the uterine lumen and partial loss of the uterine epithelium. Unmethylation of HOXA10 gene in the uterus was observed in the intron region. The present study suggested that BPA exposure to F0 mice could affect reproductive organ of F2 mice who were not exposed to BPA.

KEY WORDS: BPA, HOXA10, reproductive organ.

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Bisphenol-A (BPA) is a nonsteroidal estrogen that is ubiquitous in the environment. There are a lot of reports about effects of BPA on formation and function of female reproductive organs. Among these reports, well known are (i) that BPA exposure advanced puberty [6], (ii) that BPA exposure changed patterns of estrous cycle [11], (iii) that BPA exposure brought about the loss of uterine decidualization [13], and (iv) that BPA exposure decreased the endometrial weight and increased expressions of estrogen receptor α and progesterone receptor [7]. We previously reported that BPA exposure during implantation and placentation periods decreased the number of fetus and pups, and the survival rate before weaning [14]. These suggest that *in utero* BPA exposure altered reproductive performance and formation of reproductive organs. DNA methylation is supposed to be one of the ways that BPA induces the endocrine disruption. DNA methylation regulates gene expression that is involved with growth and development [3, 8]. There are many reports that unmethylation by BPA occurred in the promoter region of phosphodiesterase type 4 [5], that BPA induced unmethylation of agouti gene, using the viable yellow agouti (Avy) mouse [4], and that *in utero* BPA exposure brought about unmethylation of HOXA10 gene [2, 12]. These suggest that BPA exposure can also affect molecular biologically next generation. The present study is designed to understand effects of BPA exposure on the reproductive organ across generations. We analyzed the morphology of uterus and ovary, and the methylation pattern of HOXA10 gene using the next generation of the offspring that was born from mother exposed to BPA.

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ICR mice obtained from Kyudo Company (Saga, Japan) were used in this study. Mice were housed in standard polypropylene cages in a temperature-controlled room (22°C) with a 12 hr light, 12 hr dark cycle. Food and water were provided *ad libitum*. Studies were performed according to protocols for animal use, approved by the Yamaguchi University Animal Experimental Guidelines. ICR mice of 8 wks old were mated with male mice of the same strain. The day when a vaginal plug was detected was considered day 1 of gestation. Pregnant mice were then housed individually and treated with daily sc injections of BPA (Sigma Aldrich, Tokyo) in sesame oil at a dose of 0, 100, 200, 500, and 1,000 mg/kg maternal body weight on days 12–16 of gestation.

Experimental design is summarized in Fig. 1. BPA exposured mice were regarded as F0 generation, and their pups were F1. For the purpose to banish the effect of BPA from mothers including milk, lactation was performed by foster mice. F1 mice of 8 wks old were mated with normal male mice. Offspring of F1 mice was regarded as F2. F2 mice of 8wks old were sacrificed under Avertin anesthesia. The body weight was scaled, and their uterus and ovary weight were measured after removed. The left uterine horn was fixed in 10% buffered formalin for histological analysis. Sections of 4 μm in thickness were prepared, deparafnized and stained with hematoxylin-eosin.

DNA was isolated from the right uterine horn of F2 mice using the DNeasy Blood and Tissue kit (QIAGEN, Tokyo), according to the manufacturer's protocol. Sodium Bisulfite modification was performed using the Epitect Bisulfite kit (QIAGEN, Tokyo). Targeted sequences and primers are shown in Figs. 2 and 3. Primers were designed for

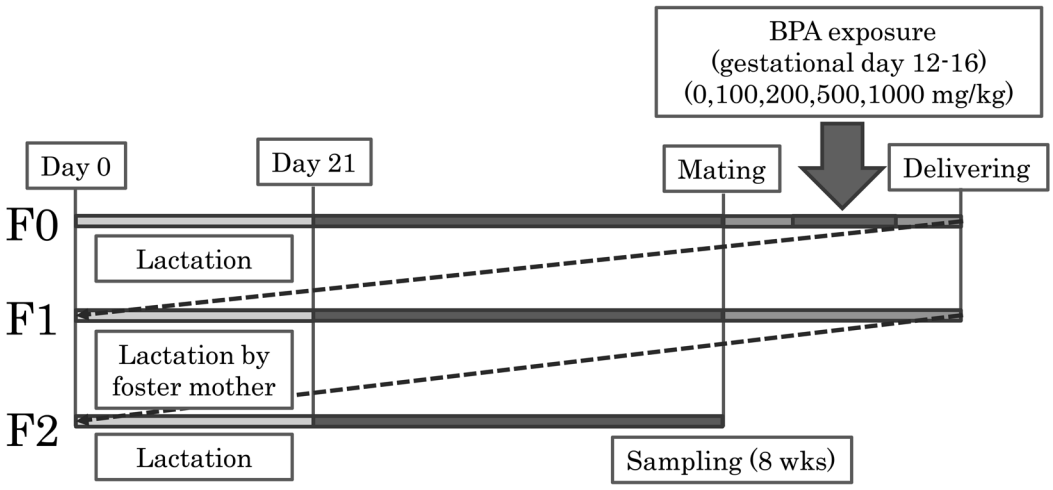

Fig. 1. Experimental protocol. BPA exposure of F0 is performed at days 12–16 of gestation. Lactation for F1 is performed by foster mice. Weaning is performed at day 21 after birth. Mating of F0 and F1, and sampling of F2 are performed at day 56 after birth.

A)HOXA10 promoter region

tgaggcagccccacagttc**cg**gcctttgagccataggtgtcagg**cg**tg**c**
gtcttctggcccatcaatacagattacatatttatatcaat**cgcg**ggctc**c**
gaggg**cg**ccctcagagag**cg**gccc**cgcg**ccta**cg**aaaccaaactggga
gtggt**cgcg**tggaaactctggct**cg**ggattggctgcaag**cg**cc**cgc**c**gcg**
gtg**cg**gggggggattgctaat**cg**tattcagcatgttttgcacaagaaatgt
cagccagaaagggctat

B)HOXA10 intron region

cagcc**cg**agtggcct**cg**acttaaccttcccctctgtattctcttaaactcca
ggcagttccaaagg**cg**aaaatgcagccaactggctcacagcaaagag**c**
ggc**cg**gaagaaa**cg**ctgcccttaca**cg**aagcaccaga**cg**ctggagctgg
agaaggagtttc

Fig. 2. Targeted sequences of the HOXA10 gene. CpG site shows bold characters. A) The 5' promoter region, containing 20 CpG sites. B) Intron region, containing 8 CpG sites.

HOXA10 5' promoter and intron-1 regions in the mouse [1]. HotStarTaq DNA polymerase (QIAGEN, Tokyo) was used. Amplification conditions were as follows: 5 min starting at 95°C; 35 cycles at 95°C for 30 sec; 53°C for 30 sec; and 72°C for 30 sec, followed by a final extension at 72°C for 10 min. PCR products were resolved by electrophoresis on a 1% agarose gel and stained with ethidium bromide. For each CpG site, a "C" was interpreted as a methylated site, whereas a "T" was interpreted as unmethylated site. PCR products amplified by primer for methylated allele were regarded as methylated, while PCR products amplified by primer for unmethylated allele were as unmethylated. PCR products amplified by both primers for methylated allele and unmethylated allele were regarded as both methylated and unmethylated. Figure 4 shows an example from results of electrophoresis.

Statistical analysis was performed with Student's *t*-test after the check homoscedasticity with F test. Values of $P<0.05$ were considered statistically signicant.

The body weights, absolute reproductive organ weights and their relative weights (organ weight/ body weight) in F2 adult females are shown in Table 1. Comparing to the vehicle (0 mg/kg) group, the body weights in 200, 500 and 1,000 mg/kg groups were increased significantly. In 100 mg/kg group, the left ovary weight, uterus weight and uterus relative weight were decreased. In 200 mg/kg group, the right ovary relative weight was decreased. In 500 mg/kg group, the left ovary weight, relative weight and right ovary rela-

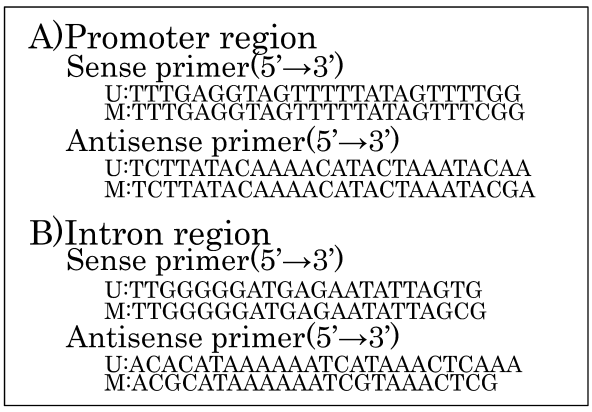

Fig. 3. Primers used for Bisulfite PCR. A) Primers for promoter region. B) Primers for intron region. M: Methylated allele, U: Unmethylated allele.

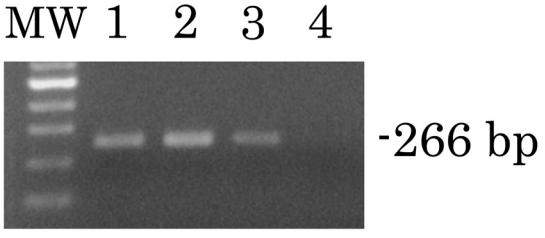

Fig. 4. Sensitivity and specificity of the PCR system using the primer for promoter region. An example from results of the 200 mg/kg group. Lanes 1–3 show methylated PCR products at 266 bp, while Lane 4 does not. MW: Molecular Weight markers.

tive weight were decreased. In 1,000 mg/kg group, the left ovary relative weight was decreased. The ovary relative weight tended to be decreased due to the increase of body weight, while the uterine relative weight did not change due to the increase of the uterine weight itself. Histology revealed abnormal morphology such as expansion or emphraxis of the uterine lumen and partial loss of the uterine epithelium (Fig. 5). Expansion of the uterine lumen was observed in 500 and 1,000 mg/kg groups, while emphraxis of the uterine lumen occurred only in 1,000 mg/kg group. Although partial loss of the uterine epithelium was observed in 200 mg/kg group, such a loss occurred at various sites of the uterus. The uterine gland appeared to be normal. Results of HOXA10 gene analysis are shown in Table 2. In the vehicle (0 mg/kg) group, both patterns representing methylation and unmethylation were observed in the promoter and intron regions. In contrast to the vehicle group, in 100 and 200 mg/kg groups unmethylation was observed in the intron region.

Table 2. Bisulfite conversion of the HOXA10 promoter and intron region

	vehicle	100 mg/kg	200 mg/kg
Promoter region			
Unmethylation	6/6	3/6	6/6
Methylation	6/6	5/6	4/6
Intron region			
Unmethylation	6/6	6/6	6/6
Methylation	6/6	0/6	2/6

Values are expressed as number/total number.

This study established that *in utero* BPA exposure during the formation period of reproductive organ in F1 mice could alter histology and methylation pattern in the reproductive organ of F2 who had no experience in BPA exposure. Since it was known that the body weight in F2 generation was increased by BPA exposure to F1 at the fetal and the lactational periods [6, 11], it is most likely that the increase of body weight in our study was due to BPA exposure. The uterus relative weight was decreased significantly, which seemed to be involved with incomplete development of the endometrium, as reported previously [7]. Furthermore, our histology in F2 mice showed at some sites abnormal morphology inducing expansion or emphraxis of the uterine lumen and partial loss of the uterine epithelium, suggesting that the constituents of F2 uterus were out of harmony in growth. Although there is a report that hyperplasia of the uterine endometrium was induced by BPA exposure [9], the report analyzed the uterus of adult F1 mice or fetal F2 mice that were different from materials in the present study. Nevertheless, we cannot rule out the possibility that abnormal morphology of the adult F2 uterus in the present study resulted from BPA exposure to F0 mice. It is well known that BPA could induce methylation in the promoter or intron regions of some genes [2, 4, 5, 12, 15]. Particular attention

Table 1. Absolute organ weights and relative organ weights in F2 generations

Group		vehicle	100 mg/kg	200 mg/kg	500 mg/kg	1,000 mg/kg
Number		12	7	7	7	7
Body weight(g)		28.73 ± 2.713	29.27 ± 2.916	31.03 ± 0.820*	31.66 ± 1.207*	32.87 ± 2.520*
Right ovary (mg)		4.42 ± 0.996	3.43 ± 1.272	3.71 ± 0.756	3.29 ± 1.496	4.86 ± 2.035
Left ovary (mg)		4.75 ± 0.622	3.71 ± 1.113*	4.14 ± 1.464	3.00 ± 1.732*	4.14 ± 0.900
Uterus (mg)		99.8 ± 37.17	66.1 ± 9.05*	99.9 ± 23.55	121.7 ± 69.70	115.3 ± 35.57
Reproductive organ	Right ovary (%)	0.02 ± 0.003	0.01 ± 0.004	0.01 ± 0.002*	0.01 ± 0.005*	0.02 ± 0.006
	Left ovary (%)	0.02 ± 0.003	0.01 ± 0.004*	0.01 ± 0.004	0.01 ± 0.006*	0.01 ± 0.002*
	Uterus (%)	0.35 ± 0.133	0.23 ± 0.042*	0.32 ± 0.075	0.38 ± 0.218	0.35 ± 0.119

Data are presented as mean ± SD. *Significant difference from the control (vehicle) group ($P<0.05$).

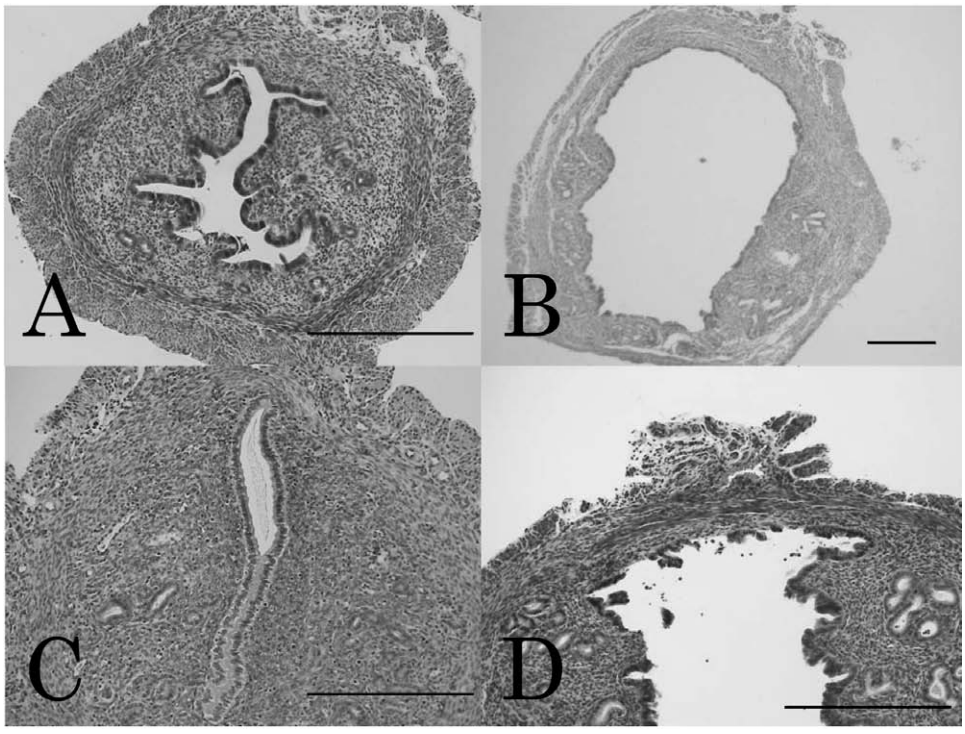

Fig. 5. Histological analysis of the uterus (HE stain). A) vehicle. B) 500 mg/kg. The uterine lumen is expanded. C) 1,000 mg/kg. Emphraxis of the uterine lumen is observed. D) 200 mg/kg. Partial loss of the epithelium is detected. Scale bars=300 μm.

was paid to HOXA10 gene that could be involved with all developmental stages in reproductive organs [2, 9, 12]. HOX genes can control the development of the oviduct, uterus, cervix, and upper vagina [15]. The alteration of methylation pattern in HOXA10 may disrupt normal formation of reproductive organs. It was reported that *in utero* BPA exposure in days 9–16 of gestation resulted in unmethylation both in the promoter and intron regions of HOXA10 gene [2]. On the other hand, the present study suggested that *in utero* BPA exposure in days 12–16 of gestation, when the reproductive organ developed, could induce unmethylation only in the intron region of HOXA10 gene. Since our F2 mice had estrus cycle until 10 weeks old, and since F2 mice used in this study were 8 weeks old, abnormal morphology of the uterus may result from delayed formation. Further studies are needed to establish effects of BPA on generation one after another.

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