Disturbed spermatogenesis in mice prenatally exposed to an endocrine disruptor, Bisphenol A

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Abstract. Bisphenol A (BPA), which has previously been shown to have estrogenic activity, was examined for its effect on spermatogenesis in offspring of mice that had been exposed to BPA during gestation. BPA (0, 1, 10, or 100 mg/kg body weight) was orally administered to pregnant mice from the 10th to the 17th day of gestation, and testes of 60- and 120-day-old male offspring were removed and processed for histological analysis. The results demonstrate that prenatal exposure to BPA brings about histopathological changes in the seminiferous epithelium of testes in mouse offspring, such as loss of the luminal space of the seminiferous tubules, accumulation of amorphous material in the tubes, reduction in the number of maturing elongate spermatids, and an aberrant distribution of spermatogenic cells within the epithelium. Electron microscopy suggested that disturbed spermiogenesis is one of the reasons for the reduction in the number of elongate spermatids, and that degeneration of somatic Sertoli cells might be responsible for the aberrant distribution of spermatogenic cells within the epithelium. These data suggest that exposure to BPA during fetal life has profound effects on spermatogenesis in the testes when the offspring become adult.

Key words: Bisphenol A, spermatogenesis, testis.

The best-characterized endocrine-disrupting chemicals are those that are able to bind to estrogen receptors (ERs) and act either as estrogen agonists or antagonists. One such chemical, Bisphenol A (BPA) [2, 2-Bis (4-hydroxyphenyl) propane], which has been proven to interact with ERs (Gould et al. 1998; Bergeron et al. 1999; Schafer et al. 1999), is a monomer of polycarbonate plastics and a constituent of epoxy and polystyrene resins that are extensively used in the food-packaging industries. BPA is also a component of dental sealant. BPA is released from the resin used to coat the interior of food cans when the cans are autoclaved, and has also been detected in the saliva of patients treated with dental sealants (Krishnan et al. 1993; vom Saal et al. 1998). Biological effects of BPA include enhancement of prolactin gene expression in pituitary cells (Steinmetz et al.

1997), promotion of hyperplasia and differentiation in the female reproductive tract epithelium of rats (Steinmetz et al. 1998), and acceleration of growth and puberty in female mice (Howdeshell et al. 1999).

It has been reported that ER gene-knockout mice are infertile and that disruption of the ER gene brings about histological abnormality of seminiferous tubules (ST) in juvenile mice and affects the quantity and quality of sperm production in adults (Eddy et al. 1996). This suggests that estrogens have direct effects on the male reproductive system and that environmental estrogen chemicals potentially can affect it as well. There is controversy, however, over the effects of prenatal BPA exposure on the male reproductive system. It has been demonstrated that prenatal exposure to BPA affected the reproductive function in male offspring including daily

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Fig. 1. Light micrographs of testes of 60 day-old-male mice prenatally exposed to 0 mg/kg (a and e), 1 mg/kg (b and f), 10 mg/kg (c, g and h), and 100 mg/kg (d and i) /BW dose of BPA. Arrows in Figs. 1b, c, and d indicate seminiferous tubules showing histopathological abnormality. Scale bars represent 100 μ m (a, b, c, d) and 25 μ m (e, f, g, h and i).

sperm production (vom Saal et al. 1998), while Cagen et al. (1999) claimed normal development of reproductive organs in CF-1 male mice prenatally exposed to BPA. In the present study, we investigated by light and electron microscopy the effects of BPA on spermatogenesis in ST

of male offspring of dams exposed to BPA from the 10th to the 17th day of gestation. This study provides the first clear evidence that prenatal exposure to BPA brings about histological abnormality of ST in male mouse offspring.

Fig. 1

Materials and methods

Bisphenol A (BPA) administration and histological observation

Investigations were conducted in accordance with the National Research Council publication, Guide for Care and Use of Laboratory Animals. BPA (Tokyo Kagaku Kogyo, Tokyo, Japan) was dissolved in corn oil (0.2 ml) and administered orally to pregnant female mice (ddY strain) from the 10th to the 17th day of gestation. Different doses of BPA (0, 1, 10, and 100 mg/kg body weight (BW)) were administered once per day. Corn oil without BPA was administered to the mice of the control group. Sixty days after birth, the body weight of the mice was recorded, and the testes were removed under ether-anesthesia and fixed in 3% (w/v) paraformaldehyde fixative, embedded in paraffin, and 5–8 μ m sections were stained with hematoxylin-eosin. The sections were observed under a light microscope (Olympus BX-50, Tokyo, Japan) and their histological images were recorded with a DP-10 digital camera (Olympus, Tokyo, Japan). To evaluate histological changes in ST of both the control group and the BPA-exposed groups, the number of ST showing normal histology and histopathological changes was counted. The number of mice examined was as follows: five for the control group, five for the 1 mg/kg BPA-exposed group, five for 10 mg/kg BPA-exposed group, and four for the 100 mg/kg BPA-exposed group. Offspring of three dams were examined in each group. More than 100 ST were examined under a microscope in at least nine randomly selected sections for each mouse.

Testes of three mice prenatally exposed to the 10 mg/kg BW dose of BPA as well as three control mice were also histologically investigated at 120 days after birth. These testes were fixed in 3% (v/v) glutaraldehyde fixative, postfixed in 1% (v/v) osmium tetroxide, dehydrated in a graded series of ethanols, and embedded in epoxy resin, as previously reported (Iida et al. 2000). Thick sections were examined under a light microscope after staining with toluidine blue. Ultra thin sections were examined under a Hitachi H-100 electron microscope after staining with uranyl acetate and lead citrate. Histopathological changes in ST were evaluated by examination of more than 100 ST for each mouse.

Statistical analysis

Statistical analysis was performed by ANOVA. Differences were considered to be significant when $P < 0.05$.

Results

Light microscopy of testes of 60-day-old mice prenatally exposed to BPA

We have previously examined the effects of BPA (0, 2, 20, 200 mg/kg BW) on spermatogenesis in Wistar adult rats. In this case, BPA was orally administered once per day to five week-old rats for seven and for 20 days, respectively, and their testes were processed for histological analysis when they were nine weeks old. In both the control and the BPA-exposed rat testes, we could not observe any histological abnormality in ST.

We next examined the effect of BPA on the testes of 60-day-old male offspring of female mice exposed to 0, 1, 10, or 100 mg/kg BW doses of BPA from the 10th to the 17th day of gestation. Neither dose of BPA had significant effects on the body weight of 60-day-old mice [0 mg: 36.6 ± 3.56 g, 1 mg: 37.7 ± 0.97 g, 10 mg: 34.4 ± 2.49 g, 100 mg: 33.0 ± 1.42 g (mean $\pm$ SD)]. To examine whether prenatal exposure to BPA has an effect on spermatogenesis in testes, paraffin-embedded, hematoxylin-eosin stained sections of testes were examined under a light microscope. As shown in Fig. 1a, pathological changes were rarely found in ST of the testes in the controls. In contrast, part of the ST of male offspring that were prenatally exposed to the 1, 10, or 100 mg/kg BW dose of BPA exhibited histological abnormality in the seminiferous epithelium, such as loss of the luminal space in the tubules and reduction in the number of maturing elongate spermatids (Figs. 1b–d). Decrease of the tubular diameter was also recognized in part of the abnormal ST (Fig. 1b). Several abnormal ST in the BPA-exposed groups as well as normal ST of the control are shown at high magnification in Figs. 1e–i. The seminiferous epithelium of the control displayed a highly organized concentric disposition of spermatogenic cells, i.e., spermatogonia, spermatocytes, round spermatids, and maturing elongate spermatids, which were disposed in order from the periphery to the luminal side of the ST (Fig. 1e). Abnormal ST showed aberrant distribution of spermatogenic cells within the epithelium (Figs. 1f, h and i), accumulation of amorphous material within the tubules (Figs. 1g, h and i), and deposits of dense cells in the center of the tubule (Fig. 1g).

These histopathological changes occurred only in part of the ST in the testes of the BPA-exposed mice. In order to examine the incidence of abnormal ST in testes, we counted the number of abnormal ST in both the control and the BPA-exposed groups. The results are summa-

Fig. 2. Incidence of abnormal seminiferous tubules in testes of male mice prenatally exposed to 0, 1, 10, or 100 mg/kg BW dose of BPA. Data represent mean $\pm$ SD (* $p < 0.02$ vs control group. ** $p < 0.001$ vs control group).

rized in Fig. 2. Histopathological changes of ST were observed at an incidence of $3.7 \pm 0.93\%$ in the control, $15.2 \pm 4.78\%$ in the 1mg/kg BPA-exposed group, $17.7 \pm 9.78\%$ in the 10 mg/kg BPA-exposed group, and $31.5 \pm 12.8\%$ in the 100 mg/kg BPA-exposed group. The data revealed that prenatal exposure to BPA significantly increased the incidence of abnormal ST in testes of male offspring, and that the effect appeared in a dose-dependent manner.

Light and electron microscopy of testes of 120-day-old mice prenatally exposed to BPA

To examine whether prenatal exposure to BPA has a prolonged and irreversible effect on spermatogenesis, testes of three mice prenatally exposed to the 10 mg/kg BW dose of BPA were examined by light and electron microscopy at 120 days after birth. As shown in Fig. 3a, BPA-exposed mice still exhibited a relatively high incidence of seminiferous epithelium abnormality, such as loss of the tubular lumen, aberrant distribution of germ cells, and reduction in the number of maturing elongate spermatids. Histopathological changes of ST were observed at an incidence of $28.3 \pm 6.6\%$ (mean $\pm$ SD),

while that of the control was $5.14 \pm 2.7\%$ (mean $\pm$ SD).

Two abnormal ST are shown at high magnification in Figs. 3b–e. One of the ST had deposits of dense cells in the center of the tubule (Fig. 3b), whose seminiferous stage seemed to be IV–VII because of the presence of round spermatids containing acrosome-associated nuclei (Fig. 3c). The ST had spermatogonia, spermatocytes, and round spermatids in the epithelium but was devoid of developing elongate spermatids (step 15–16), suggesting disturbed morphogenesis from round spermatids to elongate spermatids (spermiogenesis). Electron microscopy showed that the dense cells seen in the ST had mitochondria, membranous organelles, electron-dense materials, and lipid-like droplets but contained no nucleus (Fig. 4a). Since a flagellum associated with mitochondria was seen in the cytoplasm of the dense cells (Fig. 4a, inset), the cells seemed to be derived from spermatids. Another abnormal ST shown in Fig. 3d exhibited disorganized arrangement of Sertoli cells within the epithelium. Several Sertoli cells' nuclei, which are characterized by distinct nucleoli and homogeneous nucleoplasm, were seen in the central portion of the ST, instead of being located at the tubular periphery (Fig. 3e). These Sertoli cells are probably detached from the basal membrane and displaced to the center of the ST. The cytoplasm of the displaced Sertoli cells was occupied by a lot of vesicular and vacuolar membranous structures (Fig. 4b), which might be a sign of cellular degeneration. In the center of the ST, spermatogonia-like germ cells were also observed (Fig. 3e).

Discussion

Recent studies have demonstrated that prenatal exposure to environmental estrogens significantly affects male reproductive functions. Prenatal exposure to BPA permanently increased the size of the preputial glands and reduced the size of the epididymides (vom Saal et al. 1998). Prostate glands of male offspring of females exposed to BPA during pregnancy were significantly increased relative to untreated males (Nagel et al. 1997). In another study, prenatal exposure to a putative environmental estrogen (4-octylphenol) resulted in reduced expression of mRNA and protein for P450 in fetal Leydig cells (Majdic et al. 1996). These data suggest that the fetal male reproductive tract is very sensitive to prenatal exposure to environmental estrogens. Although it has been reported that prenatal exposure to BPA decreased the efficiency of daily sperm production (vom Saal et al.

Fig. 3. Light micrographs of testes of 120-day-old male mice prenatally exposed to the 10 mg/kg BW dose of BPA at low (a) and high (b and d) magnification. Parts of Figs. 3b and 3d are further enlarged in Figs. 3c and 3e, respectively. Note that most of the seminiferous tubules shown in Fig. 3a exhibits histological abnormality, such as loss of the tubular lumen, aberrant distribution of germ cells, and reduction in the number of maturing elongate spermatids. Arrowheads in Fig. 3c indicate acrosome-associated nuclei of round spermatids. Arrows in Fig. 3e indicate nuclei of Sertoli cells. Note that part of the Sertoli cells in the seminiferous tubule is detached from the basal membrane and displaced to the center of the tubule in which several spermatogonia-like germ cells are also seen. Scale bars represent 40 μ m (a), 20 μ m (b, d) and 10 μ m (c, e).

1998), there is little information concerning the effect of prenatal BPA exposure on germ cell differentiation in the testis of male offspring.

In this study, we histologically evaluated the effects of BPA on spermatogenesis in male offspring of mice exposed to BPA during gestation. To our knowledge, this

study provides the first clear evidence that prenatal exposure to BPA brings about remarkable histopathological abnormality in the ST of mice, such as loss of the luminal space in the tubules, reduction in the number of maturing elongate spermatids, aberrant distribution of spermatogenic cells within the epithelium, and accumu-

Fig. 3

lation of amorphous materials in the ST. Histological abnormality of ST should not be due to artefacts of poor fixation since ST of adult rats exposed to BPA did not show any detectable degeneration, and since abnormality of ST was also observed in mouse testes fixed by glutaraldehyde (Figs. 3a–e), which efficiently reduced the possibility of a fixative artefact. In some abnormal ST, spermiogenesis was disturbed (Figs. 3b, c and 4a), which seemed to be one of the reasons for reduction in the

number of maturing elongate spermatids in the tubules. Furthermore, we found that abnormal ST persisted at least for 120 days after birth, suggesting that prenatal exposure to BPA might cause permanent and irreversible abnormality of the seminiferous epithelium in male offspring. Taken together, these data suggest that exposure to BPA during fetal life has profound effects on spermatogenesis in the testes when offspring grow up to adulthood. In this context, our data is consistent with the

Fig. 4. Electron micrographs of the dense cell shown in Fig. 3c (a) and of the displaced Sertoli cell shown in Fig. 3e (b). The inset in Fig. 4a indicates a flagellum associated with mitochondria observed in the dense cell. Scale bars represent 1 μ m.

report of vom Saal et al. (1998) showing that prenatal exposure to BPA decreased efficiency of daily sperm production in male offspring. Reduction in the number of spermatozoa in the caput epididymis has also been reported in mice treated neonatally with BPA (Nakahashi et al. 2001).

Sertoli cells act as 'nurse cells' providing the structural and metabolic support for the developing germ cells.

Since a number of factors essential for germ cell development is synthesized by somatic Sertoli cells (Griswold 1995; Meehan et al. 2000), and since the number of spermatozoa produced per day is governed by the number of Sertoli cells in the ST (Amann 1970), it is thought that any agent which impairs the viability and the function of Sertoli cells during the fetal and neonatal period may have profound effects on spermatogenesis. A possible

toxic effect of BPA on Sertoli cells has been recently reported in TM4 cells, a substituted cell line for Sertoli cells (Hughes et al. 2000). Microscopic examination in this study revealed that part of the Sertoli cells within the disorganized seminiferous epithelium showed cellular degeneration (Figs. 3e and 4b). Thus it is possible that the aberrant distribution of germ cells seen in the abnormal ST might be, at least in part, due to the defect of the supporting function of Sertoli cells. It is, therefore, probable that some level of disturbed spermatogenesis observed in the BPA-exposed mice is mediated through the dysfunction of Sertoli cells. We are currently investigating in vitro whether BPA has a direct effect on the viability and the functions of Sertoli cells.

It is generally thought that estrogenic alkylphenols exert their effects after binding to ERs. ERs have been shown to be expressed in germ cells in mammalian testes (Pelt et al. 1999). In addition to germ cells, ERs are also expressed in the urogenital sinus and the Wolffian ducts during prenatal development in mice (Cooke et al. 1991; Nielsen et al. 2000) and in Sertoli cells as well as in Leydig cells in fetal testes (Pelt et al. 1999). Therefore, these organs and cell types can potentially be affected by compounds that bind to ERs, such as BPA, in the prenatal period when mothers are chronically exposed to such compounds during pregnancy. An endocrine-disrupting effect of estrogenic alkylphenols, however, is not necessarily mediated by direct interaction with ERs (Harris et al. 2000). Several studies have reported that BPA could inhibit the testis' microsomal Ca^{2+} ATPase (Hughes et al. 2000). These observations suggest that environmental estrogens may disturb male reproductive development by disturbing Ca^{2+} signaling in testicular cells rather than by direct interaction with ERs. It remains to be determined how spermatogenesis is affected by BPA exposure during fetal life.

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References

- Amann, R. P. 1970. The male rabbit. IV. Quantitative testicular histology and comparisons between daily sperm production as determined histologically and daily sperm output. *Fertility and Sterility* 21: 662–672.
- Bergeron, R. M., Thompson, T. B., Leonard, L. S., Pluta, L. and Gaido, K. W. 1999. Estrogenicity of bisphenol A in a human endometrial carcinoma cell line. *Molecular and Cellular Endocrinology* 150: 179–187.
- Cagen, S. Z., Waechter, J. M. Jr., Dimond, S. S., Breslin, W. J., Butala, J. H., Jekat, F. W., Joiner, R. L., Shiotsuka, R. N., Veenstra, G. E. and Harris, L. R. 1999. Normal reproductive organ development in CF-1 mice following prenatal exposure to bisphenol A. *Toxicological Sciences* 50: 36–44.
- Cooke, P. S., Young, P., Hess, R. A. and Cunha, G. R. 1991. Estrogen receptor expression in developing epididymis, efferent ductules, and other male reproductive organs. *Endocrinology* 128: 2874–2879.
- Eddy, E. M., Washburn, T. F., Bunch, D. O., Goulding, E. H., Gladen, B. C., Lubahn, D. B. and Korach, K. S. 1996. Targeted disruption of the estrogen receptor gene in male mice causes alteration of spermatogenesis and infertility. *Endocrinology* 137: 4796–4805.
- Gould, J. C., Leonard, L. S., Maness, S. C., Wagner, B. L., Conner, K., Zacharewski, T., Safe, S., McDonnell, D. P. and Gaido, K. W. 1998. Bisphenol A interacts with the estrogen receptor alpha in a distinct manner from estradiol. *Molecular and Cellular Endocrinology* 142: 203–214.
- Griswold, M. D. 1995. Interactions between germ cells and Sertoli cells in the testis. *Biology of Reproduction* 52: 211–216.
- Harris, R. M., Waring, R. H., Kirk, C. J. and Hughes, P. J. 2000. Sulfation of "Estrogenic" alkylphenols and 17 β -estradiol by human platelet phenol sulfotransferases. *Journal of Biological Chemistry* 275: 159–166.
- Howdeshell, K. L., Hotchkiss, A. K., Thayer, K. A., Vandenbergh, J. G. and vom Saal, F. S. 1999. Exposure to bisphenol A advances puberty. *Nature* 401: 763–764.
- Hughes, P. J., McLellan, H., Lowes, D. A., Khan, S. Z., Bilmen, J. G., Tovey, S. C., Godfrey, R. E., Michell, R. H., Kirk, C. J. and Michelangeli, F. 2000. Estrogenic alkylphenols induce cell death by inhibiting testis endoplasmic reticulum Ca^{2+} pumps. *Biochemical and Biophysical Research Communications* 277: 568–574.
- Iida, H., Kaneko, T., Tanaka, S. and Mori, T. 2000. Association of the developing acrosome with multiple small Golgi units, the Golgi satellites, in spermatids of the musk shrew, *Suncus murinus*. *Journal of Reproduction and Fertility* 119: 49–58.
- Krishnan, A. V., Stathis, P., Permuth, S. F., Tokes, L. and Feldman, D. 1993. Bisphenol-A: an estrogenic substance is released from polycarbonate flasks during autoclaving. *Endocrinology* 132: 2279–2286.
- Majdic, G., Sharpe, R. M., O'Shaughnessy, P. J. and Saunders, P. T. 1996. Expression of cytochrome p450 17 α -hydroxylase/C17-20 lyase in the fetal rat testis is reduced by maternal exposure to exogenous estrogens. *Endocrinology* 137: 1063–1070.
- Meehan, T., Schlatt, S., O'Bryan, M. K., de Kretser, D. M. and Loveland, K. L. 2000. Regulation of germ cell and Sertoli cell development by activin, follistatin, and FSH. *Developmental Biology* 220: 225–237.
- Nagel, S. C., vom Saal, F. S., Thayer, K. A., Dhar, M. G., Boechler, M. and Welshons, W. V. 1997. Relative binding affinity-serum modified access (RBA-SMA) assay predicts the relative in vivo bioactivity of the xenoestrogens bisphenol A and octylphenol.

- Environmental Health Perspectives 105: 70–76.
- Nakahashi, K., Matsuda, M. and Mori, T. 2001. Vitamin A insufficiency accelerates the decrease in the number of sperm induced by environmental disruptor, Bisphenol A, in neonatal mice. *Zoological Science* 18: 819–821.
- Nielsen, M., Bjornsdottir, S., Hoyer, P. E. and Byskov, A. G. 2000. Ontogeny of oestrogen receptor α in gonads and sex ducts of fetal and newborn mice. *Journal of Reproduction and Fertility* 118: 195–204.
- Pelt, A. M. M., Rooij, D. G., Burg, B., Saag, P. T., Gustafsson, J. and Kuiper, G. G. J. M. 1999. Ontogeny of estrogen receptor- β expression in rat testis. *Endocrinology* 140: 478–483.
- Schafer, T. E., Lapp, C. A., Hanes, C. M., Lewis, J. B., Wataha, J. C. and Schuster, G. S. 1999. Estrogenicity of bisphenol A and bisphenol A dimethacrylate in vitro. *Journal of Biomedical Materials Research* 45: 192–197.
- Steinmetz, R., Brown, N. G., Allen, D. L., Bigsby, R. M. and Ben-Jonathan, N. 1997. The environmental estrogen bisphenol A stimulates prolactin release in vitro and in vivo. *Endocrinology* 138: 1780–1786.
- Steinmetz, R., Mitchner, N. A., Grant, A., Allen, D. L., Bigsby, R. M. and Ben-Jonathan, N. 1998. The xenoestrogen bisphenol A induces growth, differentiation, and c-fos gene expression in the female reproductive tract. *Endocrinology* 139: 2741–2747.
- vom Saal, F. S., Cooke, P. S., Buchanan, D. L., Palanza, P., Thayer, K. A., Nagel, S. C., Parmigiani, S. and Welshons, W. V. 1998. A physiologically based approach to the study of bisphenol A and other estrogenic chemicals on the size of reproductive organs, daily sperm production, and behavior. *Toxicology and Industrial Health* 14: 239–260.

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