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Analysis of Gene Expression in the Testes of Mice Exposed to Bisphenol A and Nonylphenol

Jung-Hwa Oh[1], Moon-Ju Oh[2], Han-Jin Park[1], Seung Jun Kim[2], Se-Myo Park[1], Hea-Jin Yoon[1], Jae-Woo Cho[3], Seung Yong Hwang[2] & Seokjoo Yoon[1]

[1]Toxicogenomics Team, [3]Clinical Pathology Team, Korea Institute of Toxicology, 19 Shinsung-ro, Yuseong, Daejeon 305-343, Korea
[2]GenoCheck Co., Ltd. & Hanyang University, Sa 3-dong, Sangnok-gu, Ansan, Gyeonggi-do 426-791, Korea
Correspondence and requests for materials should be addressed to S. Yoon (sjyoon@kitox.re.kr)

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Abstract

Estrogenic environmental compounds (xenoestrogens) have adverse effects on male reproductive systems, including decreased sperm counts and problems with reproductive development. Although the male reproductive toxicity induced by xenoestrogens has been investigated, the molecular mechanisms by which estrogenic compounds induce toxicity in testes are unclear. This study used a microarray analysis to examine testicular toxicity and gene expression profiles in mice after 30 days of exposure to two estrogenic compounds, bisphenol A (BPA) and nonylphenol (NP). In total, 275 and 729 genes were identified as being either up- or down-regulated, with over 1.5-fold changes, in the testes of the BPA- and NP-treated groups, respectively. Differentially expressed genes were classified using a *k*-means clustering algorithm, and their biological functions and canonical pathways were further analyzed using Ingenuity Pathways Analysis (IPA). Toxicological function analysis characterized the mode of action according to BPA and NP. Pathway analysis identified genes involved in gluconeogenesis and calcium signaling in the BPA-treated group and genes involved in Wnt/β-catenin and estrogen receptor signaling in the NP-treated group. In addition, several differentially expressed genes that may play a role in spermatogenesis, such as *Odf1* and the *Sox* family, were identified. Collectively, these data help to elucidate the molecular mechanism of reproductive toxicity induced by xenoestrogens.

Keywords: Xenoestrogens, Bisphenol A, Nonylphenol, Gene expression profiling, Reproductive toxicity

Introduction

Recently, significant public concern has been raised about the potential endocrine-disrupting effects of environmental compounds, including industrial by-products, pesticides, and pharmaceuticals, in humans and many wildlife species. Xenoestrogens, or endocrine disruptors with estrogen activity, mimic or disturb the function of endogenous estrogen by binding the estrogen receptor[1-3]. Estrogens play important roles in regulating the development, differentiation, and function of both the male and female reproductive systems[4-6]. There is growing interest in the potential adverse effects of environmental xenoestrogens on male reproductive ability. Xenoestrogens induce testicular dysgenesis syndrome and increase the risk of seminomas in adults by disrupting the programming of reproductive development[7]. Much of what is known of the effects of xenoestrogens on male infertility and reproductive malformation has been obtained in animal studies[8-10]. Although there are many well-documented observations of the male reproductive toxicity induced by xenoestrogens, the precise molecular mechanisms of the toxicity or adverse effects induced by xenoestrogens are poorly understood.

Genomic approaches have been applied extensively to elucidate the molecular mechanisms of toxicological phenomena and to assess toxicity[11-14]. Using microarray analysis, changes in gene expression associated with xenoestrogen exposure *in vivo*[15-17] or *in vitro*[18-20] have been reported; however, the investigations of male reproductive toxicity induced by xenoestrogens are relatively limited. This study analyzed the gene expression profiles in reproductive toxicity induced by the xenoestrogens 2,2-bis(4-hydroxyphenyl)propane (bisphenol A, BPA) and nonylphenol (NP), which are likely endocrine-disrupting chemicals. BPA, an alkylphenol derivative, is widely used in the manufacture of polycarbonate plastics. Several studies have reported that BPA induces male reproductive toxicity *in vitro* and *in vivo*[21-23]. NP, an alkylphenol ethoxylate, is commonly used in the production of plastics and for household applications such as in lubricating oil additives and surface-active agents. Recent studies have shown that NP induces reproductive abnormalities and disrupts reproductive development in animal models[24,25]. We used microarray analysis to examine the changes in gene expre-

Table 1. Toxicological data on the BPA- and NP-treated mice.

	BPA				NP			
	Control (Corn oil)	Low (20 mg/kg)	Intermediate (100 mg/kg)	High (200 mg/kg)	Control (Corn oil)	Low (19 mg/kg)	Intermediate (29 mg/kg)	High (57 mg/kg)
Body weight								
Initial (g)	41.97±2.77	41.09±0.40	41.25±0.82	40.71±0.33	41.28±4.64	43.17±0.54	42.70±0.58	42.95±0.41
Terminal (g)	44.39±2.12	45.32±1.67	44.64±0.78	42.09±1.21	43.64±3.48	41.88±1.34	42.79±1.05	43.26±2.04
Relative organ weight								
Right testis (%)	0.35±0.03	0.28±0.06[a]	0.31±0.04	0.27±0.02[b]	0.33±0.03	0.34±0.03	0.29±0.03	0.28±0.03
Right epididymis (%)	0.14±0.02	0.12±0.02	0.14±0.03	0.12±0.02	0.14±0.01	0.17±0.02[a]	0.14±0.03	0.14±0.02
Epididymal sperms								
Motility (%)	76.25±14.48	82.33±9.37	83.50±3.87	85.75±3.40	89.09±3.69	94.95±5.72	43.95±25.53[a]	67.75± 27.48
Sperm counts ($\times 10^6$)	7.3±2.4	5.0±1.0	6.0±2.4	5.4±1.8	7.2±0.7	6.4±1.9	5.4±0.6[a]	3.3±1.6[b]

The results are expressed as the mean±SD.
[a]$P<0.05$, [b]$P<0.01$, compared with the control group (Student's *t*-test).

ssion in the testis after the administration of xenoestrogens, especially alkylphenol derivatives, in order to obtain new molecular insights into the reproductive toxicity induced by BPA and NP.

Results and Discussion

Morphological Changes of BPA- and NP-treated Mice

As shown in Table 1, we first examined the changes in body weight, relative organ weight, epididymal sperm motility, and sperm counts of the BPA- and NP-treated groups after exposure for 30 days. The relative weight of the right testis in the low- and high-dose BPA-treated groups was reduced slightly, although the overall body weight and relative organ weight including the right testis and epididymidis were not changed significantly after the administration of BPA or NP. As the terminal body weight was similar to the initial body weight, the administration of BPA and NP might have suppressed any expected weight gain. A reduction in sperm motility was observed in the NP-treated groups but not in the BPA-treated groups. The sperm counts in the NP-treated groups were also reduced significantly at intermediate and high doses, whereas the sperm counts in the BPA-treated groups were reduced slightly, without significance. Histopathological observations of the testis and epididymis showed no discernable differences between the controls and the BPA-treated groups, although the relative weight of the testis decreased slightly. By contrast, the NP-treated groups showed some evidence of reproductive toxicity; this included tubular dilatation, tubular atrophy, and sloughing of cells in the testis lumen, and oligospermia and cell debris in the epididymis, although the changes were slight and subject to individual variation (data not shown). These toxicological results indicate that the administration of BPA did not cause severe reproductive toxicity and that NP was weakly toxic in the male testis and epididymis of mice.

The reproductive toxicity of BPA at relatively low doses is controversial. Some studies have reported no effect on male reproductive organs[26-28], whereas others have reported that BPA causes reproductive toxic effects in rodent models[29-31]. The doses used in our study were determined from previous reports on the testicular toxicity of BPA[32]. The observed decreases in the relative weight of the testis and the epididymal sperm counts in BPA-treated mice suggest that BPA has a slight effect on the testis. Although no pathological lesions were observed, we postulate that the changes in gene expression associated with the early response to testicular toxicity occur before the onset of the histopathological changes.

Differentially Expressed Genes in the Testes of BPA- and NP-treated Mice

To elucidate the adverse effects of environmental xenoestrogens on the testis and to characterize the transcriptional changes, microarray analyses of BPA- and NP-treated mice were performed using the Agilent mouse whole genome array. Changes in gene expression in the BPA- and NP-treated groups compared with the control groups were analyzed. Differentially expressed genes in the BPA- and NP-treated groups were selected based on changes greater than 1.5-fold compared with the corresponding controls, in all independent experiments. In the BPA-treated groups,

275 genes were differentially expressed, including 68, 138, and 181 genes in the low-, intermediate-, and high-dose groups, respectively. In the NP-treated groups, 729 genes were differentially expressed, including 398, 358, and 454 genes in the low-, intermediate-, and high-dose groups, respectively. Overall, fewer genes were differentially expressed in the BPA-treated groups than in the NP-treated groups, which concurs with the histopathological observations showing fewer toxicological changes in the BPA-treated groups compared with the NP-treated groups.

In detail, the 896 differentially expressed genes in the BPA- or NP-treated groups were analyzed using *k*-means clustering (Figure 1) to show specific gene expression patterns, including genes that were up- or down-regulated in common or in a chemical-specific manner. Common to both groups, 46 genes (Cluster 1) and 269 genes (Cluster 5) were down-regulated, with the genes belonging to Cluster 1 being down-regulated more strongly. In contrast, 123 genes (Cluster 7) were up-regulated in common, and 223 genes (Cluster 2) were up-regulated slightly in the BPA-treated groups but highly up-regulated in the NP-treated groups. Genes belonged to Clusters 3, 4, 6, and 8 showed specific expression patterns according to the unique modes of action of each chemical or the degree of toxicity, as the toxicological phenotypes of the BPA- and NP-treated groups differed.

From among the genes up- or down-regulated in common, the top 20 genes that were significantly altered in the BP- or NP-treated groups were selected and are listed in Table 2. Many genes involved in signal transduction or cell cycle regulation, such as *D0H6S2654E*, *Olfr1509*, *LOC545217*, *1500034J01Rik*, and *Stard10*, were up-regulated. In addition, genes related to protein modification (*Gnas*), cell structure (*Krt1-c29*), DNA binding (*Hist1h2ai* and *Mlf1*), protein polymerization (*Ropn1l*), and transport (*Bat3*) were up-regulated. Many of the down-regulated genes had no annotated functions, although the down-regulated genes included genes involved in receptor activity or transport, such as *Slc22a20*, *Ryr1*, *Ryr3*, *Stx1b2*, and *Plxna3*, DNA binding (*11100851B16Rik*), cell proliferation (*Plekhk1*), Zn-ion binding (*1300004G08Rik*), the intercellular junction (*Ahnak*), metabolism (*6820-429M01*), and stress response or movement (*Dnahc1*) in the BPA- and NP-treated groups.

Among the genes regulated in common in both the BPA- and NP-treated groups, *Odf1* (outer dense fiber of sperm tail 1), which is a component of the outer fiber of the sperm tail, was up-regulated in both groups. Defects in the protein encoded by *Odf1* cause abnormal sperm morphology or motility disorders[33]. *Ddx25*, a member of the DEAD-box family, plays an important role in spermatogenesis and post-transcriptionally regulates components of the spermatid, such as *Odf1*[34]. The expression of *Ddx25* was not changed in our analysis, although that of *Ddx5*, another DEAD-box family member that is involved in estrogen receptor signaling, was up-regulated more than 1.5-fold in both the BPA- and NP-treated groups (data not shown). This suggests that *Ddx5* regulates the transcription of genes involved in spermatid development. Moreover, three *Sox* (SRY-related HMG box) genes, *Sox10*, *Sox11*, and *Sox30*, were differentially express-

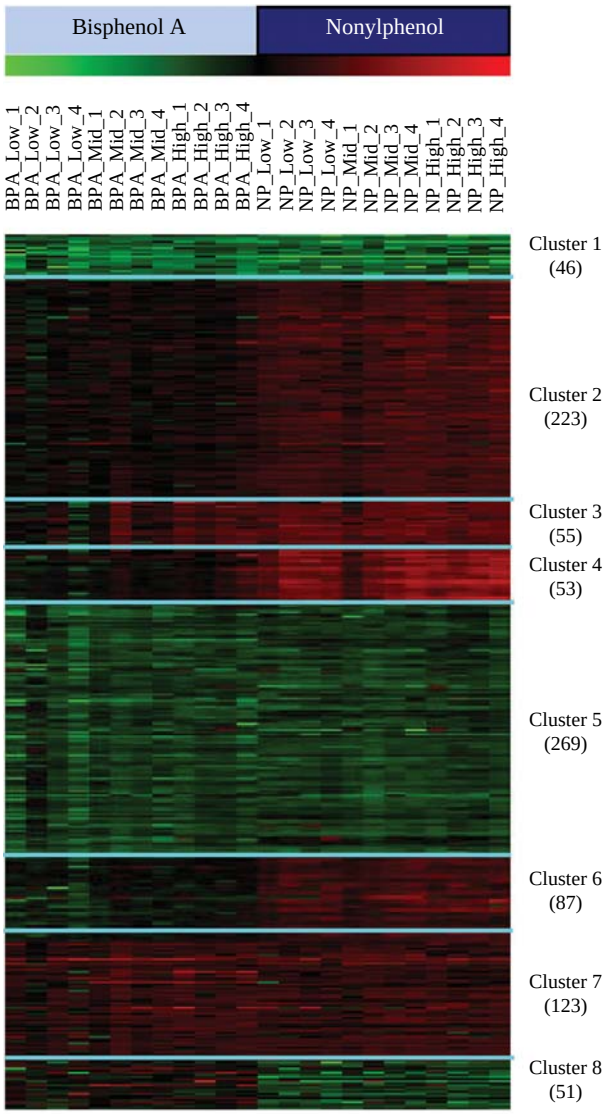

Figure 1. The *k*-means clustering of differentially expressed genes in the testes of the BPA- and NP-treated groups. As described in the Methods section, we performed *k*-means clustering by setting $k=8$ (total clusters=8). The eight clusters are highlighted with colored lines.

Table 2. Differentially expressed genes in the testes of BPA- and NP-treated mice.

Gene_symbol/ID	Gene_title	Acc. No.	Fold change (Log 2)	
			BPA	NP
Up-regulated genes in the BPA- and NP-treated group				
D0H6S2654E	DNA segment, human D6S2654E	BC049659	1.05	1.63
Gnas	guanine nucleotide binding protein, alpha stimulating	BC062654	0.85	1.60
Krt1-c29	keratin complex-1, acidic, gene C29	NM_010666	0.88	1.43
Olfr1509	olfactory receptor 1509	NM_020514	1.27	1.37
LOC545217	similar to F-box/LRR-repeat protein 17	XM_619480	0.93	1.34
Ap2s1	adaptor-related protein complex 2, sigma 1 subunit	NM_198613	0.92	1.25
Hist1h2ai	histone 1, H2ai	NM_178182	1.27	1.21
A_52_P257377	unknown	ENSMUST00000090782	0.85	1.19
Mlf1	myeloid leukemia factor 1	NM_010801	0.76	1.16
1500034J01Rik	RIKEN cDNA 1500034J01 gene	BC034701	1.02	1.16
Stard10	START domain containing 10	NM_019990	0.76	1.15
Mrpl28	mitochondrial ribosomal protein L28	NM_024227	1.00	1.13
Dkkl1	dickkopf-like 1	NM_015789	0.67	1.12
Krtcap3	keratinocyte associated protein 3	NM_027221	0.97	1.11
E130201H02Rik	RIKEN cDNA E130201H02 gene	XM_488078	0.70	1.11
Odf1	outer dense fiber of sperm tails 1	NM_008757	0.98	1.08
Bat3	HLA-B-associated transcript 3	NM_057171	1.10	1.01
Ropn1l	ropporin 1-like	NM_145852	0.75	1.00
Down-regulated genes in the BPA- and NP-treated group				
Ryr3	ryanodine receptor 3	XM_619795	−1.39	−2.35
Slc22a20	solute carrier family 22 (organic anion transporter), member 20	AK053009	−1.58	−2.25
Ryr3	ryanodine receptor 3	XM_619795	−1.26	−2.24
Ryr3	ryanodine receptor 3	XM_619795	−1.23	−2.13
Syne2	synaptic nuclear envelope 2	XM_619002	−1.08	−1.61
1110051B16Rik	RIKEN cDNA 1110051B16 gene	NM_183389	−1.27	−1.60
Ryr1	ryanodine receptor 1, skeletal muscle	NM_009109	−0.88	−1.49
Plekhk1	pleckstrin homology domain containing, family K member 1	AK045134	−1.12	−1.41
Slp	sex-limited protein	NM_011413	−1.42	−1.40
Stx1b2	syntaxin 1B2	NM_024414	−0.92	−1.39
A230059G12Rik	RIKEN cDNA A230059G12 gene	NM_177037	−1.77	−1.35
1300004G08Rik	RIKEN cDNA 1300004G08 gene	AK054549	−1.46	−1.35
Ahnak	AHNAK nucleoprotein (desmoyokin)	NM_009643	−1.18	−1.33
6820429M01	hypothetical protein 6820429M01	NM_172749	−2.26	−1.32
Aldh1l2	aldehyde dehydrogenase 1 family, member L2	NM_153543	−0.98	−1.32
A930009L07Rik	RIKEN cDNA A930009L07 gene	AK039650	−1.24	−1.31
A_52_P772350	unknown	TC1509035	−0.94	−1.28
LOC434512	similar to variable region of immunoglobulin kappa light chain	XM_486347	−1.14	−1.28
Dnahc1	dynein, axonemal, heavy chain 1	Z83815	−1.79	−1.27
Plxna3	plexin A3	NM_008883	−0.76	−1.24

The fold change was calculated as the relative average value of four arrays in each group compared with the pooled controls, with the values expressed as natural logarithms.

ed. SOX proteins are involved in spermatogenesis, and these genes seem to be differentially expressed at distinct steps during spermatogenesis[35]. *Sox30* was highly expressed in spermatocytes, while *Sox5* and *Sox17* were highly expressed in early post-meiotic cells (round spermatids) and spermatogonia, respectively[36,37]. In the present study, *Sox10* and *Sox 30* were up-regulated more than 1.5-fold in the BPA-treated groups and especially in the NP-treated groups (data not shown). By contrast, *Sox11*, which is involved in nervous system development[38], was down-regulated in the NP-treated groups. It is thought that

Table 3. Functional classification of differentially expressed genes in the BPA- and NP-treated groups.

Bisphenol A		Nonylphenol	
Functions	No. of genes	Functions	No. of genes
Molecular and cellular functions			
Cellular assembly and organization	21	Cellular function and maintenance	18
Cell death	7	Cellular assembly and organization	32
Cellular function and maintenance	14	Drug metabolism	4
Cellular development	23	Small molecule biochemistry	15
Amino acid metabolism	2	Cell cycle	33
Physiological system development and function			
Embryonic development	10	Hair and skin development	3
Reproductive system development	12	Tumor morphology	6
Organismal development	21	Organismal development	40
Skeletal and muscular system	17	Cell-mediated immune response	16
Tissue morphology	13	Hematological system development	31

The top functional categories for significantly ($P < 0.05$) differentially expressed genes in both the BPA- and NP-treated groups. *P* values were calculated by comparing the number of molecules of interest relative to the total number of occurrences of these molecules in all functional annotations stored in the Ingenuity Pathways knowledge base (Fisher's exact test with the *P*-value adjusted using the Benjamin-Hochberg multiple testing correction).

these differentially expressed *Sox* genes regulate the differentiation of male germ cells at distinct steps.

Functional Classification and Toxicological Interpretation of Differentially Expressed Genes

To classify and characterize the biological or toxicological functions of the differentially expressed genes, the 275 and 729 genes in the BPA- and NP-treated groups, respectively, were analyzed using IPA. As shown in Table 3, the biological functions of differentially expressed genes were analyzed according to two categories: 1) molecular and cellular functions and 2) physiological system development. Based on molecular and cellular function, many genes involved in cellular assembly and organization and cellular function and maintenance were identified in both the BPA- and NP-treated groups. In terms of physiological system development, the top-ranked functions differed slightly between the BPA- and NP-groups. In the BPA-treated group, genes involved in embryonic and reproductive system development were identified; genes involved in hair and skin development and tumor morphology were identified in the NP-treated group.

The toxicological functions of genes differentially expressed between the BPA- and NP-treated groups were compared, and the results are shown in Figure 2. Genes involved in positive acute-phase response proteins (*C4b* for BPA and NP), the cytochrome P450 panel (*Cyp2fa* for BPA; *Cyp2d6* and *Cyp3a4* for NP), and fatty acid metabolism (*Acsl1* and *Cyp2fa* for BPA; *Aldh1b*, *Cyp2d6*, *Cyp3a4*, and *Slc27a1* for NP) were common to the BPA- and NP-treated groups. On the other hand, genes related to oxidative stress (*Gpx3*) and PPARα/RXR activation (*Gnas*) were identified only in the BPA-treated groups, while genes associated with hypoxia-inducible factor signaling (*Eifa*, *Hras*,

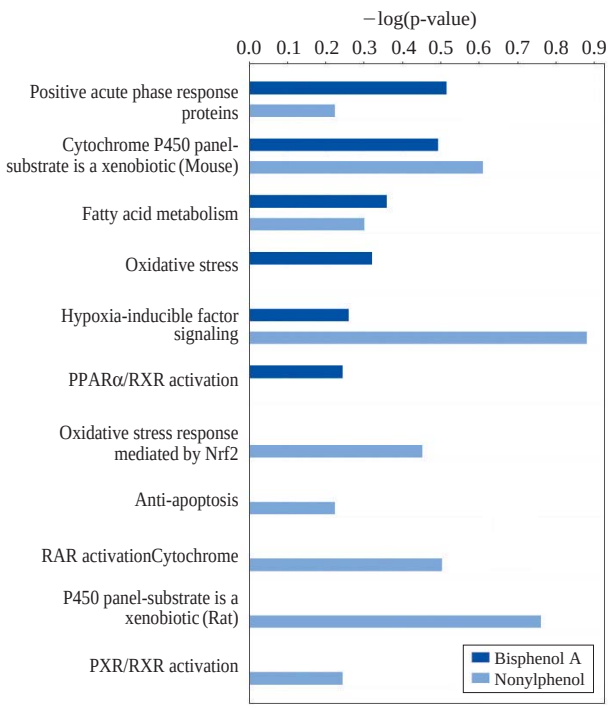

Figure 2. Toxicological function analysis of differentially expressed genes in the BPA- and NP-treated groups. Interesting categories of mode of action were selected and are represented. The dark blue and light blue histograms indicate the BPA- and NP-treated groups, respectively.

Table 4. Canonical pathway analysis of the differentially expressed genes in the BPA- and NP-treated groups.

Canonical pathways	Ratio	Genes
Bisphenol A		
Glycolysis/Gluconeogenesis	4/97 (0.041)	*Acsl1, Aldoc, Hk1, Ldha*
Calcium signaling	6/175 (0.034)	*Cabin1, Calm3, Myh6, Ryr3, Ryr1, Trpc6*
Axonal guidance signaling	9/364 (0.025)	*Ablim2, Gnas, Gng7, Klk1, Plxna3, Shh, Stk36, Wnt10b, Wnt5b*
α-adrenergic signaling	4/91 (0.044)	*Calm3, Gnas, Gng7, Phkg2*
Glutamate receptor signaling	3/55 (0.055)	*Calm3, Gng7, Slc17a7*
Nonylphenol		
Wnt/β-catenin signaling	11/161 (0.068)	*Csnk1a1, Csnk1g2, Csnk2b, Dkk1, Dkkl1, Kremen1, Ppp2cb, Sox10, Sox11, Wnt10b, Wnt5b*
CCR3 signaling in eosinophils	8/110 (0.073)	*Calm2, Calm3, Gnas, Hras, Jmjd7-Pla2g4b, Pak1, Pik3cg, Ppp1cc*
Estrogen receptor signaling	8/112 (0.071)	*Ddx5, H3f3a, H3f3b, Hist2h3d, Hras, Smarca4, Taf4, Taf9*
Synaptic long term depression	8/144 (0.056)	*C7orf16, Gna13, Gnas, Gucy1b2, Hras, Ppp2cb, Ryr3, Ryr1*
IL-4 signaling	5/67 (0.075)	*Hla-Dqa1, Hras, Il4, Jak3, Pik3cg*

The top ranked canonical pathways of differentially expressed genes in the BPA- and NP-treated groups are shown. The ratio was calculated using IPA as follows : the number of genes in a given pathway that meet the cutoff criteria, divided by the total number of genes that make up the pathway.

Ube2d3, and *Ube2n*), the oxidative stress response mediated by Nrf2 (*Cyp3a4*, *Dnaja4*, *Hras*, *Jund*, *Maff*, *Pik3cg*, and *Sod3*), anti-apoptosis (*Bag1*), and PXR/RXR activation (*Cyp3a4* and *Prkar2a*) were identified only in the NP-treated groups.

The toxicological function analysis of the differentially expressed genes characterized specific modes of action for BPA and NP. Regarding xenobiotic metabolism, *Cyp2f1* was up-regulated in the BPA-treated groups, whereas *Cyp2d6* and *Cyp3a4* were down-regulated in the NP-treated groups in the mice testes. BPA has been reported to induce the activity of *Cyp19a* in a breast cancer cell line[39]. However, the down-regulation of *Cyp3a4* observed in the NP-treated groups in the present study is in contrast to a previous finding that nonylphenol increased the expression of *Cyp3a4* in the liver[40]. Perhaps the expression of *Cyp3a4* is different in the testes of NP-treated mice. No transcriptional changes in the genes encoding P450 isozymes in the testis have been reported. In the NP-treated groups, genes involved in oxidative stress, especially *Nrf2*, were identified. El-dakdoky and Helal observed oxidative stress in testis tissue following NP exposure, as indicated by significant increases in the glutathione level and superoxide dismutase activity[41]. Our gene expression profiling identified the involvement of *Cyp3a4*, *Dnaja4*, *Hras*, *Jund*, *Maff*, *Pik3cg*, and *Sod3* in this process.

To understand the molecular functions of each gene set in detail, canonical pathways were also analyzed. As shown in Table 4, gluconeogenesis, calcium signaling, axonal guidance signaling, α-adrenergic signaling, and glutamate receptor signaling were highly ranked in the BPA-treated groups. Wnt/β-catenin signaling, CCR3 signaling in eosinophils, estrogen receptor signaling, synaptic long-term depression, and IL-4 signaling were ranked highly in the NP-treated groups. Interestingly, genes that are associated with estrogen receptor signaling, such as *Ddx5*, *Hras*, and *Smarca4*, and genes for the histone complex and *Tif* families were indentified in the NP-treated groups. In the BPA-treated groups, these genes were not identified because their fold changes fell below the cutoff value (1.5-fold), although they were expressed similarly (data not shown).

It was recently reported that BPA inhibits testicular Ca^{2+}-ATPase, and it has been suggested that BPA disrupts testicular development by inhibiting Ca^{2+} homeostasis[23]. In the present study, significant ($P < 0.05$) involvement of calcium signaling was detected using IPA, and genes such as *Cabin1*, *Calm3*, *Myh6*, *Ryr3*, *Ryr1*, and *Trpc6* were deregulated in the BPA-treated groups. In addition, cross-talk between the Wnt and estrogen signaling pathways is reported to play important roles in controlling a wide range of biological processes via the functional interaction between β-catenin and estrogen receptor[42]. In the NP-treated groups, the high ranking of Wnt/β-catenin signaling and estrogen receptor signaling suggests that the xenoestrogen NP causes the differential expression of genes involved in estrogen receptor signaling and that Wnt/β-catenin signaling interacts functionally in this process.

Conclusions

Although estrogens are regarded as female sex hormones, they also play an important role in regulating the development of the reproductive system in males.

Increasing numbers of studies have demonstrated that environmental compounds with estrogenic activity affect male reproduction, producing effects such as a decline in semen quality. These toxic effects have triggered investigations into the adverse effects of environmental xenoestrogens on male reproductive health in human and animal models. To evaluate the mode of action of xenoestrogens and to identify the molecular targets participating in these processes, toxicogenomic approaches are essential for screening the significant gene set involved in signal pathways for reproductive toxicity. In this study, we examined the effects of exposure to two xenoestrogens, BPA and NP, on gene expression changes in the testes of male mice. Using microarray analysis, we obtained a comprehensive gene expression profile during reproductive toxicity after exposure to the xenoestrogens BPA and NP. We identified differentially expressed gene sets in both xenoestrogen-treated groups and analyzed their toxicological function and canonical pathways. This information may aid in understanding the mechanisms of reproductive toxicity induced by BPA and NP and support the risk assessment of environmental estrogenic compounds.

Materials and Methods

Animals and Chemicals

Approximately 10-week-old Crj : CD-1 (ICR) male mice (SLC, Japan) were kept under a 12-h light/dark cycle in an animal room at a controlled temperature and humidity for 2 weeks before the experiment. The mice were provided standard food pellets and water *ad libitum*. BPA and NP were obtained from Sigma-Aldrich Chemicals (USA). BPA dissolved in dimethyl sulfoxide (DMSO)/corn oil (0.5 : 9.5, v/v) was injected subcutaneously at 20 (low), 100 (intermediate), or 200 (high) mg/kg body weight. NP dissolved in corn oil was administered intraperitoneally at 19 (low), 29 (intermediate), or 57 (high) mg/kg body weight. The chemicals were administered every other day for 30 days. The control mice were given the corresponding quantities of the vehicle. The mice were sacrificed after 30 days and were dissected carefully in order to count the sperm and collect tissue samples. Five or six animals were used in each experiment. All experiments were approved by the Institutional Animal Care and Use Committee and conducted in accordance with Association for Assessment and Accreditation of Laboratory Animal Care international guidelines.

Organ Weights and Histopathology

The control and experimental groups were killed at the same times. At necropsy, both of the testes and epididymides were collected, and the right ones were weighed individually. Relative organ weight was calculated as the ratio of the right organ weight to body weight. The right testis and epididymis were used to observe the histopathological changes, and the left ones were used for the microarray analysis and to evaluate sperm motility and sperm count. For histopathology, the right testis and epididymis were fixed in Bouin's solution, embedded in paraffin, sectioned at 4-μm thickness, and stained with hematoxylin and eosin (H & E). The histopathological changes were analyzed using light microscopy.

Epididymal Sperm Motility and Sperm Count

The cauda epididymidis was obtained from the left epididymis and weighed. Immediately, epididymal sperm were collected by chopping the cauda epididymidis in 1 mL of CO_2-independent medium (pH 7.4-7.6, Invitrogen, USA) with 5 mg/mL bovine serum albumin, and the sperm were incubated for 5 min at 37°C. Approximately 10 μL of this solution were placed on a glass slide set on a warming plate, and motile and non-motile sperm were counted. Sperm motility was reported as the percentage of motile sperm to the total sperm counted. For sperm counting, chopped cauda epididymides were homogenized to remove tissue debris and sperm tails. The diluted sperm suspension was placed in a hemocytometer, and the sperm heads were counted using phase contrast microscopy.

Microarray Analysis

Four testes were selected from the BPA- and NP-treated groups and corresponding control groups. Using TRIzol (Invitrogen, USA), total RNA was isolated from the testis tissue according to the manufacturer's instructions. Total RNA from the control groups was pooled, and 5-μg RNA samples were used for the microarray analysis. The Agilent mouse whole genome, containing more than 41,000 probes, was used. Sample labeling, microarray hybridization, washing, and scanning were performed according to the manufacturer's protocols (Agilent Technologies, USA). The preprocessing procedure and subsequent microarray data analyses were performed using GenPlex software (Istech, Korea). The data were normalized using Lowess normalization. Subsequently, 11,620 genes that were missing data were removed, and 29,380 genes were further analyzed. The differentially expressed genes were selected based on 1.5-fold changes in all of the treated groups compared with the levels in the pooled controls. The selected differentially expressed genes were analyzed using a *k*-means clustering algorithm based on Euclidean distances (k=8). The biofunctional and canonical path-

way analysis of differentially expressed genes was performed using Ingenuity Pathways Analysis (IPA).

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