This article investigated a group of testicular genes dysregulated by prenatal exposure to bisphenol A (BPA), an endocrine disruptor which is known to affect the development of reproductive system. In 1990s, it was reported that BPA was one of the important chemicals used principally as a monomer in the manufacture of plastics and other products and have been detected in food and water consumed by animals and people (Krishnan et al., 1993; Brotons et al., 1995). Previous studies have shown that oral and subcutaneous exposure of male animals (mouse or rat) to BPA resulted in abnormalities of Sertoli cells (Takahashi and Oishi 2001; Toyama et al., 2004) and spermatids (Toyama et al., 2004) and a decrease in weights of testis, epididymis and accessory reproductive...
tive organs (Takahashi and Oishi, 2001, 2003) and serum testosterone level (Takao et al., 1999). Sperm production was also decreased by BPA exposure (> 25 μg/kg) (Al-Hiyasat et al., 2002). The mechanism underlying the effects of direct exposure to chemical substances on the male bodies may differ to that of maternal exposure on the male offspring. It has also been reported that oral exposure of pregnant animals to a low dose of BPA decreased reproductive function of male offspring, with significant decrease in testis weight (Kawai et al., 2003) and sperm production (vom Saal et al., 1998; Tinwell et al., 2002). However, it was also reported that the selective effect of BPA on reproductive system in the developmental period have been unclear (Cagen et al., 1999a, 1999b; Ashby et al., 1999), therefore a reliable and useful method for evaluating the effect is required.

Although one of the major concerns about testicular toxicology is the effect of prenatal exposure to chemical substances including BPA as well as phthalates (Culty et al., 2008; Hotchkiss et al., 2004), pentachlorobiphenyl (Wakui et al., 2010), tobacco smoke (Jensen et al., 2005), diesel exhaust (Yoshida et al., 2002; Yoshida et al., 2006; Watanabe, 2005; Ono et al., 2007; Xu et al., 2009; Umezawa et al., 2011a) and nanoparticle (Takeda et al., 2009; Yoshida et al., 2010, Ema et al., 2010, Umezawa et al., 2011b), the present reproduction/developmental toxicity screening test is expensive and time-consuming. The standard method to evaluate the reproductive and developmental toxicity of chemical substances is the Organisation for Economic Co-operation and Development (OECD) Guidelines for Testing of Chemicals. Since it is expensive and time-consuming to carry out, an inexpensive and fast method that can provide predictive information on the potential toxicity of chemical substances would be beneficial. To provide a method for the needs in reproductive toxicology, we examined the male reproductive system after prenatal exposure to BPA based on a gene expression analysis in the testis. The analysis was conducted using a tissue-specific microarray, Mouse Testis2 Array, combined with Medical Subject Headings (MeSH) terms annotation. The Testis2 array has selected probes for genes which are known to be relevant to testis components or reproductive functions. MeSH is a vocabulary indicative of cellular components and biological functions produced by the National Library of Medicine and used for indexing, cataloging, and searching for biomedical and health-related information and documents. Recent studies have suggested that MeSH is proposed to be a useful complementary tool for the interpretation of gene expression data. A method for analyzing data combined with MeSH annotations was based on the principles proposed by Nakazato et al. (2008, 2009) with a minor modification as employed by subsequent studies (Umezawa et al., 2009; Shimizu et al., 2009; Takahashi et al., 2011). The aim of the present study was to generate a functional overview and to extract a gene group of interest related to testicular toxicity by maternal exposure to BPA. Additionally, we propose the approach of MeSH annotation analysis is useful to identify gene groups altered by in utero exposure to chemical substances.

MATERIALS AND METHODS

Bisphenol A

Corn oil and BPA were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). BPA was dissolved at 0.5 or 5 mg/ml in corn oil.

Animals and treatments

Eighteen female ICR mice of the first day of pregnancy were purchased from Japan SLC Inc. (Shizuoka, Japan) and housed under controlled temperature (23 ± 1°C), humidity (55 ± 5%) and light (12 hr light/12 hr dark cycle with light on at 8:00 a.m.) with ad libitum access to chow and water. Mice were divided into three groups of six mice each: BPA (5 and 50 mg/kg/time) and control groups. BPA was administered subcutaneously to pregnant mice on days 7 and 14 of pregnancy. Mice in the control group received only corn oil, on the same schedule as the treated groups. BPA doses were based on the lowest observed adverse effect level (LOAEL) of chronic oral BPA intake (50 mg/kg/day) in rats (U.S. NTP, 1982). After parturition, the number of pups per litter was adjusted randomly to ten on postnatal day (PND) 4. Pups were weaned on PND 21. At 6 weeks old, they were weighed and sacrificed under ether hyperanesthesia to obtain blood and tissue samples of testis, epididymis, accessory reproductive organs (prostate, seminal vesicles and coagulating glands) (n = 10 offspring/group). One or two offspring per dam were used to perform the experiments. All animals were handled in accordance with institutional and national guidelines for the care and use of laboratory animals.

Organ weight and treatment of testis tissue

The weight of testis, epididymis and accessory reproductive organs was bilaterally measured for each animal. After weighing, a piece of the right testis was fixed with Bouin’s fluid and embedded in paraffin for histological observation. The remainder of the right testis was immediately frozen in liquid nitrogen and stored at -80°C until used for RNA extraction. The left testis was frozen at -80°C until thawing for sperm counts.
Sperm morphology and motility
Sperm suspension was collected from the cauda epididymis by mincing in HEPES-buffered TYH medium (pH 7.4) and prepared for examination of sperm motility and morphology as previously described (Oshio et al., 1990). To quantitate the sperm motility, 20 μl sample aliquots were placed on a hemocytometer and more than 200 sperm were observed and counted under a phase contrast microscope (BX51, Olympus Co., Tokyo, Japan) and a video system. The samples were kept at 37°C using a counting chamber Standard Count 2 Chamber Slide 20 micron (Leja, Nieuw Vennep, Netherland) and a constant-temperature unit MP-10 (Kitazato Supply Co. Ltd., Shizuoka, Japan). Percentage motile sperm was defined as Nm/Ns × 100 (%), where Nm is the number of forward motile and submotile sperm and Ns is the number of sperm in the view area.

Daily sperm production
Testicular tissue was thawed at room temperature and weighed after removal of any extra capsular materials from the testis. Samples were homogenized for 2 min in 1 ml of saline containing 0.05% Triton X-100 (Nacalai Tesque, Inc., Kyoto, Japan) and 0.2% Eosin Y (Merck & Co., Inc., Whitehouse station, NJ, USA). The concentration of sperm nuclei in each suspension was determined using a hemocytometer and daily sperm production (DSP) was calculated using the following formula as previously described (Ono et al., 2007, 2008; Yoshida et al., 2010):

\[ DSP = \frac{\text{sperm count/ml}}{4.84/\text{g testis weight}} \]

where 4.84 is a coefficient for calculating sperm production in mice.

Testicular morphology and sertoli cell counting
Testis fixed in Bouin’s solution were embedded in paraffin and cut into 6-μm thick sections. Sections were stained with hematoxylin and eosin (HE) and observed under light microscopy. A total of five seminiferous tubules exhibiting a round shape were randomly selected in samples of each mouse, and the number of Sertoli cells was counted.

ELISA
The serum testosterone concentration was measured by an enzyme-linked immunosorbent assay (testosterone ELISA kit, Alpha Diagnostic Intl, Inc., San Antonio, TX, USA).

Statistical analysis
The Steel-Dwass test was performed to compare data in three groups. A P value of less than 0.05 was considered to be significant.
RESULTS

Effects of maternal BPA exposure on body and reproductive organ weights, sperm count and quality and testicular morphology

To determine the general toxicity of maternal exposure to BPA, body and reproductive organ weights were measured and they were not significantly altered among any groups (Table 2). Sperm counts indicating the levels of sperm production, epididymal sperm morphology and motility were dose-dependently lower in mice maternaly treated with BPA (Fig. 1). BPA (both 5 and 50 mg/kg doses) injected into pregnant mice induced a shedding of immature germ cells in the seminiferous tubules in the testis of offspring male mice (Fig. 2). This observation indicated a disruption of sperm differentiation in the treated groups. The number of Sertoli cells was significantly decreased in the maternal BPA exposure groups (5 and 50 mg/kg) compared to the control group (Fig. 3).

Effects of maternal exposure to BPA on gene expression in testes

From the 2,482 genes printed on Mouse Testis2 Array, 38 and 32 genes were found to be dysregulated in testis
Fig. 1. Impact of maternal exposure to BPA on epididymal sperm count, morphology and motility. Pregnant mice were treated by BPA (twice administered 0, 5, 50 mg/kg) at gestational day 7 and 14 and epididymis tissue was collected from 6-week-old male offspring. The levels of (A) daily sperm production, (B) normal sperm morphology and (C) sperm motility were shown. Data are presented as mean ± S.E.M ($n = 10$ offspring/group, One or two offspring per dam). *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ vs control.

Fig. 2. Impact of maternal exposure to BPA on testicular morphology. Pregnant mice were treated by twice administered (A) corn oil (control); (B) BPA, 5 mg/kg; (C) BPA, 50 mg/kg at GD7 and 14. Testes were collected from 6-week-old male offspring. HE-stained images of seminiferous tubules are shown. Arrows and arrowheads indicate shedding of immature germ cells and disruption of Sertoli cell structure, respectively. Scale bar indicates 50 μm.
of the low-dose (5 mg/kg) and the high-dose (50 mg/kg) BPA groups, respectively. Functional analysis of the data with gene annotation showed that out of all the MeSH categories, 2 categories showed enriched dysregulation by the low-dose BPA, and 4 categories by the high-dose BPA (Table 3). A MeSH of “Sertoli Cells” did not show any enrichment of dysregulated gene sets by any comparison in this study, but did show significant enrichment from a set of downregulated genes in the high-dose BPA (50 mg/kg) group (Table 3). The downregulated genes related to Sertoli cells in the BPA (50 mg/kg) group were Msi1h, Ncoa1, Nid1, Hspb2, and Gata6 (Table 4, Fig. 4). The genes dysregulated in testis of the BPA exposure groups were categorized with enriched MeSH and were shown in Table 4.

Effects of maternal exposure to BPA on serum testosterone concentration

Serum testosterone level was not significantly altered by exposure of pregnant mice to BPA (data not shown).

DISCUSSION

The present study focused on the effects of prenatal BPA exposure on gene expression in testis of male offspring. Previous studies showed that oral exposure of pregnant rats to BPA increased testicular expression of platelet-derived growth factor receptors (PDGF-Rs) (Thuillier et al., 2003), Hsp90, and Hsc70 (Wang et al., 2004). In the present study, we determined a group of testicular genes dysregulated by prenatal BPA exposure by a testis-specific microarray and combined with MeSH annotation. The difference in bioavailability of BPA between subcutaneous injection and oral administration was already reported (Pottenger et al., 2000). The doses of BPA were set based on LOAEL of orally administered BPA (U.S. NTP, 1982) to observe changes in testicular toxicity under conditions with obvious pathological change, and at a lower dose. The present study showed that maternal exposure to two doses of BPA (< 50 mg/kg/time) did not affect serum testosterone level but decreased the number of Sertoli cells, the ratio of normal morphology in sperm and sperm motility. Although the schedule and the route of BPA administration differed between the present study and some previous studies, the data showed that...
analyses using these doses of BPA are useful for investigating the mechanisms of testosterone-independent toxicity on the testes.

In the present study, the effects of maternal exposure to BPA on testicular function were analyzed based on gene expression patterns generated from a microarray. As a result, a MeSH of “Spermatozoa” was extracted from both BPA-treated groups, as expected. Although the number of dysregulated genes in the testis was similar between the two BPA-treated groups, functional analysis using MeSH showed that dysregulated genes in the high-dose BPA group (twice administered 50 mg/kg) were more highly enriched in MeSH related to androgen and Leydig cells than those in the low-dose BPA group (twice administered 5 mg/kg). The result indicated that the mechanisms underlying testicular toxicity may be different between low- and high-dose of prenatal BPA treatment. BPA treatment in the present study did not induce

<p>| Table 3. | Significantly enriched MeSH categories of dysregulated genes in each treated group |</p>
<table>
<thead>
<tr>
<th>MeSH enriched of dysregulated genes</th>
<th>Total</th>
<th>BPA (5 mg/kg)</th>
<th>BPA (50 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td># of genes</td>
<td># of relevant genes</td>
<td>P value</td>
</tr>
<tr>
<td>Androgen Receptors</td>
<td>283</td>
<td>4</td>
<td>0.21</td>
</tr>
<tr>
<td>Androgens</td>
<td>318</td>
<td>5</td>
<td>0.19</td>
</tr>
<tr>
<td>Leydig Cells</td>
<td>243</td>
<td>4</td>
<td>0.21</td>
</tr>
<tr>
<td>Sperm Immobilizing Agents</td>
<td>3</td>
<td>1*</td>
<td>0.05</td>
</tr>
<tr>
<td>Spermatozoa</td>
<td>735</td>
<td>17*</td>
<td>0.02</td>
</tr>
</tbody>
</table>

<p>| Table 4. | Differentially expressed genes within relevant MeSH categories |</p>
<table>
<thead>
<tr>
<th>MeSH category</th>
<th>Upregulated gene</th>
<th>Downregulated gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Androgen Receptors</td>
<td>Erh, Ncoa4</td>
<td>Cyp1b1, Ncoa1, Neu1, Nid1, Psca</td>
</tr>
<tr>
<td>Androgens</td>
<td>Gast, Gm169, Hoxa2, Serpinb9c</td>
<td>Cyp1b1, Gata6, Hspb2, Ncoa1</td>
</tr>
<tr>
<td>Antispermatogenic Agents</td>
<td>Epha4</td>
<td></td>
</tr>
<tr>
<td>Leydig Cells</td>
<td>Erh, Gast, Serpinb9c</td>
<td>Cyp1b1, Gata6, Gjb2, Ncoa1</td>
</tr>
<tr>
<td>Sertoli Cells</td>
<td>Gata6, Hspb2, Msi1h, Ncoa1, Nid1</td>
<td>Epha4</td>
</tr>
<tr>
<td>Sperm Agglutination</td>
<td>Epha4</td>
<td></td>
</tr>
<tr>
<td>Sperm Immobilizing Agents</td>
<td>Epha4</td>
<td></td>
</tr>
<tr>
<td>Spermatocidal Agents</td>
<td>Epha4</td>
<td></td>
</tr>
<tr>
<td>Spermatozoa</td>
<td>Low-dose: Blvrh, Cbx5, Cflar, Glrbrh, Itpr1, Pfg, Smpd1, Spdef, Tff2, Tfpi, Vsig2</td>
<td>High-dose: Btlh14h, Epha4, Gba, Pdh1</td>
</tr>
<tr>
<td></td>
<td>High-dose: Gast, Map2k1ip1, Prs11, Qtr1, Serpinb9c, Pdh1</td>
<td>Both groups: Stk10</td>
</tr>
</tbody>
</table>

*P < 0.05 by a Fisher’s exact test with hypergeometric distribution.
abnormality of serum testosterone level and Leydig cell morphology, but altered expression levels of genes related to androgen and Leydig cells. Significant enrichment of androgen-related categories (Androgens and Androgen Receptors) in the maternal BPA-treated group was also consistent with the estrogenic and anti-androgenic activity of BPA (Witorsch, 2002; Richter et al., 2007). Our results also showed that sperm motility was decreased by prenatal BPA exposure but a MeSH of “Sperm Motility” was not enriched of dysregulated genes in the treated groups. The results indicated that sperm motility was decreased by relatively indirect effects of prenatal exposure to BPA. MeSH of “Antispermatogenic Agents”, “Sperm Agglutination”, “Sperm Immobilizing Agents” and “Spermaticidal Agents” were significantly enriched with only one relevant gene, Epha4, in the low-dose BPA group but not in the high-dose BPA group. These MeSH terms did not show any gene group related to prenatal BPA exposure, and therefore they were considered not to have any specific biological meaning. Moreover, the enrichment data from downregulated genes was informative. A MeSH category “Sertoli cells” was extracted from downregulated genes in the high dose BPA-treated group. Downregulation of Msilh (Saunders et al., 2002) and Gata6 (Ketola et al., 1999, 2003), which is expressed in Sertoli cells from fetal life to adulthood, and Ncoa1, which is expressed in Sertoli cells and is dispensable for primary organogenesis (Mark et al., 2004), may be a marker of prenatal exposure to BPA, which affects the development and functions of Sertoli cells. A previous study showed that the genes dysregulated by doxorubicin treatment were broadly enriched in some categories including Sertoli cells, Leydig cells and androgens as well as germ cells, fertility and infertility (Takahashi et al., 2011). It should be noted that the category “Sertoli Cells” was enriched of the genes downregulated by BPA but not enriched of the genes dysregulated in the present study. Expression of the genes related to Sertoli cell function was decreased with a dose-dependent manner, but a decrease in the number of Sertoli cells in the testes did not show dose-dependency. A group of genes related to Sertoli cells may be useful as a marker of testicular toxicity of prenatal exposure to BPA and other chemical substances. The relationship of the gene group with the effect of prenatal exposure to low-dose BPA (Cagen et al., 1999a, 1999b; Ashby et al., 1999) and estrogen-related receptor gamma (Esrrg), a receptor strongly-bound to BPA (Takayanagi et al., 2006), is future interest.

Overall, the present study showed gene expression changes related to the spermatic and histological abnormalities induced by in utero exposures to BPA. The results showed a group of genes related to Sertoli cells as a potential marker of testicular toxicity related to prenatal BPA exposure. This article concludes that an analysis using a testis-specific microarray and MeSH annotation can extract common terms among dysregulated genes associated with testicular dysfunctions and spermatogenetic impairments. It has been reported that microarray and gene network analyses detected a similarity of the genetic targets altered after fetal and prepubertal phthalate exposure (Lahousse et al., 2006). The method described in the present study using MeSH annotation can provide a biomedical interpretation of the gene expression changes followed by prenatal BPA exposure. We propose that this method is useful for rapid interpretation of gene expression changes associated with effects of prenatal exposure to chemical substances on testis and male reproductive system.

ACKNOWLEDGMENTS

The authors thank Dr. Tomomi Hishinuma (Research Center for Health Sciences of Nanoparticles, Research Institute for Science and Technology, Tokyo University of Science) for assistance in the microarray experiments. This work was supported by a Grant-in-Aid from the Private University Science Research Upgrade Promotion Business Academic Frontier Project. The funder had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

REFERENCES


Culty, M., Thuillier, R., Li, W., Wang, Y., Martinez-Anguilles, D.B., Benjamin, C.G., Triantafilou, K.M., Zirkin, B.R. and...
Microarray analysis of toxicity of prenatal BPA exposure using MeSH


